

DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR QUANTIFICATION OF DOCETAXEL IN PALM-BASED NANOEMULSION AEROSOLS

(Pembangunan dan Validasi Kaedah KCPT untuk Kuantifikasi Docetaxel di dalam Nanoemulsi
Aerosol Berasaskan Sawit)

Azren Aida Asmawi¹, Norazlinaliza Salim^{1,2}, Emilia Abdulmalek^{1,2}, Mohd Basyaruddin Abdul Rahman^{1,2*}

¹*Integrated Chemical BioPhysics Research, Faculty of Science*

²*Department of Chemistry, Faculty of Science
Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*

*Corresponding author: basya@upm.edu.my

Received: 20 November 2019; Accepted: 3 March 2020

Abstract

Lung cancer is the leading cause of cancer-related deaths worldwide. Among the most potent of chemotherapeutic drugs used for lung cancer treatment are the taxanes, including docetaxel. However, the drugs' efficacy in combating the disease is reduced due to their poor solubility, low stability and high toxicity. Inhalation therapy of lipid-based carrier has great potential in direct target towards the respiratory diseases. Hence, application of aerosolized palm-based nanoemulsion system is one approach to alleviate the drawbacks. This study describes the development and validation of method that can quantitate the amount of docetaxel nanoemulsion formulation via high-performance liquid chromatography (HPLC). Chromatographic analysis was conducted using a reversed phase C₁₈ column with a mixture of acetonitrile and water (50:50) adjusted to pH 3.0 as the mobile phase. The flow rate was 1.0 mL/min and the detection was carried out by ultraviolet-visible detector at 228 nm. The developed method was validated in terms of specificity, linearity, accuracy and precision accordance with the International Conference on Harmonization (ICH) guidelines. The calibration curve was linear ($r^2 = 0.999$) over the concentration range from 62.5 to 1000 µg/mL with lower limit of detection (LOD) of 9.88 µg/mL and lower limit of quantification (LOQ) of 29.93 µg/mL. The percentage relative standard deviation for both intra and inter-day precision was less than 2%, while percentage recovery was more than 90%, indicating the precision and accuracy of the study. The developed HPLC method was proved as suitable and reliable for its intended application.

Keywords: aerosols, docetaxel, high-performance liquid chromatography, nanoemulsion, lung cancer

Abstrak

Kanser paru-paru adalah punca utama kematian akibat kanser di seluruh dunia. Antara ubat kemoterapi yang berkesan digunakan untuk rawatan kanser paru-paru ialah taxane, termasuk docetaxel. Walau bagaimanapun, keberkesanan ubat dalam merawat penyakit ini berkurangan disebabkan oleh kelarutannya yang kurang baik, kestabilan yang rendah dan ketoksikan yang tinggi. Oleh itu, penggunaan sistem nanoemulsi berasaskan sawit adalah satu pendekatan untuk mengurangkan kelemahan tersebut. Kajian ini menerangkan pembangunan dan validasi yang boleh mengkuantifikasi jumlah docetaxel di dalam rumusan nanoemulsi melalui kaedah kromatografi cecair prestasi tinggi (KCPT). Analisis kromatografi dijalankan menggunakan turus fasa terbalik C₁₈ dengan campuran asetonitril dan air (50:50) diselaraskan kepada pH 3.0 sebagai fasa bergerak. Kadar aliran adalah 1.0 mL/min dan pengesanan dilakukan oleh pengesan ultralembayung-nampak pada 228 nm. Kaedah yang dibangunkan telah disahkan dari segi kekhususan, kelinearan, kejutuan, ketepatan dan kepersisan berdasarkan garis panduan persidangan pengharmonian antarabangsa (ICH). Plot tentukan adalah linear ($r^2 = 0.999$) di atas kepekatan antara 62.5 hingga 1000 µg/mL dengan had pengesanan (LOD) iaitu 9.88 µg/mL dan had kuantifikasi (LOQ) sebanyak 29.93 µg/mL. Peratusan sisihan piawai relatif untuk kedua-dua kepersisan intra dan inter-hari adalah kurang daripada 2%, manakala purata kejutuan adalah lebih

daripada 90%, menunjukkan kepersisan dan ketepatan kajian. Kaedah KCPT yang dibangunkan terbukti sesuai dan boleh digunakan untuk aplikasi yang dirancang.

Kata kunci: aerosol, docetaxel, kromatografi cecair prestasi tinggi, nanoemulsi, kanser paru-paru

Introduction

Docetaxel (DTX) is a chemotherapeutic drug derived from a natural inactive precursor of 10-deacetyl baccatin III, extracted from the Yew plant (*Taxus* genus). DTX is approved by the Food and Drug Administration (FDA) for the treatment of non-small-cell lung cancer (NSCLC) as a mitosis inhibitor in cell division *via* stabilization of the polymerization and de-polymerization of microtubules [1, 2]. However, poor water solubility, low stability and high toxicity of DTX has reduced its therapeutic index in combating the disease [3, 4]. Therefore, increasing the bioavailability and minimizing the side effects of DTX remain challenges in the lung cancer therapy.

Inhalation therapy of lipid-based carrier system has gained considerable attention for local non-invasive drug delivery. This system is a suitable alternative to deliver high local drug concentration to the desired region in the lungs, while significantly reduces the toxicity towards healthy cells [5–7]. Hence, research focus has shifted to the use of encapsulated DTX in palm-based nanoemulsion aerosols for pulmonary drug delivery to overcome the challenges. Investigations on aerosolization properties of the formulation using multistage cascade impactor was essential to quantitate the amount of DTX deposited and to predict the site of deposition in the lungs' region.

In this paper, the development and validation of a simple, precise and accurate reversed-phase high performance liquid chromatography-ultra violet (RP-HPLC-UV) analytical method to detect DTX in palm-based nanoemulsion aerosols was investigated. The validation method was accomplished according to the International Conference on Harmonisation (ICH) Q2 (R1) guidelines. Additionally, the method can be used for quantification of DTX in other type of nanoparticles.

Materials and Methods

Materials

Docetaxel (DTX) (98% purity) and trifluoroacetic acid (99% extra purity) were purchased from Acros Organics, UK. Lecithin Lipoid S75 was purchased from Lipoid via GmbH, Germany. Safflower seed oil, Tween® 85 and Span® 85 were purchased from Sigma-Aldrich, Germany. Palm kernel oil esters (PKOEs) was synthesized enzymatic alcoholysis of oleyl alcohol and palm kernel oil (3:1) catalysed by Lipzyme RM IM [8]. HPLC-grade acetonitrile and glycerol were purchased from J.T. Baker, USA while HPLC-grade methanol was purchased from Merck, Germany. Deionized water was generated using Milli-Q gradient water system (Billerica, USA).

Instrument and chromatographic conditions

Chromatographic analysis for detection of DTX was performed using the high-performance liquid chromatography (HPLC) (Waters, USA) equipped with Breeze software, dual GM150 gradient mixers in the 1525 pump, waters 2489 UV/Visible detector and C₁₈ analytical column with particle size of 5 µm and dimension of 4.6 x 150 mm (Eclipse plus, Agilent Technologies, USA). The mobile phase was composed of filtered acetonitrile and deionized water (50:50, v/v) adjusted to pH 3.0 with trifluoroacetic acid. The analysis was performed at room temperature by isocratic elution at flow rate of 1.0 mL/min, with UV-vis detector set at 228 nm and injection volume of 10 µL. The sample solutions were filtered using polyvinylidene difluoride (PVDF) 0.45 µm prior to injection.

Preparation of nanoemulsion sample

A stable of DTX-loaded nanoemulsion was prepared with 0.1% DTX, 5.0 wt.% palm kernel oil esters (PKOEs) and safflower seed oil (9:1, w/w), 2.5 wt.% lecithin, 2.0 wt.% Tween® 85 and Span® 85 (9:1), 2.5 wt.% glycerol, and 87.9 wt.% water using high energy emulsification techniques. Lecithin was first dissolved in a mixture of PKOEs and safflower seed oil at 40 °C for 30 minutes before being mixed with DTX and mixture of Tween 85 and Span 85 for 30 minutes. The coarse emulsion was prepared by adding the oil phase dropwise into the aqueous phase containing deionized water and glycerol for 30 minutes at 350 rpm using overhead stirrer. The coarse emulsion was then further subjected to high shear homogenizer for 15 minutes at 12,000 rpm.

Preparation of standard solution

Stock solution of DTX was prepared by accurately weighed 10 mg of DTX and was added into a 5 mL volumetric flask. The flask was filled and dissolved with methanol to the mark to get a 2000 µg/mL stock concentration. The two-fold serial dilution factor in the range 62.5-1000 µg/mL was prepared by serially reducing the concentration by one half. The HPLC methods in quantification of DTX solutions were validated using specificity, linearity, sensitivity, repeatability and accuracy tests according to the guidelines from International Conference on Harmonisation.

Specificity and system suitability

Specificity is the ability of the developed method to distinguish and separate analyte and other component in the sample matrix [9]. A volume of 0.5 mL blank nanoemulsion formulation was spiked with 0.5 mL of DTX solution at concentration of 125 µg/mL. The mixture was vortexed vigorously and analysed using HPLC method as described above. The system suitability including asymmetric peak, tailing factor and number of theoretical plates were determined.

Linearity test

Five standard concentrations of 62.5, 125, 250, 500 and 1000 µg/mL were used in the preparation of standard plot of DTX. Three replicates (n = 3) at each concentration of DTX were analysed using HPLC method as described above. The slope and intercept of calibration plot was calculated for regression analysis in order to determine the linearity of the plot and the significance of linear regression was confirmed by one-way ANOVA test.

Sensitivity test

The sensitivity for detection of DTX was determined by evaluating the limit of detection (LOD) and limit of quantification (LOQ) of the analyte. According to ICH guidelines, the LOD of the analyte response is the lowest amount of analyte in the sample can be detected while the analyte response (signal) at the LOQ should be at least 10 times the response compared to blank response (noise) with suitable accuracy and precision. The LOD and LOQ of the response analyte were calculated using the following equations:

$$\text{LOD} = 3.3 \times \sigma/S \quad (1)$$

$$\text{LOQ} = 10 \times \sigma/S \quad (2)$$

where, σ is the standard deviation of intercept of calibration plot and S is the average of the slope of the corresponding calibration plot.

Precision and repeatability test

Intra-day (n = 3) and inter-day (n = 3) variation studies were determined by evaluating precision and repeatability over one and three days, respectively. Standard concentrations of DTX were prepared accordance with the method as described in preparation of standard solution section and were analysed using HPLC method as described above. The percentage of coefficient of variation (%CV) was calculated for each sample analysed in order to evaluate the closeness of the analytes response obtained from the same measurement procedures and conditions. The %CV was calculated using the following formula:

$$\%CV = (\sigma/\mu) \times 100 \quad (3)$$

where, σ is the standard deviation and μ is the sample mean.

Accuracy test

The recovery studies were carried out in triplicates by adding 62.5 µg/mL of analyte to four standard concentrations of 125, 250, 500 and 1000 µg/mL; thus, the final nominal concentrations were 93.75, 156.25, 281.25 and 531.25 µg/mL. The concentration of DTX in prepared solutions was analysed using HPLC method as described above and the mean percent recovery was then calculated. According to the ICH guidelines, accuracy is the closeness of agreement between the analyte of known concentration and the values found. The acceptance mean value should be within 15% of the nominal values.

Cascade impactor analysis

The evaluation of aerosolization performance of the palm-based nanoemulsion formulation was performed using eight –stage Andersen cascade impactor (ACI; Copley Scientific Ltd, UK). A sample of 3 mL was nebulized via OMRON MicroAIR nebulizer into the ACI that connected to vacuum pump with flow rate of 15 L/min on a continuous mode. The drug content on each stage of ACI including inlet cone, induction port, mouthpiece and adapter were quantitated using HPLC method as described above. The aerosolization properties including emitted dose (ED), deposited dose (DD) and fine particle dose less than 5 μm ($\text{FPD}_{<5\ \mu\text{m}}$) were calculated. The ED depicted as the cumulative percentage of DTX content collected from induction port, all stages (0 – 7) and the filter, while DD was the cumulative percentage of DTX content deposited on all stages (0 – 7) and the filter. The $\text{FPD}_{<5\ \mu\text{m}}$ was the percentage of DTX deposited on stage 3 to filter.

Results and Discussion

The HPLC method was developed to select chromatographic conditions including stationary phase, mobile phase and wavelength for detection. For this purpose, preliminary trials were performed by varying the conditions. After the optimization, the chromatographic conditions as described in previous section were used for the method validation. Specificity was indicated by the absence of any significant interfering peak in the chromatograms of blank sample and blank sample spiked with the analyte. From the chromatogram of blank palm-based nanoemulsion sample, selectivity was proved as no interfering endogenous peaks were found at the retention time of DTX, which was eluted at 3.12 minutes (Figure 1). The asymmetry (A_s) of DTX peak was found to be nearly symmetrical in shape with A_s value close to 1 and tailing factor value equal to 1 representing a Gaussian peak distribution [10]. Moreover, the number of theoretical plates which is used to measure column efficiency was higher than 1000. The results of system suitability are summarized in Table 1.

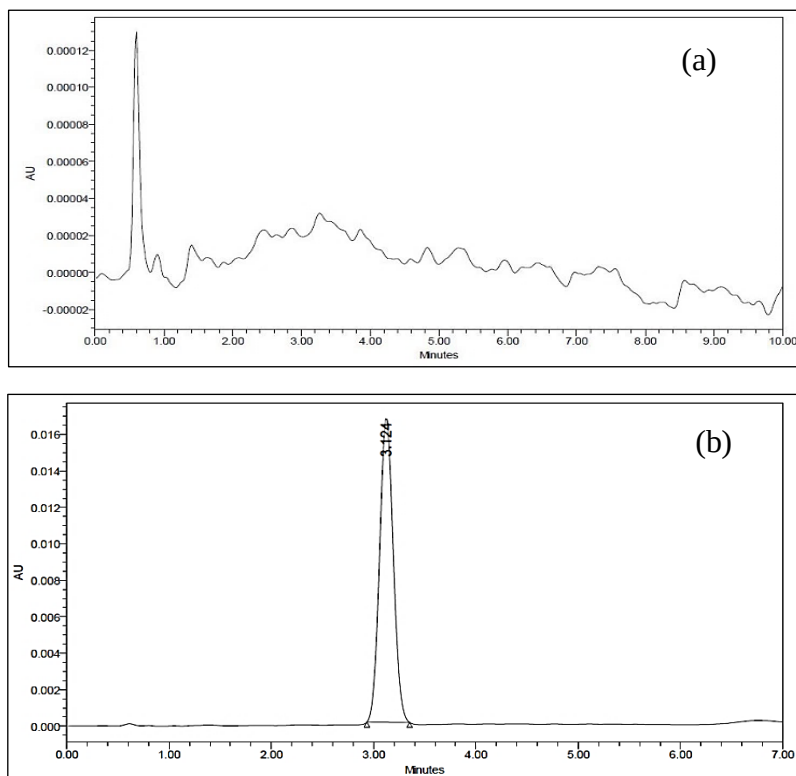


Figure 1. Chromatogram for (a) blank palm-based nanoemulsion sample and, (b) blank palm-based nanoemulsion sample spiked with DTX at 62.5 $\mu\text{g/mL}$

Table 1. Results of system suitability

Validation Parameter	
Retention time (min)	3.12
Asymmetry of peak	1.01
Tailing factor	1.00
Number of theoretical plates	1936

Calibration curve was constructed using the five-standard concentration of DTX in the range of 62.5 µg/mL and 1000 µg/mL against the mean peak area of DTX. A good linear relationship with equation of $y = 624.94x - 341.33$ was established between the peak area and DTX concentrations over the range tested (Figure 2). It was confirmed by the regression correlation coefficient value close to 1 ($R^2 = 0.9999$) indicating of strong positive linear correlation between the DTX concentration and the obtained peak area. ANOVA analysis also showed that the positive regression model is statistically significant ($p < 0.05$) to predict the response. The model F value also implies that the model is significant and less than 0.01% chance that the model F -value could occur due to noise (Table 2). The LOD and LOQ of DTX determined from the standard deviation of the regression line were found to be 9.88 µg/mL and 29.93 µg/mL, respectively.

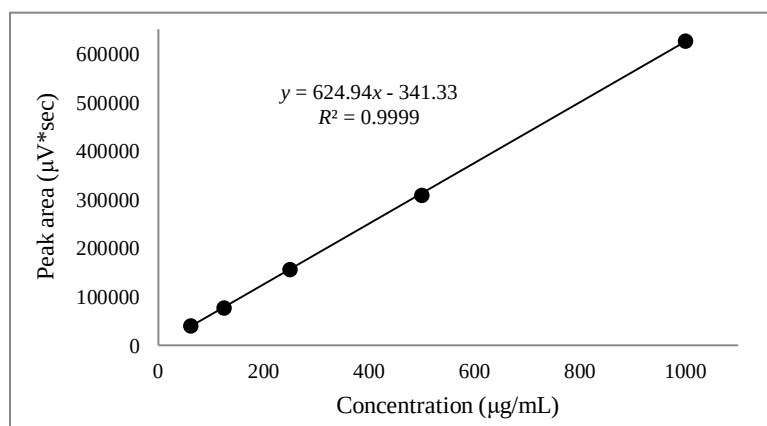


Figure 2. Calibration curve of DTX in concentration range of 62.5 – 1000 µg/mL

Table 2. Results of ANOVA analysis for calibration curve of DTX

ANOVA ^b	Sum of squares	df	Mean square	F	Significance
Regression	2.28E11	1	2.28E11	1.49E4	<0.0001 ^a
Residual	4.58E6	3	1.53E6		
Total	2.28E11	4			

^aPredictor: (constant), conc, ^bdependent variable: area

The intra-day and inter-day precision and repeatability for DTX were both found to be between 0.8-1.9%. In both cases, the percentage of CV values was found to be within the acceptable limits of ICH specification which is less than 2%. Therefore, the method is demonstrated to be precise and reproducible with less variation in the results regardless of whether the quantification of DTX (similar experiment) is performed several times within-day (intra-day) or between-day (inter-day). The percentage of CV value for precision and repeatability is summarized in Table

3. The results of accuracy study (Table 4) demonstrated of high rate of recovery from 93.3 to 99.9% over its therapeutic range. Furthermore, the CV value between the measurements in all concentrations did not exceed 5%. The method was proven to be accurate in quantitating DTX where the measured concentrations were close to the nominal concentrations.

Table 3. Results of precision and repeatability for determination of DTX

Concentration ($\mu\text{g/mL}$)	Intra-day (n = 3)			Inter-day (n = 3)		
	MC ($\mu\text{g/mL}$)	SD	CV (%)	MC ($\mu\text{g/mL}$)	SD	V (%)
62.50	64.31	0.97	1.5	62.67	1.20	1.9
125.00	124.67	0.94	0.8	124.07	1.84	1.5
250.00	252.38	4.88	1.9	252.81	3.92	1.6
500.00	494.44	4.66	0.9	490.13	4.12	0.8
1000.00	1008.33	15.65	1.6	1014.35	16.25	1.6

MC: mean concentration, SD: standard deviation, CV: coefficient of variation

Table 4. Results of accuracy for determination of DTX

Initial Concentration ($\mu\text{g/mL}$)	Added Concentration ($\mu\text{g/mL}$)	Nominal Concentration ($\mu\text{g/mL}$)	Recovery Concentration ($\mu\text{g/mL}$)	Accuracy (%)
62.50	62.50	62.50	59.77	95.6
125.00	62.50	93.75	87.45	93.3
250.00	62.50	156.25	156.44	99.9
500.00	62.50	281.25	266.17	94.6
1000.00	62.50	531.25	507.06	95.5

Successful drug delivery into the lungs *via* inhalation depends greatly on the aerosolization performance of its carrier system, which related directly to its aerodynamic properties. The use of ACI benefits the preliminary screening on DTX-loaded palm-based nanoemulsion formulations to possibly correlate the lung deposition based on mass of the deposited drug. In this study, most of DTX was deposited on stage 3 to 6 (Table 5), representing aerodynamic diameter cutoff of 4.53, 2.88, 1.51 and 0.96 μm , respectively [11]. Moreover, studies have demonstrated that, nanocarrier with aerodynamic diameter of less than 5 μm has a capability to deposit in the lower airways including alveolar region, where the tumors are normally found and absorption mainly occurred [7, 12, 13].

Table 6 summarized the aerosolization properties of nebulized DTX-loaded nanoemulsion calculated based on the amount of DTX deposited on stages of ACI. The aerosolized nanoemulsion emitted 85.4% of loaded drug while remaining drug might be trapped in the sampling apparatus including nebulizer. It was also found that, more than 75% of the loaded drug able to deposit on ACI stages (stage 0 – 7 and filter) which mimic the pulmonary region of human lungs. On top of that, a high deposition of drug in the lower stage of cascade impactor was obtained, as almost 71.8% of total drug deposited on stage 3 and below ($\text{FPD}_{<5\mu\text{m}}$).

Table 5. Amount of DTX deposited on stages of Anderson cascade impactor

Site of Deposition	Amount of DTX Deposited (μg)
Nebulizer	241.12 $\pm$ 36.80
Induction port	13.17 $\pm$ 6.74
Inlet cone	ND
Stage 0	34.74 $\pm$ 9.04
Stage 1	31.47 $\pm$ 16.41
Stage 2	86.73 $\pm$ 20.88
Stage 3	289.73 $\pm$ 27.71
Stage 4	757.91 $\pm$ 118.51
Stage 5	823.73 $\pm$ 59.60
Stage 6	212.70 $\pm$ 101.15
Stage 7	71.09 $\pm$ 22.60
Filter	ND

ND: not detected

Table 6. Aerosolization properties of nebulized DTX-loaded nanoemulsion

Parameter	
Emitted dose (DD), %	85.4 $\pm$ 3.8
Deposited dose (ED), %	75.8 $\pm$ 2.5
Fine particle dose (FPD _{<5μm}), %	71.8 $\pm$ 2.1

Conclusion

The proposed HPLC method was successfully developed and validated according to ICH guidelines and was found to fulfill the requirements and exhibited acceptable values of recovery. The method was found to be simple, fast, precise and highly accurate for analysis of DTX in palm-based nanoemulsion sample and can readily be used for quantitative analysis of DTX in many studies including stability, drug entrapment, and drug release.

Acknowledgement

This work was supported by the Universiti Putra Malaysia (UPM) and LRGS NanoMITe-Ministry of Higher Education, Malaysia (RU029-2014/5526306).

References

1. Gubens, M. A. and Wakelee, H. A. (2010). Docetaxel in the treatment of non-small cell lung carcinoma: an update and analysis. *Lung Cancer (Auckland)*, 1: 63-76.
2. Varshosaz, J., Taymouri, S., Hassanzadeh, F., Javanmard, S. H. and Rostami, M. (2015). Self-assembly micelles with lipid core of cholesterol for docetaxel delivery to B16F10 melanoma and Hepg2 cells. *Journal of Liposome Research*, 25(2): 157-165.
3. Zhang, L., Tan, L., Chen, L., Chen, X., Long, C., Peng, J. and Qian, Z. (2016). A simple method to improve the stability of docetaxel micelles. *Scientific Reports - Nature*, 6 (36957): 1-10.
4. Liu, Z., Liu, D., Wang, L., Zhang, J. and Zhang, N. (2011). Docetaxel-loaded Pluronic P123 polymeric micelles: in vitro and in vivo evaluation. *International Journal of Molecular Sciences*, 12(3): 1684- 1696.

5. Nesamony, J., Shah, I. S., Kalra, A. and Jung, R. (2014). Nebulized oil-in-water nanoemulsion mists for pulmonary delivery: Development, physico-chemical characterization and in vitro evaluation. *Drug Development and Industrial Pharmacy*, 40(9): 1253-1263.
6. Xia, D., Sun, W. K., Tan, M. M., Zhang, M., Ding, Y., Liu, Z. C., Su, X. and Shi, Y. (2015). Aerosolized amphotericin B as prophylaxis for invasive pulmonary aspergillosis: A meta-analysis. *International Journal of Infectious Diseases*, 30: 78-84.
7. Ngan C. L. and Asmawi, A. A. (2018). Lipid-based pulmonary delivery system: A review and future considerations of formulation strategies and limitations. *Drug Delivery and Translational Research*, 8 (5): 1527-1544.
8. Keng, P. S., Basri, M., Zakaria, M. R. S., Rahman, M. B. A., Ariff, A. B., Rahman, R. N. Z. A. and Salleh, A. B. (2009). Newly synthesized palm esters for cosmetics industry. *Industrial Crops and Products*, 29(1): 37-44.
9. Che, H. L., Tan, D. M. Y., Meganathan, P., Gan, Y. L., Abdul Razak, G. and Fu, J. Y. (2015). Validation of a HPLC/FLD method for quantification of tocotrienols in human plasma. *International Journal of Analytical Chemistry*, 2015: 1-7.
10. Le, T. H. H., Phung, T. H. and Le, D. C. (2019). Development and validation of an HPLC method for simultaneous assay of potassium guaiacolsulfonate and sodium benzoate in pediatric oral powder. *International Journal of Analytical Chemistry*, 2019: 1-9.
11. Shah, K., Chan, L. W. and Wong, T. W. (2017). Critical physicochemical and biological attributes of nanoemulsions for pulmonary delivery of rifampicin by nebulization technique in tuberculosis treatment. *Drug Delivery*, 24(1): 1631-1647.
12. Shin, S. W., Song, I. H. and Um, S. H. (2015). Role of physicochemical properties in nanoparticle toxicity. *Nanomaterials*, 5(3): 1351-1365.
13. Ezzati N. D. J., Hamishehkar, H. and Valizadeh, H. (2015). Development of dry powder inhaler formulation loaded with alendronate solid lipid nanoparticles: Solid-state characterization and aerosol dispersion performance. *Drug Development and Industrial Pharmacy*, 41(9): 1431-1437.