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Development of a liquid chromatography-tandem mass spectrometry method for the analysis of docetaxel-loaded Poly(lactic-co-glycolic acid) nanoparticles

Amir Khajavinia[a], Mehran Yarahmadi, Anas El-Aneed[a], Azita Haddadi[a, *]

[a] College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, SK Canada

[*]Corresponding author. Tel.: +1 3069666495

Email address: azita.haddadi@usask.ca (A. Haddadi)

Abstract

Docetaxel is among the most effective chemotherapeutic agents used for the treatment of solid tumors, such as breast cancer. Targeting docetaxel to the tumor site would increase the safety and efficacy of the treatment. The focus of this work was to develop an efficient liquid chromatography tandem mass spectrometry (LC-MS/MS) method to quantify docetaxel entrapped in optimized poly lactic-co-glycolic acid (PLGA) nanoparticles.

Several nanoparticle formulations were prepared to optimize the nanoparticles based on their size and yield percentage using a modified solvent evaporation technique. The MS/MS fingerprints of docetaxel and paclitaxel (as internal standard) were used to identify diagnostic product ion for developing a multiple reaction monitoring (MRM) LC-MS/MS method for the quantification of docetaxel in the PLGA nanoparticles. A triple quadrupole linear ion trap instrument (AB Sciex 4000 QTRAP) equipped with electrospray ionization was used. The optimized nanoparticles had a zeta potential of -23.2 ± 1.4 mV and mean particle sizes of 202.2 ± 4.7 nm and 251.7 ± 8.2 nm before and after freeze-drying, respectively. Polydispersity index values of the nanoparticles confirmed their uniform size distribution. The developed LC-MS/MS method could quantify docetaxel in the PLGA matrix with accuracy and precision covering a broad linear range of 15.6 to 4000 ng/mL. Method validation was conducted using the regulatory guidelines of the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) and showed acceptable values for all the tested criteria. The developed LC-MS/MS method with the novelty of using a phenyl column will be beneficial for future analysis of docetaxel loaded polymeric nano-delivery systems.

Keywords: Docetaxel; Nanoparticle; PLGA; LC-MS/MS; Quantification

1. Introduction

Breast cancer is one of the most prevalent types of cancer with nearly 1 in 8 women being diagnosed with invasive breast cancer over the course of their lifetime; in addition,1 in 39 women will die from breast cancer based on latest statistics (1). It has been established, however, that chemotherapeutic regimens that contain a taxane, such as paclitaxel or docetaxel have higher disease-free survival rates compared to therapies without a taxane (2). Paclitaxel is naturally found in *Taxus* plants while docetaxel is a semi-synthetic derivative compound. The success of taxanes stems from their ability to inhibit a fundamental process involved in cell proliferation, namely microtubule depolymerization. These drugs exert their action by binding to the β-tubulin subunit in the structure of the microtubules, leading to mitotic arrest and eventual cell death (2). Despite their advantages, the clinical use of these medications are limited due to their poor aqueous solubility, rapid elimination, low selective distribution, and severe adverse effects (3). Taxane-associated toxicities frequently require reduction of the dose or therapy cessation, despite desirable clinical outcomes.

To overcome the limitations of chemotherapy, nano-carriers have been utilized to enhance the dissolution rate of the encapsulated drug which, in turn, enhances its bioavailability and absorption (4). Nanoparticles can also be employed to prolong the effects of a drug by providing more protection against metabolism and renal clearance (5). Moreover, nanoparticles can improve the tissue distribution of a drug by enhancing its delivery to certain tissues (i.e., targeted drug delivery); this can, in turn, lower side effects of the drug (6). Due to its desirable properties, PLGA polymer has been extensively used to prepare novel drug delivery systems, especially nanoparticles (7). PLGA is completely biocompatible and biodegradable, has controllable properties, and is also approved by the FDA for human therapy (7). After formulating the nanoparticles, it is essential to determine the loading parameters prior to *in vivo* testing to assess the properties of the particles in the animal models. Different analytical methods have been applied to quantify docetaxel in polymeric or biologic samples (8). High performance liquid chromatography coupled with ultraviolet detector (HPLC-UV), or tandem mass spectrometry (HPLC-MS/MS) are the most widely used techniques for the analysis of docetaxel. While UV-

based analysis is simpler, MS-based methods provide higher selectivity and lower quantification limits. In a recently published study, the limit of quantification for docetaxel was 700 ng/mL in a PLGA nanoparticle matrix using HPLC-UV (9), while MS-based methods can have a quantification limit lower than 10 ng/mL (10).

HPLC-MS/MS has been adapted to analyze docetaxel in different matrices, such as plasma, serum, and urine (11). HPLC columns used in all published studies are either C8 (12) or C18 (13). To the best of our knowledge, a phenyl column has never been applied for the quantification of docetaxel in a polymeric formulation matrix. The stationary phase in a Nova-Pak® phenyl column is made up of silica particles with hydrophobic phenyl hexyl chains (-$(CH_2)_8$-C_6H_5) that can interact with analytes (14). In a sample of PLGA polymetric nanoparticle formulation, the main impurity is PLGA, a linear polymer which will have minor interactions with the stationary phase of a phenyl column. Therefore, PLGA molecules that has not been eliminated from the sample during extraction will be separated early in the analysis. On the other hand, docetaxel and paclitaxel have hydrophobic phenyl rings in their structures, allowing for pi-pi interactions with the phenyl groups of the stationary phase, unlike PLGA molecules. Such differences in the interactions of the analyte and the polymer with the stationary phase will theoretically result in good separation of the drug molecules from PLGA impurities, potentially increasing the sensitivity of the method. Previous studies reported quantification limits as low as 1 ng/mL for docetaxel (15); however, these methods did not undergo full method validation per regulatory guidelines for clinical and pre-clinical applications (16, 17). In addition, the linearity range for docetaxel was about 100 folds (18), which limits the application of the calibration curve and mandates developing several standard curves for quantification over different concentration ranges.

In this study, a novel LC–MS/MS method is developed for the quantification of docetaxel entrapped in a polymeric nanoparticle formulation. A 250-fold calibration range is achieved for docetaxel.

2. Materials and methods

2.1. Materials

Ester-terminated PLGA (50:50) with an inherent viscosity of 0.15-0.25 deciliters per gram (dL/g) was purchased from Birmingham Polymers (LA, USA). Paclitaxel (>99.5% purity) and docetaxel (>99%) were from LC laboratories (Woburn, USA). Polyvinyl alcohol (PVA) was from Sigma-Aldrich Co. (St Louis, USA). Methanol and acetone were LC-MS grade, purchased from Fisher Scientific (Fairlawn, NJ, USA), and formic acid was from BDH Chemicals (Toronto, ON, Canada).

2.2. Nanoparticle preparation

PLGA nanoparticles were prepared based on method established previously in our laboratory using the solvent evaporation technique (19). Briefly, 10% w/v ester ended PLGA, and 3% w/v docetaxel were dissolved in ethyl acetate to serve as the organic phase. Polyvinyl alcohol (PVA) 2.2% w/v was added as the aqueous phase (organic phase to aqueous phase ratio 1 to 6.7) and vigorously mixed followed by high energy sonication for two minutes using a digital tip-sonifier (Branson digital sonifier 250, Danbury, USA). The resulting suspension was stirred at room temperature to allow for the evaporation of the organic solvent, and the prepared nanoparticles were washed with the addition of excess amount of deionized water followed by ultracentrifugation to remove the extra PVA and free drug residues. Finally, sucrose was added as a cryoprotectant, and the nanoparticles were freeze dried for 48 hours (Labconco Co., Kansas City, USA) and stored at -20°C. To measure the particle size, zeta potential and polydispersity index (PdI), 1 mg of the dried formulation was resuspended in 5 mL deionized water and the suspension was transferred to a folded capillary cell, before being analyzed using Malvern Zetasizer (Nano series, Montreal, Canada) at 25°± 1 C (n=3). The yield percentage of the process was calculated using the following equation:

$$\text{Yield (\%)} = \frac{\text{weight of the obtained particles}}{\text{the initial weight of polymer,drug and other ingredients}} \times 100 \quad \text{(Equation 1)}$$

2.3. LC-MS quantification of encapsulated docetaxel

2.3.1. Standard, Quality control (QC), and experimental sample preparation

Stock solutions of docetaxel (4 µg/mL) and paclitaxel (2 µg/mL) were prepared in methanol. Paclitaxel was used as the internal standard (IS) in the analysis with a final concentration of 400 ng/mL. All stock solutions were prepared fresh before each experiment. Working solutions of the standards were made by serial dilution of the stock solution in methanol to prepare eight concentrations, as follows: 15.6, 31.2, 62.5, 250, 1000, 2000, 3500, 4000 ng/mL. 500 µL from each standard working solution plus 100 µL from the IS stock solution were transferred to into 2 mL tubes and vortexed for 60 seconds. The solvent was evaporated using a centrifugal vacuum concentrator (Labconco, USA) and 0.5 mg drug-free nanoparticles (plain nanoparticles) were added to the tube to prepare the standard samples. Each prepared standard was then extracted using the method described in section 2.3.2.

Quality control (QC) samples, for method validation, were prepared at four concentration levels. According to the FDA guidelines (17), the high-quality control (HQC) sample should be at least 75% of the upper limit of quantification (ULOQ); the middle quality control (MQC) sample should be in the mid-range; the low-quality control (LQC) sample should be within 3 times the concentration of LLOQ; and the LLOQ as the fourth QC level (17). A stock solution of 3000 ng/mL was used to prepare a QC set of 15.6, 40, 1500, and 3000 ng/mL. Half a milliliter of each solution and 100 µL of the IS stock solution were transferred to 2mL tubes and vortexed for 60s. The solvent was evaporated, and 0.5 mg drug-free nanoparticles were added to the tube. Each prepared QC sample was then extracted using the method described in section 2.3.2.

To prepare experimental samples with unknown concentrations, 100 µL of IS stock solution was placed in 2 mL tubes. The solvent was evaporated, and 0.5 mg drug-loaded nanoparticle formulation was placed in the tube. Each sample was extracted using the method described in section 2.3.2. Samples were diluted 20 times with blank (i.e., zero calibrator), which is an extracted matrix sample to which the IS had been added, before analysis. An analytical run included the following samples: double blank, which is an extracted matrix sample without the analyte (i.e., matrix blank), blank, eight calibration standards, four levels of QC, and unknown samples.

2.3.2. Docetaxel extraction from nanoparticles

To quantify docetaxel, samples were extracted prior to injection in the mass spectrometer. An extraction method established previously in our laboratory (19) was used with some modifications. Paclitaxel was added at the beginning of the extraction process as the IS at a concentration of 400 ng/mL.

Half a milliliter of acetone was added to 0.5 mg drug-loaded or drug-spiked nanoparticles to dissolve both the polymer and the drug. The obtained mixture was vigorously shaken (VWR Mini Vortexer, USA) for one minute and was subjected to bath sonication (Branson 5510 bath sonicator, USA) for 30 minutes. It was then centrifuged for 30 minutes at 20,000 g (Beckman J2-21 Ultracentrifuge, USA). The supernatant was separated and transferred to new ependorf tubes. The same procedure was repeated by dissolving the precipitate in acetone. The collected supernatants from the first and second centrifugation steps were mixed, and the solvent was evaporated. Methanol (0.5 mL) was added to the residue after evaporation, vortexed for one minute and centrifuged for 30 minutes at 20,000 g. The supernatant was transferred to HPLC vials (Figure 1).

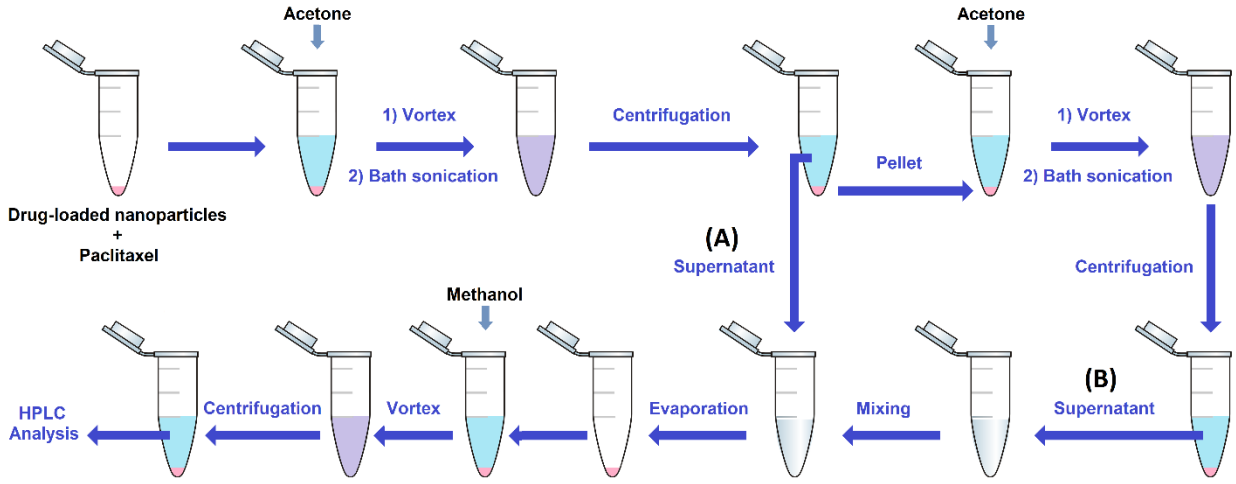

Figure 1. Illustration of the steps of docetaxel extraction from nanoparticles. The process of disintegrating the nanoparticles in acetone and collecting the supernatant was performed twice (A and B) to ensure the maximum extraction of the entrapped docetaxel.

2.3.3. LC-MS/MS analysis

A new LC-MS/MS method was developed for the quantification of docetaxel in the nanoparticle matrix. Chromatographic separation was achieved on a 1260 Agilent Infinity II (Waldbronn, Germany) High Performance Liquid Chromatography (HPLC) system. A sample volume of 5 µL was injected using the 1260 Agilent autoinjector set to 4°C for the separation on a Nova-Pak® Phenyl column (60 Å, 4 µm, 3.9 mm × 75 mm) maintained at 40°C. A gradient binary mobile phase composed of (A) 0.1% (v/v) formic acid in water and (B) 0.1% (v/v) formic acid in methanol was set to a flow rate of 0.3 mL/min for a run time of 10 min. The gradient program started with 70% B for the first 0.5 min and changed to 90% B over 6.5 min. The initial composition was restored within the next 0.5 min and was held constant for an additional 2.5 min for equilibration.

A Valco® switching valve was used to deliver the flow to the column between time-points of 4 min and 7 min. At time periods of 0 to 4 min and 7 to 10 min, the flow was diverted into waste to decrease the contamination of the ion source and increase sensitivity. System control and data processing were achieved on Analyst® software, version 1.7.0 (AB Sciex, ON, Canada).

After HPLC separation, column effluent was directed to a Hybrid Triple Quadrupole-linear Ion trap mass spectrometer (AB Sciex 4000 QTRAP MS/MS system, Framingham, MA, US) equipped with a Turboionspray® interface. Quantitation was achieved in the multiple reaction monitoring (MRM) mode in positive ionization using electrospray ionization (ESI) source. Abundant and diagnostic ions were selected for each compound based on our previous MS/MS analysis study (20). The monitored precursor ion → product ion transitions for docetaxel as quantifier and qualifier transitions were *m/z* 808.4 → *m/z* 527.3 and *m/z* 808.4 → *m/z* 226.1, respectively. The monitored transition for paclitaxel was *m/z* 854.4 → *m/z* 569.3 and *m/z* (Table 1, **Error! Reference source not found.**).

The source temperature was set at 400°C, ion spray voltage (ISV) 5500V, curtain gas (CUR) 40, nebulizer gas (GS1) 40, heater gas pressure (GS2) 40 and an entrance potential of 10 was used for all transitions. The declustering potentials (DP), collision energy (CE), and collision cell exit potential (CXP) were optimized for each MRM transition to ensure ion abundance and stability (Table 1). Dwell time for all transitions was 50ms at unit resolution. The interface heater was on and nitrogen was used as the gas for all cases.

After the determination of the amount of drug encapsulated in the nanoparticles, drug loading and encapsulation efficiency of nanoparticles were calculated as follows:

$$\text{Drug loading (\%)} = \frac{\text{weight of the drug in the particles}}{\text{weight of the particles}} \times 100 \quad \text{(Equation 2)}$$

$$\text{Encapsulation efficiency (\%)} = \frac{\text{weight of drug in particles}}{\text{initial weight of drug added}} \times 100 \quad \text{(Equation 3)}$$

Table 1. MRM transitions for docetaxel and paclitaxel (IS) and optimized MS conditions.

	Docetaxel		**Paclitaxel**
	808.4 → 527.3 (Quantifier)	808.4 → 226.1 (Qualifier)	854.4 → 569.3
De-clustering potential (DP)	76	76	101
Collision energy (CE)	15	21	15
Cell exit potential (CXP)	14	14	16

2.4. Method validation

Validation of the method was conducted according to the Guidance for Bioanalytical Method Validation issued by the FDA (17) and the EMA Guidelines on Bioanalytical Method Validation (16).

2.4.1. Accuracy and precision

The four levels of QCs were used in sextuplicates during the same day (intra-day) and on three consecutive days (inter-day). For each sample, the accuracy was calculated as the difference in percentage between its measured and nominal concentrations. Precision was determined by calculating the coefficient of variation (CV).

To accept the accuracy from a single run, the mean calculated value should be within ±15% of the nominal value of each QC level, except the accuracy for LLOQ which can be within ±20%. The acceptance criteria for precision is a CV% of less than 15% for each QC level, except for the LLOQ where it should not be more than 20% (16, 17).

2.4.2. Linearity

An 8-point standard curve was constructed by plotting known concentrations of docetaxel in the range of 15.625 ng/mL to 4000 ng/mL against the peak area ratio of docetaxel to paclitaxel. A linear regression analysis was implemented. A double blank sample and a blank sample were also analyzed with each set of calibration standards. The regression coefficient (R^2), slope, intercept, and equation of the resulting standard curves were determined.

2.4.3. Limit of detection and limit of quantification

The standard that has a signal to noise ratio of 3:1 was considered as the limit of detection (LOD). The limit of quantitation (LOQ) of the method was measured based on a signal-to-noise ratio of 10:1 with the acceptable precision and accuracy (within ±20% of the nominal value).

2.4.4. Selectivity

To assess the method selectivity, double blank solution (n=6) was injected, and the peak area observed at channels of the analyte and the IS at their expected retention times were compared to the mean peak area of the LLOQ. The method was considered selective if the observed interference is less than 20% of the LLOQ for the analyte and less than 5% for the IS (16).

To ensure that there is no interference between the analyte and the IS, six blank solutions (an extracted solution containing only IS) were injected. The observed interference at the analyte channel was measured and compared to the mean peak area of the LLOQ. An interference of less than 20% of the LLOQ was considered acceptable (16). In addition, the transitions of the analyte and the IS were monitored in a blank sample (a matrix containing IS), and an analyte solution without the IS to observe any interference between the analyte and the IS.

2.4.5. Matrix effects

EMA guidelines were used to evaluate matrix effects (16). Peak areas were compared between post-spiked samples at two concentration levels (LQC and HQC) and samples prepared in neat solvent. The same procedure was performed for the IS at the concentration of 400 ng/mL.

Six extracted double blank samples were spiked with the analyte or the IS. An equivalent set of samples was also prepared in methanol (n=6 at each level of concentration). The matrix factor (MF) and the IS-normalized MF were calculated according to equations (4) and (5), respectively (16). The CV% was determined for each factor. Based on the EMA guidelines, the CV% of the IS-normalized MF should not be greater than 15%.

$$\text{Matrix Factor (MF)} = \frac{\text{Peak area}_{(\text{post-extraction spiked sample})}}{\text{Peak area}_{(\text{non-extracted neat solvent sample})}} \quad \text{(Equation 4)}$$

$$\text{IS-normalized MF} = \frac{\text{MF}_{\text{analyte}}}{\text{MF}_{\text{IS}}} \quad \text{(Equation 5)}$$

2.4.6. Extraction procedure efficiency

To determine the efficiency of the extraction method, the peak areas were compared between pre-spiked samples and samples prepared in neat solvent, at three concentration levels (LQC, MQC, and HQC). The same procedure was performed for the IS at a concentration of 400 ng/mL.
Six samples of drug-free nanoparticles were spiked with the analyte or the IS and extracted (section 2.3.2). An equivalent set of samples was also prepared in methanol (n=6 at each level of concentration). The extraction efficiency percentage was calculated using equation (6).

$$\text{Extraction Efficiency \%} = \frac{\text{Peak area}_{(\text{pre-extraction spiked sample})}}{\text{Peak area}_{(\text{non-extracted neat solvent sample})}} \times 100 \quad \text{(Equation 6)}$$

2.4.7. Extraction Recovery

To determine the extraction recovery, the peak areas were compared between pre-spiked samples and post-spiked samples, at three concentration levels (LQC, MQC, and HQC). The same procedure was performed for the IS at a concentration of 400 ng/mL.
Six double blank samples were spiked with the analyte or the IS. An equivalent set of samples was also prepared by spiking the drug-free nanoparticles with the analyte or the IS (n=6 at each QC level). The second set was also extracted and then analyzed with LC-MS. The extraction recovery percentage was calculated according to equation (7).

$$\text{Extraction Recovery \%} = \frac{\text{Peak area}_{(\text{pre-extraction spiked sample})}}{\text{Peak area}_{(\text{post-extraction spiked sample})}} \times 100 \quad \text{(Equation 7)}$$

2.4.8. Carry-over effects

The FDA and EMA guidelines were used to calculate the carry-over effects for the analyte (16, 17) and EMA guidelines were used to calculate this parameter for the IS (16). To minimize the carry-over effects, a double blank sample was injected after each highly concentrated sample (ULOQ, HQC, or unknown samples with an expected high concentration) (16). Equations 8 was used to assess the carry-over effect of the analyte and the IS. A double blank sample was injected after the ULOQ to determine the peak areas of the analyte and the IS.

$$\text{Carry-over Effect } \% = \frac{\text{Peak area of the double blank after ULOQ}}{\text{Peak area of the LLOQ}} \times 100 \quad \text{(Equation 8)}$$

Carry over was deemed insignificant if it was less than or equal to 20% of the LLOQ for the analyte (16, 17), and less than or equal to 5% of the LLOQ for the IS (16).

2.4.9. Dilution integrity

Dilution integrity was carried out to make sure that diluting samples did not affect the quantitative results. Nanoparticles were spiked with the IS (400 ng/mL) and docetaxel to yield a final concentration of 20,000 ng/mL. The sample was extracted (section 2.3.2) and diluted 10, 20, and 40-folds with the blank solution (n=6 at each dilution level). The test was accepted if the accuracy and precision were between 85% and 115% (16).

2.4.10. Stability tests

Stability tests were conducted at conditions encountered during analysis using QC samples to ensure that the steps taken during sample preparation and analysis have no effect on the stability of the analyte.

Although the analyte and the IS stock solutions were prepared freshly for each experiment, we assessed the stock solution stability stored at -20 C for future studies. A stock solution of the analyte was stored at $-20\,℃$ for one month and used to prepare two calibration standards (n= 6 at each level); four times the concentration of LLOQ (62.5 ng/mL) and 50% of ULOQ (2000

ng/mL) which were then analyzed against a freshly prepared standard curve. A sample was considered stable if its actual concentration was within ±15% of its nominal concentration (17).

To assess the stability of the IS stock solution, six samples were prepared at a concentration of 400 ng/mL form a one-month-old stock solution stored at −20 ℃. The peak areas of these samples were compared to the peak areas of the samples prepared from a fresh IS stock solution.

Autosampler stability was assessed by extracting (section 2.3.2) LQC and HQC samples (n=6 at each level) and analyzing them after being placed for 24 h in the autosampler at 4 ℃. The results were compared against a freshly prepared standard curve. Samples were considered stable if their actual concentrations were within ±15% of their nominal concentration (17).

One month stability at −20 ℃ was assessed by analyzing a set of six replicates of LQC and HQC samples stored at −20 ℃ for one month. Fresh IS solution was added, then samples were extracted (section 2.3.2) and analyzed against a freshly prepared standard curve. Samples were considered stable if accuracy was within ±15% of the nominal value (17).

2.5. Statistical analysis

Data were analyzed by descriptive statistics calculating the mean and standard deviation (mean±SD) for continuous variables. Analysis of variance (ANOVA) with multiple comparison (Tukey's and Pairwise Comparison) tests were performed to demonstrate statistical difference at an α-level of 0.05 using SPSS software. The paired student's t-test were used to evaluate the differences when we had two groups with a level of significance set at $P<0.05$.

3. Results and discussion

Nano-encapsulation is one of the most studied methods to overcome the side effects of chemotherapeutic agents, such as docetaxel used in the treatment of breast cancer (4). In order to prepare nanoparticle formulations with optimum particle size and yield percentage, it was essential to adjust different parameters such as Polyvinyl alcohol (PVA) solution volume, PLGA amount, centrifugation force and time, and ultra-sonication amplitude. In addition, a quantification method was needed to measure the entrapped drug within the nanoparticles. Therefore, a liquid chromatography (LC)-MS/MS method was developed to quantify the encapsulated drug using a phenyl column for the first time.

3.1. Preparation and characterization of the nanoparticles

There are two main steps in the preparation of polymeric nanoparticles using the solvent evaporation technique (21). The first step is to form an o/w emulsion by dispersing an organic phase (polymer dissolved in an organic solvent) in an aqueous phase (surfactant dissolved in water). The second step involves the subsequent evaporation of the organic solvent to form a nanoparticle suspension. A variety of organic solvents, such as ethyl acetate (22) and chloroform (23) have been used to prepare polymeric nanocarriers. In this work, we evaluated the use of these two organic solvents to choose the optimal one for the preparation of PLGA nanoparticles. Measurement of the size of the plain formulations prepared using chloroform showed larger particles (198.0 nm) than the nanoparticles that were prepared by ethyl acetate (170.2 nm). Chloroform has higher toxicity than ethyl acetate (24), meaning that ethyl acetate is a more favorable solvent than chloroform. Therefore, ethyl acetate was chosen as the organic solvent to prepare the nanoparticles due to its relatively high safety in comparison to chloroform, as well as its ability to produce smaller particles.

Changes in the production procedure can change the characteristics of the resulting nanoparticle formulations. In order to optimize the preparation process of the PLGA nanoparticles, different technical parameters were investigated to reach an optimum formulation with the lowest particle size and highest yield percentage. Twenty-seven different formulations were assessed (data not shown) to evaluate the effects of centrifugation force and sonication amplitude on the particle size and yield of the nanoparticles.

The condition for the production of the formulation with the optimum size and yield percentage was PLGA 16.7 % (% w/v in oil phase), PVA 2.2 % (% w/v in aqueous phase), 2 minutes of ultra-sonication, and freeze-drying using 1.7% sucrose solution. The optimum drug-free formulation had a z-average of 170.2 ± 4.2 nm and a poly-dispersity Index of 0.274 ± 0.032. Z-average is the mean hydrodynamic size of the collection of particles in a suspension measured by dynamic light scattering (25), and Poly-dispersity Index (PdI) is a measure of heterogeneity of size and shows the size distribution of the particles in a sample . A suspension with a PdI of more than 0.7 is commonly considered to have a broad size distribution of particles (25); therefore, the prepared formulations had a narrow and acceptable size distribution. After freeze-drying (FD), the particle size and PdI values changed to 232.0 ± 5.1 nm and 0.289 ± 0.025, respectively. The formulation yield percentage was measured to be 63.8%. Drug-loaded nanoparticles showed a larger mean

particle size of 202.2±4.7 nm and a PdI of 0.237±0.005 before FD. The freeze-dried nanoparticles had also a larger particle size (251.7±8.2 nm) and PdI (0.320±0.038) than plain nanoparticles. The *in vivo* fate of a nano-sized particle is highly dependent on its size (26). For example, the size of a nanoparticle has a strong correlation with its recognition by the macrophages (27) or its pharmacokinetic properties (28). Very small-sized particles (less than 20-30 nm) will undergo renal excretion and get eliminated quickly from the body (29) before exerting their therapeutic effects, while large particles are taken up by macrophages remarkably higher compared to smaller particles (30). In this study, the aim was to prepare nanoparticles with a size around 250 nm to avoid high phagocytosis by the macrophages, while maintaining the ability of the nanoparticles to cross the discontinuous endothelium of the tumor vessels with fenestration sizes of 200-780 nm (31). The intracellular uptake of the nanoparticles with a size of around 500 nm has been confirmed in many studies (32).

The plain nanoparticles showed a zeta potential of -23.2 ± 1.4 mV, and the drug loaded nanoparticles had a zeta potential of -22.5 ± 0.8 mV. This surface charge will allow for long circulation times in the blood (33). In addition, the medium-range zeta potential of the particles led to the formation of a colloidal suspension with a stability enough for the purpose of the study. Saxena et al. reported zeta potential values ranging from -7.2 ± 1.0 to -16.3 ± 1.5 for indocyanine green-loaded PLGA nanoparticles (34). Statistical analysis of the zeta potential measurements showed no significant difference between plain and docetaxel-loaded nanoparticles or before and after FD.

3.2. LC-MS/MS method development

The formation of an o/w emulsion is the first step towards the production of nanoparticles using the solvent evaporation technique. It means that drug molecules would be partitioned between the organic and the aqueous phases with the latter containing a surfactant. After the evaporation of the solvent and the formation of a nano-suspension, the drug molecules whichhave been solubilized in the aqueous phase will be removed in the washing step (i.e., the ultracentrifugation process). Therefore, only drug molecules that enter the organic phase will have the chance to become encapsulated in the nanoparticles. As a result of losing some drug molecules, the loading efficiency of an emulsion-based preparation is always below 100%. To further biologically assess the produced PLGA nanoparticles, there is a need to measure the amount of the drug that has been entrapped within the nanoparticles, meaning that a quantification method needs to be established.

A LC-MS/MS method was developed for the quantification of docetaxel in the nanoparticle matrix using the MRM mode to attain the needed specificity and selectivity. Paclitaxel, a structural analogue of docetaxel, was used as the IS at a final concentration of 400 ng/mL. IS was added to all standard curve, quality control (QC), and experimental samples prior to analysis.

Based on our recent investigation of the MS/MS spectra and the fragmentation patterns of docetaxel and paclitaxel (20), two diagnostic and relatively abundant product ions were selected and monitored to ensure the identity of the target compounds. **Error! Reference source not found.** Figure 2 shows the structures of the selected product ions and the monitored MRMs. Different ratios of methanol and water were evaluated to examine the possibility of having an isocratic elution during method development. The efforts were not successful because the analyte eluted from the column very early (< 1 min) when using an isocratic solvent system (data not shown). Since a reverse-phase stationary column was used, water-soluble impurities eluted from the column at the beginning of the chromatographic run, resulting in a significant baseline noise that interferes with the analysis. Therefore, a gradient solvent system is adopted to ensure adequate separation from interferences. To account for the matrix effects and the variations in the ion current response observed when using ESI, paclitaxel was used as the IS in the analysis. As seen in Figure 2, the IS eluted from the phenyl column shortly after the analyte meaning that the two compounds were subjected to very similar chromatographic conditions. This justifies choosing paclitaxel, a structural analogue, as the IS for the analysis of docetaxel. In addition, the peaks of the analyte and IS were observed at the mid-range of the run, which allowed for directing the chromatographic flow to waste from time 0 to 4 min protecting the MS instrument from unwanted impurities, yielding a low limit of detection (LOD).

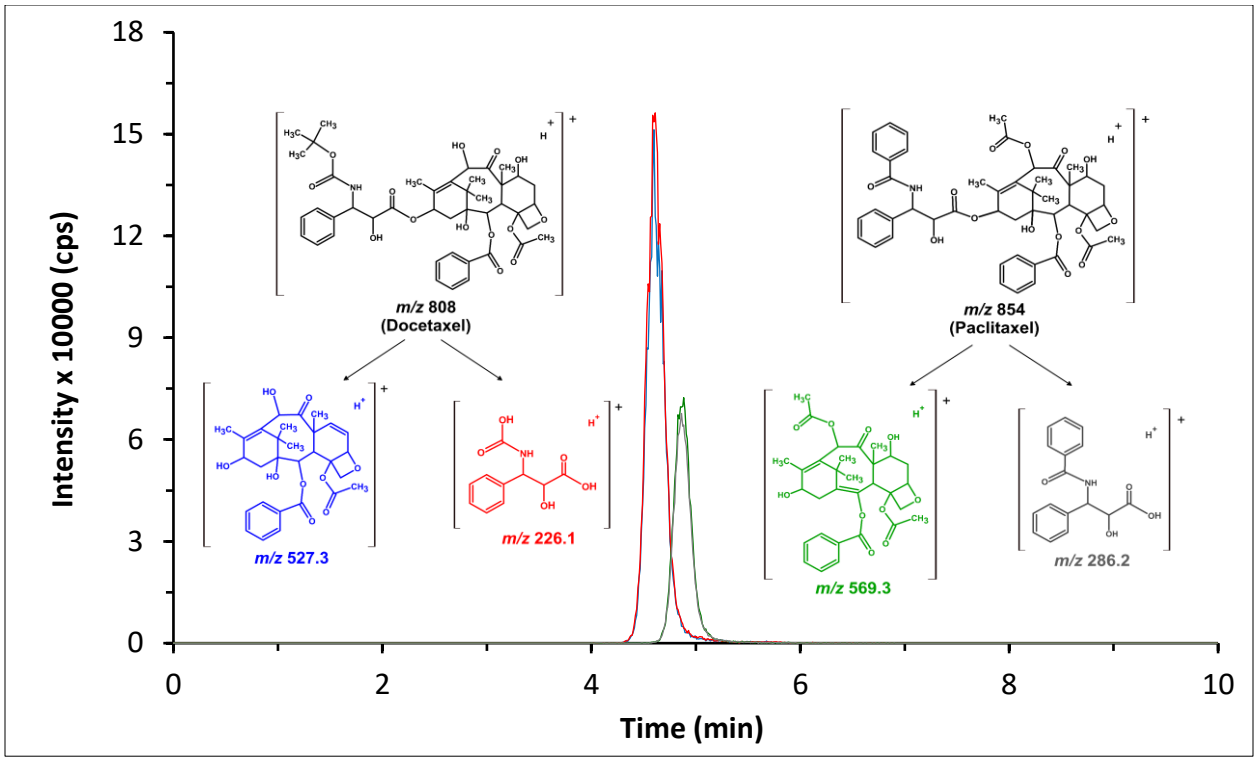

Figure 2. Representative LC-MS/MS chromatogram of a calibration standard sample (1000 ng/mL) and the structures of the monitored ions with the analyte (Docetaxel) and its IS (Paclitaxel).

Despite its potential benefits, a phenyl column has never been used to quantify docetaxel. In a previous study in our lab (19), docetaxel was quantified in a PLGA nanoparticle matrix using a flow-injection-MS/MS (FIA-MS/MS) method. The LOD and LLOQ were 62.5 ng/mL and 125 ng/mL, respectively. A phenyl column, on the other hand, significantly enhanced the sensitivity of the quantification and resulted in a LOD and LLOQ values of 4 ng/mL and 15.6 ng/mL, respectively. This difference between the two methods can be attributed to the effective separation capability of the phenyl column. Smaller LLOQ values will help quantify the amount of encapsulated docetaxel in formulations with low loading efficiency. In addition, the method can be adopted as a starting point for biological assessment of the formulation in which sensitivity will inherently be needed.

3.3. Method validation

3.3.1. Accuracy and precision

Intraday precision and accuracy were determined by analyzing four sets of QCs (LLOQ, LQC, MQC, and HQC) in three separate days, with six replicates at each QC level. Accuracy was

calculated as the percent of the theoretical value. The calculated accuracies ranged from 101.2 to 117.1% for LLOQ and 90.4 to 112.0 for other QCs (Table 2). The precision, reported as CV%, did not exceed 15% for the four QCs as reported in Table 2.

The inter-day accuracy and precision, using 18 replicates of each QC level, yielded accuracy ranging from 103.1 to 107.7% and a precision between 7.0 and 10.9% (Table 3).

Table 2. Intra-day accuracy and precision.

QC	Replicates	Analysis day (#)	Observed Concentration (mean ± SD; ng/mL)	Precision (CV%)	Accuracy (%)
LLOQ	6	1	16.37 ± 2.16	13.2	104.8
(15.625 ng/mL)	6	2	15.81 ± 1.86	11.8	101.2
	6	3	18.30 ± 178	9.7	117.1
LQC	6	1	43.50 ± 3.54	8.1	108.8
(40 ng/mL)	6	2	43.97 ± 5.42	12.3	109.9
	6	3	38.63 ± 5.52	14.3	96.5
MQC	6	1	1648.45 ± 87.50	5.3	109.9
(1500 ng/mL)	6	2	1402.99 ± 163.12	11.6	93.5
	6	3	1650.37 ± 48.06	2.9	110.0
HQC	6	1	3360.56 ± 268.54	8.0	112.0
(3000 ng/mL)	6	2	2712.13 ± 403.29	14.9	90.4
	6	3	3210.41 ± 214.91	6.7	107.1

Table 3. Inter-day accuracy and precision.

QC	Concentration (ng/mL)	Replicates	Observed Concentration (mean ± SD; ng/mL)	Precision (CV%)	Accuracy (%)
LLOQ	15.625	18	16.83 ± 1.31	7.8	107.7
LQC	40	18	42.03 ± 2.95	7.0	105.1

MQC	1500	18	1567.27 ± 142.27	9.1	104.5
HQC	3000	18	3094.37 ± 339.43	10.9	103.1

3.3.2. Linearity

A standard curve with eight calibration standard points was constructed by plotting the peak area ratio of the analyte to IS versus the analyte concentration and a linear regression analysis was performed to fit a linear model to the data with a weighting factor of $1/x^2$. Figures S1 to S3 (Supplementary Data) show the standard curves generated on days one to three of validation as well as their residual plots before and after the application of the weighting factor. A series of concentrations from 1 to 16000 ng/mL were analyzed to identify the linear range. Linearity was attained from 15.6 ng/mL to 4000 ng/mL (quantification range) and the average correlation coefficient was found to be 0.9984 (n=3). The LOD was 4 at ng/mL.

3.3.3. Selectivity, matrix effects, and carry-over

The assessment of selectivity of the method for docetaxel and paclitaxel as per regulatory requirements (17) showed that the interference observed at the analyte channel was 4.16 ± 2.33 % of the LLOQ (for the analyte) and the interference at the IS channel was 0.07 ± 0.02 %, which is within the acceptable range of below 20% and 5% of the LLOQ, respectively.

The analysis of double blank revealed no interferences in the analyte channel or the IS channel (Supplementary Data, Figure S4) at the retention time of the analyte or IS. Monitoring the transitions of the analyte and the IS in a blank sample that is a matrix containing IS, and an analyte solution without the IS showed no interference between the analyte and the IS (Supplementary Data, Figures S5 and S6).

The matrix factor (MF) experiment was performed in replicates of 6 and the calculated MF (0.6 at three QC levels) showed ion suppression (Supplementary data, Table S1). Ion suppression, in this case, could be attributed to the competition for charge between the analyte and the co-eluting species during ionization. However, the observed matrix effect did not undermine the quantification of the analyte for the intended application with the needed high sensitivity. It can

be argued that matrix effects are balanced with the high extraction recovery percentage (97.98% ± 5.27 at LQC level and 100.28% ± 5.40 at HQC level, **Error! Reference source not found.**).

Table 4. Evaluation of the extraction recovery percentage of the developed HPLC-MS/MS method for the quantification of docetaxel in the nanoparticle matrix at three QC levels (LQC, MQC, HQC) and IS level.

	Extraction recovery %	**CV%**
LQC (n=6)	97.98 ± 5.27	5.4
MQC (n=6)	81.67 ± 10.50	12.8
HQC (n=6)	100.28 ± 5.40	5.4
IS (n=6)	87.32 ± 3.92	4.5

To minimize carry over, a needle-washing step with methanol was added between injections to avoid cross-contamination between samples. In addition, a double blank sample was injected after each sample with a high concentration, namely ULOQ, HQC, and expected experimental samples with high concentration as recommended by the EMA guidelines (16). The carry-over effect after the ULOQ was calculated to be 15.698 ± 0.653 % of the LLOQ for the analyte and 4.888×10^{-5} % ± 3.711×10^{-7} for the IS, indicating negligible carry-over effects during the analysis of a samples, meeting regulatory guidelines (16).

3.3.4. Extraction efficiency and recovery

Extraction efficiency values ranged from 60.70% to 71.73% at three QC levels (**Error! Reference source not found.**). Statistical analysis showed no significant differences between the three tested concentrations.

Table 5. Evaluation of the extraction efficiency percentage of the developed HPLC-MS/MS method for the quantification of docetaxel in the nanoparticle matrix at three QC levels (LQC, MQC, HQC) and IS level.

	Extraction Efficiency %	**CV%**
LQC (n=6)	71.73 ± 8.44	11.8
MQC (n=6)	60.70 ± 7.58	12.5

HQC (n=6)	68.78 ± 4.34	6.3
IS (n=6)	89.81 ± 9.60	10.7

In addition to extraction efficiency, the evaluation of recovery (Equation 7) is important since the extraction efficiency does not take matrix effects into account. As shown in **Error! Reference source not found.**, the extraction recovery percentage ranged from 81.67% to 100.28% across all QC levels. The difference between the observed extraction recoveries was not statistically significant. Such high recovery values show the high efficiency of the extraction method and ensures that the majority of the analyte is extracted from the matrix. A higher recovery percentage contributes to the quantification of lower analyte concentrations.

3.3.5. Dilution integrity

Three different dilution factors were chosen to ensure that the dilution integrity test will cover the dilution applied to the experimental samples (Table 6). All tested samples met the FDA guidelines for accuracy and precision of ±15% for accuracy and CV% (17) . Since the samples with an unknown concentration were 20-fold diluted with blank, the observed results indicate that diluting the samples does not affect the analytical results during the analysis of the nanoparticle formulations loaded with docetaxel.

Table 6. Evaluation of dilution integrity using double blank for the developed HPLC-MS/MS method for the quantification of docetaxel in the nanoparticle matrix at three levels (10-, 20-, and 40-times dilution).

	Replicates	**Observed Concentration (mean±SD; ng/mL)**	**Precision (CV%)**	**Accuracy (%)**
10 × Diluted (2000 ng/mL)	6	2215.17± 201.39	9.1	110.8
20 × Diluted (1000 ng/mL)	6	923.33 ± 137.6	14.9	92.3
40 × Diluted (500 ng/mL)	6	504.16 ± 70.01	13.9	102.1

3.3.6. Stability evaluation

Stability tests were designed and performed based on the conditions that were encountered during sample handling and analysis. For example, a freeze-thaw test was not necessary because the samples were stored in the freezer in a powder form. All the prepared samples for the stock solution stability test (at two concentration levels for the analyte, i.e. 4×LLOQ and 50% of ULOQ, and one concentration for the IS, i.e. 400 ng/mL) met the accuracy limits as specified in the FDA guidelines (17) (Table 7). This implies that the stock solutions of docetaxel and paclitaxel can be stored at -20℃ for one month and remain stable.

Auto-sampler stability for 24h at 4℃ passed the required accuracy limits showing that the analyte was stable during data acquisition (Table 7). In addition, storing LQC and HQC samples at -20℃ for one month before extraction showed accurate measurement of the concentration of the samples with accuracy values of 112.4% and 95.5%, respectively. This indicates that nanoparticle samples can be stored at the mentioned conditions without any concerns of analyte degradation.

Table 7. Stability data of the developed method in different conditions used for the preparation and HPLC-MS/MS analysis of the docetaxel-loaded nanoparticles.

Stability test	Condition	Sample (n=6)	Concentration (ng/mL)	Observed Concentration (mean ± SD; ng/mL)	Accuracy (%)
Stock solution stability	30 days, −20 ℃	4×LLOQ	62.5	59.43 ± 6.05	95.1
		50% of ULOQ	2000	2200 ± 207.65	110
		IS	400	358.83 ± 37.55	89.7
Auto-sampler stability	24 h, 4 ℃	LQC	40	34.53 ± 3.55	86.3
		HQC	3000	2745.17 ± 168.76	91.5
Storage stability	30 days, −20 ℃	LQC	40	44.97 ± 8.47	112.4
		HQC	3000	2864.50 ± 274.21	95.5

3.3.7. Application of the method for the quantification of docetaxel in nanoparticle formulation

The developed LC-MS/MS method was applied for the quantification of docetaxel in the optimized PLGA nanoparticles. The concentration of the drug was determined in the extracted samples of the nanoparticle formulation and the encapsulation efficiency of the formulation was calculated to be 52.28% ± 10.18 (n=4). This means that the developed method for the preparation of PLGA nanoparticles was able to encapsulate 52.28% of the initial amount of docetaxel with which the preparation was started. The drug loading (%) was determined to be 4.28% ± 0.27 indicating that the loaded docetaxel accounts for 4.28% of the weight of the final nanoparticle formulation. The drug loading results were comparable to the results of other studies using polymeric nanoparticles as a docetaxel delivery system (35). The formation of an o/w emulsion is the first step towards the production of nanoparticles using the solvent evaporation technique. It means that the drug molecules would be partitioned between the organic and the aqueous phases with the latter containing a surfactant. After the evaporation of the solvent and the formation of a nano-suspension, the drug molecules that have been solubilized in the aqueous phase with the aid of the surfactants will be removed in the washing step (ultracentrifugation process). Therefore, only drug molecules that enter the organic phase will have the chance to become encapsulated in the nanoparticles. As a result of losing some drug molecules, the loading efficiency of a preparation method cannot be 100%. High loading efficiency of the prepared nanoparticles will allow for the injection of low amounts of the nanoparticles in *in vivo* studies in the future. Injecting a lower amount of nanoparticle formulation has the advantage of decreasing the injection volume, lowering the viscosity of the mixture, and reducing the adverse effects caused by possible impurity residues in the formulation (such as PVA or free drug).

4. Conclusion

Several nanoparticle formulations were prepared to optimize the conditions for the fabrication of the docetaxel loaded PLGA nanoparticles. A new and reliable LC-MS/MS method was developed for the quantification of the encapsulated drug. A phenyl column was applied as the LC column for the first time; and the chromatogram showed a desirable close elution of the IS and the analyte. In addition, the limit of detection and the limit of quantification was lower compared to the previously developed flow-injection method and a broader linearity range was achieved in comparison with other LC-MS studies. The method was fully validated in agreement with the FDA and EMA guidelines and ensured the sensitive, robust, and reliable quantification of the docetaxel in a polymeric matrix.

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Conflict of interests

The authors have no conflict of interests to declare.

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