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Development and validation of an analytical method for the determination of nanostructured lipid carrier's cinchonine used direct method modified by liquid-liquid extraction using high-performance liquid chromatography

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ABSTRACT: Background: cinchonine nanostructured lipid carrier is a preparation with a nanoparticle lipid delivery system. Analysis of levels (efficiency of entrapment) of cinchonine lipid nanostructure is critical to ensure the accuracy of drug dosage. Objective: development and validation of an analytical method for the direct assay of nanostructured lipid carrier's cinchonine modified by liquid-liquid extraction using high-performance liquid chromatography. Methodology: The research was started by determining the maximum wavelength and validation parameters of selectivity, linearity, sensitivity, precision (intraday and inter days), accuracy, and assay (entrapment efficiency) of nanostructured lipid carrier's cinchonine directly with modification of liquid-liquid extraction. Results: maximum wavelength of cinchonine 289 nm; selectivity (RF 4.096 ± 0.30 min and TF 1); linearity (R^2) 0.9998; sensitivity (LoD 1.2016 mg/L and LoQ 4.0054 mg/L); precision ($RSD_{intraday}$ and $RSD_{inter\ days}$ <2%); accuracy (recovery 99.52 – 99.87%), entrapment efficiency using direct assay of 94.85 ± 1.91% and indirect assay of 93.35 ± 0.22%. Conclusion: The analysis method for the direct assay of nanostructured lipid carrier's cinchonine modified by the liquid-liquid extraction method using high-performance liquid chromatography is effective, efficient, and specific with high validity to determine the concentration (entrapment efficiency) of cinchonine from nanostructured lipid carrier's cinchonine.

KEYWORDS: Cinchonine; Liquid-liquid extraction; Validation method; Nanostructured lipid carrier.

1. INTRODUCTION

Cinchonine is an alkaloid isolated from *Cinchona succirubra*. Alkaloids have an essential role in the treatment and immune system of organisms. The number of alkaloids ranges from 15 to 20% of the secondary metabolites in plants. In plants, alkaloids play a role in protecting from predators and regulating growth. Alkaloids have pharmacological activities such as anesthetics, cardioprotective, and anti-inflammatory [1]. Characteristics of cinchonine (melting point 264 °C) are insoluble in water, slightly soluble in chloroform, and alkaline [2]. Cinchonine has three main molecular group units: quinoline aromatic ring, quinuclidine ring, and methylene alcohol group. The nitrogen atom in the ring of quinuclidine and methylene alcohol is a functional group that plays an essential role in pharmacological activity [3–5].

Cinchonine has activity as a hair growth stimulant, [6] thus requiring a nanostructured lipid carrier (NLC) delivery system to reach hair follicles and dermal papillae [7–11]. NLC is a second-generation lipid nanoparticle delivery system from solid lipid nanoparticles (SLN). Cinchonine has good solubility in NLC-forming lipids. The use of NLC as a cinchonine delivery system provides several advantages, including suppressing the occurrence of side effects [12], improving drug stability [13], high drug trapping capacity [14], increasing drug permeability [15], increasing drug penetration [16], improve drug solubility and bioavailability [17]. An appropriate assay analysis method is needed to determine an efficient, effective, and

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selective drug dose. The chemical characteristics of cinchonine, which are alkaline, became the basis for developing an analytical method for determining the concentration of cinchonine by modifying the determination of the adsorption efficiency directly with the liquid-liquid extraction method [18–21]. This study aims to develop and validate an analytical method for the direct assay of NLC Cinchonine with modified liquid-liquid extraction using high-performance liquid chromatography (HPLC).

2. RESULTS AND DISCUSSION

2.1 The maximum wavelength (λ_{max}) of cinchonine

Determination of the maximum wavelength of cinchonine was carried out using a UV-Vis spectrophotometer. Cinchonine has a chromophore group with a conjugated system that has a pi bond in the aromatic quinoline ring [22]. When the analyte is exposed to UV-Vis light, some of the light will be absorbed, reflected, and transmitted, so the data obtained from this analysis will be absorption or transmittance. Absorption of UV-Vis light will cause electronic transitions, where electrons in lower energy orbitals are excited to higher energy orbitals. The maximum wavelength (λ_{max}) is determined based on the response or maximum absorbance given by the standard cinchonine solution, which is at 289 nm (Fig. 1) [23,24].

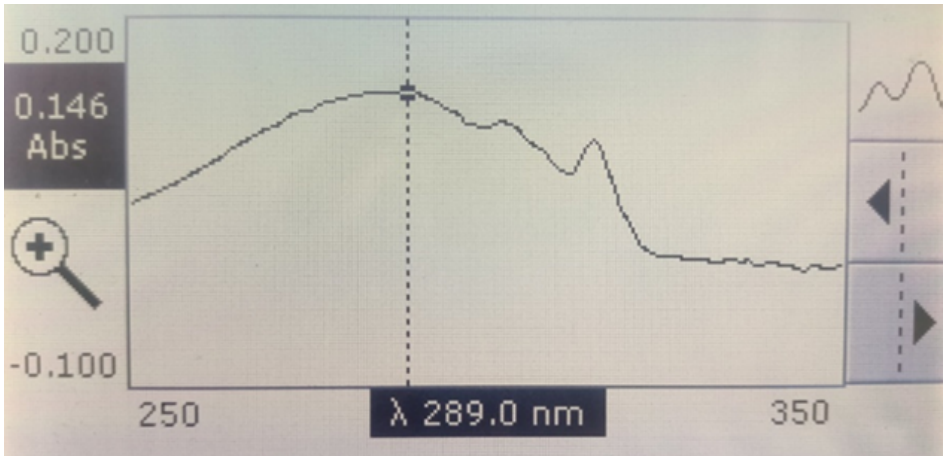

Figure 1. The maximum wavelength of cinchonine is 289 nm.

2.2 Selectivity

The direct assay procedure of NLC cinchonine showed good selectivity. The data (Fig. 2) show that the analyte can be separated selectively from the solvent/medium. The solvent is HCl (pH 1) used in the liquid-liquid extraction process that has been diluted using Aquadest. Aquadest were detected on the chromatogram with codes 1 (RF: 2.1 – 2.7 minutes) and 2 (RF: 3.2 – 3.8 minutes). HCl used in the liquid-liquid extraction process was detected on the chromatogram with code 4 (RF: 4.4 – 4.9 minutes). Cinchonine was seen on a chromatogram with code 3 (RF: 3.9 – 4.2 minutes) and separated selectively with distilled water and HCl medium. The average cinchonine RF obtained from each analysis ranged from 4.096 ± 0.30 minutes with a tailings factor (Tf) of 1 [24].

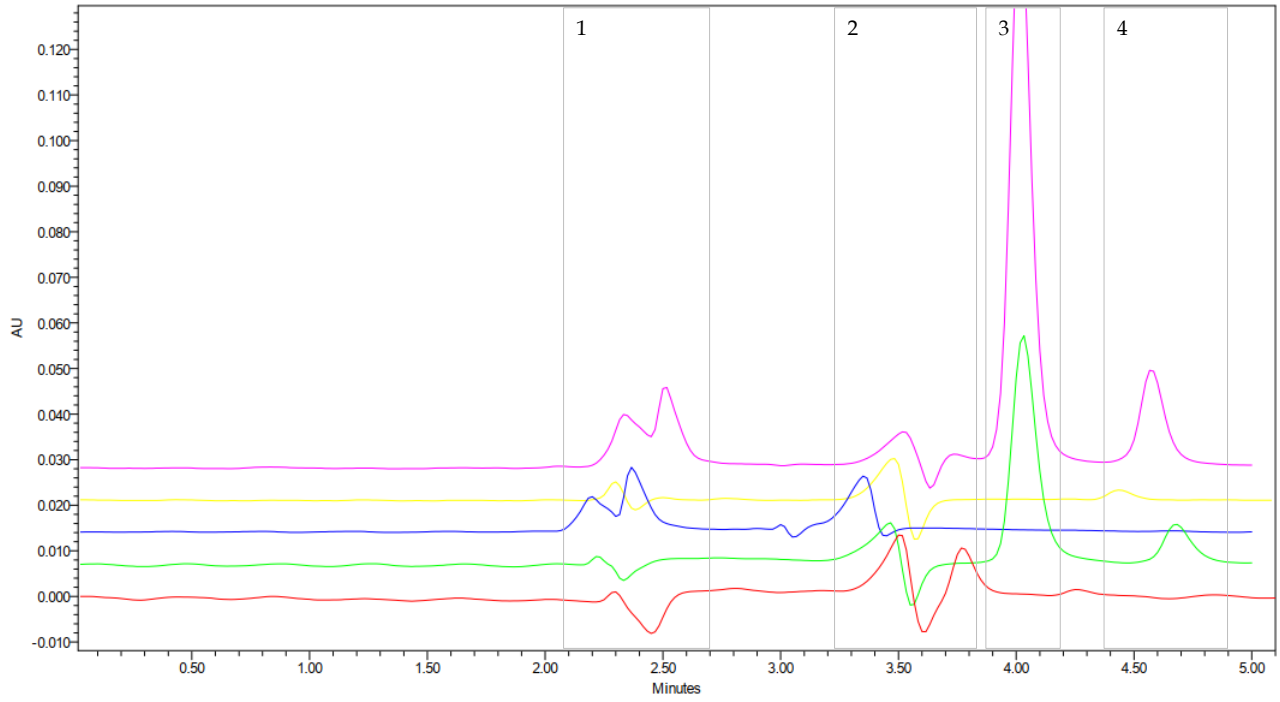

Figure 2. Chromatogram [(Red: Aquadest/solvent used for dilution, Blue: Blank NLC, Yellow: HCl pH 1, Green and purple: NLC-Cinchonine; 1, 2: Aquadest/solvent (RF: 2.1 – 2.7 minutes, RF: 3.2 – 3.8 minutes), 3: Cinchonine (RF: 3.9 – 4.2 minutes), 4: HCl (RF: 4.4 – 4.9 minutes)]

2.3 Linearity

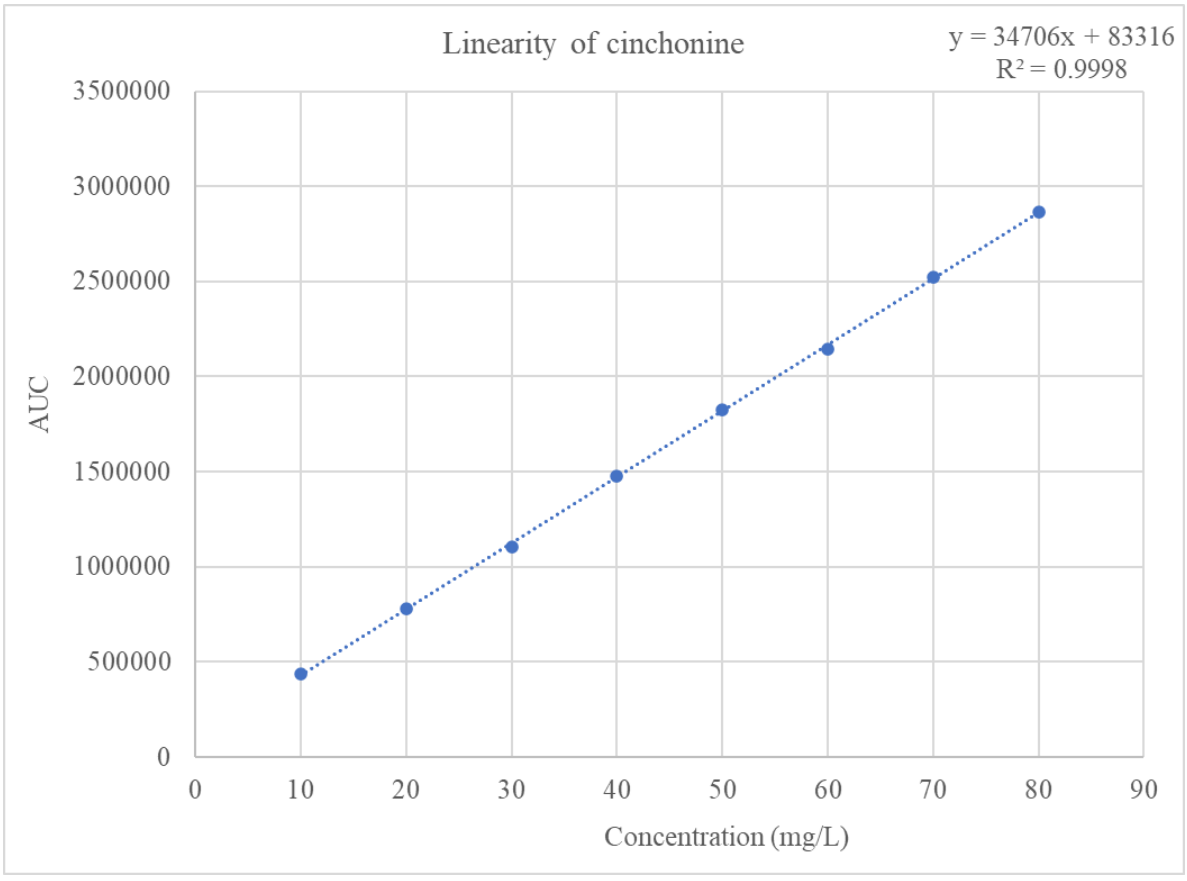

Figure 3. Calibration curve of cinchonine (λ_{max}: 289 nm).

Linearity is the analytical method's ability to respond to changes in the concentration of the sample analyte proportionally. The proportional relationship between the response (y) to changes in concentration variation (x) can be written in the equation y = bx + a. The ideal relationship between response and concentration variation occurs when the value of b = 0 and r = +1/-1. While the value of a is the sensitivity parameter of the instrument used. From (Fig. 3) shows that the analytical method gives a proportional linear response to the variation of analyte concentration which is indicated by the value of R^2: 0.9998 with the regression equation y = 34706x + 83316 [24–27].

2.4 Limit of detection (LoD) and limit of quantitation (LoQ) cinchonine

The limit of detection (LoD) is the smallest amount of sample analyte that can be detected and is significantly different compared to the blank. The limit of quantitation (LoQ) is the smallest quantity of analyte in the sample that can still meet the criteria of accuracy and precision. LoD and LoQ are parameters that show the method's sensitivity. Table 1 shows that this method has a detection limit (LoD) of 1.2016 mg/L and a quantization limit (LoQ) of 4.0054 mg/L. This data indicates that the process has good sensitivity in analyzing cinchonine levels [24,25].

Table 1. Value of LoD and LoQ cinchonine.

C (mg/L)	AUC (y)	x	y'	(y-y')	$(y-y')^2$
10	438003	10.22	430376	7627	58171129
20	781906	20.13	777436	4470	19980900
30	1105307	29.45	1124496	-19189	368217721
40	1478169	40.19	1471556	6613	43731769
50	1825432	50.20	1818616	6816	46457856
60	2143965	59.37	2165676	-21711	471367521
70	2524579	70.34	2512736	11843	140256649
80	2863150	80.10	2859796	3354	11249316
Total					1159432861
$S(y/x)^2$					193238810
S(y/x)					13901.04
LOD					1.2016
LOQ					4.0054

2.5 Precision.

Precision is measured as standard deviation (SD) or relative standard deviation (RSD). Accuracy can be expressed as repeatability or reproducibility. Repeatability is the method's accuracy if it is carried out repeatedly by the same analyst under the same conditions and in short time intervals. The data in Table 2 and Table 3 shows that the accuracy value reaches 99.68-101.26%, with a coefficient of variation (RSD) between 0.9-1.84% [24,26].

Table 2. Intraday Precision.

Rep	CT (mg/L)	AUC	C'	Recovery (%)	Average (%)	SD (%)	RSD (%)
1	6	289649	5.95	99.09	99.97	1.80	1.80
2	6	284954	5.81	96.83			
3	6	295730	6.12	102.01			
4	6	292587	6.03	100.50			
5	6	293139	6.05	100.76			
6	6	292932	6.04	100.66			
1	30	1130070	30.16	100.54	101.16	1.83	1.81
2	30	1150311	30.74	102.48			
3	30	1121897	29.93	99.75			
4	30	1125181	30.02	100.07			
5	30	1122886	29.95	99.85			
6	30	1168912	31.28	104.27			
1	50	1824056	50.16	100.31	101.26	1.86	1.84
2	50	1828685	50.29	100.58			
3	50	1818908	50.01	100.02			
4	50	1818640	50.00	100.00			
5	50	1850552	50.92	101.84			
6	50	1901933	52.40	104.80			

Table 3. Inter days precision.

Rep	CT (mg/L)	AUC	C'	Recovery (%)	Average (%)	SD (%)	RSD (%)
1	6	289949	5.95	99.23	99.68	1.29	1.30
2	6	285955	5.84	97.31			
3	6	291130	5.99	99.80			
4	6	292556	6.03	100.48			
5	6	293231	6.05	100.81			
6	6	292537	6.03	100.47			
1	30	1130161	30.16	100.54	100.69	1.78	1.76
2	30	1120391	29.88	99.61			
3	30	1121888	29.92	99.75			
4	30	1125241	30.02	100.07			
5	30	1123786	29.98	99.93			
6	30	1168822	31.28	104.26			
1	50	1824146	50.16	100.32	100.28	0.90	0.90
2	50	1827691	50.26	100.52			
3	50	1818816	50.01	100.01			
4	50	1818549	50.00	100.00			
5	50	1850039	50.91	101.81			
6	50	1801955	49.52	99.04			

2.6 Accuracy (Recovery).

Accuracy (recovery) is a measure that shows the degree of closeness of the analyst's results with the actual analyte content. Accuracy is expressed as the percent recovery of the added analyte. Table 4 shows that the analytical procedure gives a recovery of ±99% [24,25].

Table 4. Accuracy (recovery) of NLC cinchonine.

Rep	CT (mg/L)	AUC	C'	Recovery (%)	Average (%)	SD (%)	RSD (%)
1	20	769801	19.78	98.90	99.81	0.53	0.53
2	20	774453	19.91	99.57			
3	20	779728	20.07	100.33			
4	20	777732	20.01	100.04			
5	20	779171	20.05	100.25			
6	20	776001	19.96	99.79			
1	40	1501509	40.86	102.16	99.87	1.38	1.38
2	40	1472315	40.02	100.05			
3	40	1478876	40.21	100.53			
4	40	1447389	39.30	98.26			
5	40	1458554	39.63	99.06			
6	40	1459852	39.66	99.16			
1	60	2135884	59.14	98.57	99.52	1.17	1.18
2	60	2177834	60.35	100.58			
3	60	2125770	58.85	98.08			
4	60	2165880	60.01	100.01			
5	60	2186222	60.59	100.99			
6	60	2142395	59.33	98.88			

2.7 The entrapment of efficiency

The entrapment of efficiency using the direct method modified by liquid-liquid extraction is shown that the determination of the entrapment efficiency of NLC cinchonine by the direct method modified by liquid-liquid extraction was 94.85 ± 1.91% (Table 5) compared to the indirect method of 93.35 ± 0.22% (Table 6). These data show that the analysis procedure for direct and indirect assay of cinchonine with modified liquid-liquid extraction is correlated (comparable).

Table 5. The entrapment efficiency of NLC cinchonine using the direct method.

Rep	w (mg)	CT (mg/L)	V sample (µL)	AUC	C	FP	FP	Total FP	C′	EE (%)
1	18	1800	500	767408	19.7111	5	17	85	1675.44	93.08
2	18	1800	500	795403	20.5177	5	17	85	1744.00	96.89
3	18	1800	500	778408	20.028	5	17	85	1702.38	94.58
Average ± SD										94.85 ± 1.91

Table 6. The entrapment efficiency of NLC cinchonine using the indirect method.

Rep	w (mg)	CT (mg/L)	V sample (µL)	AUC	C	FP	C′	(CT-C′)	EE (%)
1	18	1800	500	755816	19.3771	6	116.262	1683.74	93.54
2	18	1800	500	800420	20.6622	6	123.973	1676.03	93.11
3	18	1800	500	769578	19.7736	6	118.642	1681.36	93.41
Average ± SD									93.35 ± 0.22

2.8 The summary of validation parameters.

The summary of validation parameters is shown in **Table 7.**

Table 7. Summary of validation parameters.

Parameters	Results	RSD	Acceptance criteria
Maximum wavelength (nm)	289		
Selectivity			
Retention time factor (RF)	4.096 ± 0.30		3 – 5 minutes
Tailings factor (Tf)	1		1
Linearity (n = 8)			
Slope	34706		
Intercept	83316		
Correlation coefficient (R^2)	0.9998		1
Sensitivity			
LoD (mg/L)	1.2016		Sensitive
LoQ (mg/L)	4.0054		Sensitive
Precision (n = 6)			
Intra-day precision			
6 mg/L	99.97	1.80	
30 mg/L	101.16	1.81	< 2%
50 mg/L	101.26	1.84	
Inter-day precision			
6 mg/L	99.68	1.30	
30 mg/L	100.69	1.76	< 2%
50 mg/L	100.28	0.90	
Accuracy (n = 6)			
Average recovery (%)			
20 mg/L	99.81	0.53	
40 mg/L	99.87	1.38	100%
60 mg/L	99.52	1.18	
Entrapment efficiency of NLC cinchonine			
Direct method (%)	94.85 ± 1.91		100%
Indirect method (%)	93.35 ± 0.22		

4. CONCLUSION

The analysis method for the direct assay of NLC cinchonine modified by the liquid-liquid extraction method using HPLC is effective, efficient, and specific with high validity to determine the efficiency of cinchonine entrapment from NLC cinchonine formulation. The method can be used for the analysis of entrapment efficiency in NLC formulation (similar formulation with alkaline active ingredients) and the stability test.

5. MATERIALS AND METHODS

5.1 Materials.

Cinchonine (PT. Sinkona Indonesia Lestari), stearic acid, oleic acid, tween 80, glycerine, deionized water (Amidis), acetonitrile (Merck), aqua pro injection (IPHA), KH_2PO_4, HCl pH 1, chloroform (Merck), all materials used are pharmaceutical grades.

Analytical Balance (mg) Metler Toledo ME 204, Magnetic stirrer IKA RT 15, Ultraturax T18 IKA Digital, bath sonicator falc, Probe Sonicator (CY-500 Ultrasound Homogenizer), Beckman pH Meter, Amicon (Centrifugal Filters Ultracel, cut off 10kD), Beckman Spectrophotometer Coulter DU 720, HPLC Waters 2487, Silversil column C18 (250 x 4.6 mm) and glassware that is often used in laboratories.

5.2 Methods.

Cinchonine standard solution

500 mg/L of cinchonine was prepared. Cinchonine (50 mg) was dissolved with HCl pH 1 (5 mL), added with distilled water to 100 mL, and vortexed for 5 minutes.

HPLC system

All samples were analyzed using HPLC, at a predetermined λ_{max} (289 nm), using column C18 using 25% acetonitrile mobile phase in 0.1M phosphate buffer pH.3 (KH_2PO_4 – H_3PO_4) with UV detector at the maximum wavelength (λ_{max}), sample volume: 50 µL, at a rate of 1 mL/min, and cinchonine retention time ±3-5 minutes.

Cinchonine and blank NLC formulations (NLC without cinchonine)

The lipid phase (1.8%b/v stearic acid, 0.2%b/v oleic acid, and 0.18%b/v cinchonine) and the aqueous phase (tween 80 3.5%b/v, glycerine 2.5%b/v, and deionized water up to 100%) were heated to a temperature of 70°C. The aqueous phase was added to the lipid phase while homogenizing with a magnetic stirrer for 20 minutes (70°C, 800 rpm). A hot microemulsion was formed after the homogenization with ultra turrax at 8000 rpm for 2 minutes, followed by bath sonication (frequency 59 kHz, for 15 minutes), solidification at a temperature of 2-3°C (30 minutes), and probe sonication for 15 minutes (pulse on-off 45:15 seconds, amplitude 60%). The NLC cinchonine formed was stored overnight in the refrigerator and then characterized.

Liquid-liquid extraction

500µL of the sample was mixed with 3 mL of chloroform, followed by 8 mL of HCl (pH 1), and vortexed for 5 minutes to form 2 immiscible liquid layers. The aqueous phase (containing cinchonine HCl) was analyzed using HPLC at the maximum wavelength.

5.3 Validation

Method validation was performed according to the guidelines set by the international conference on harmonization ("Validation of Analytical Procedures: Text and Methodology Q2(R1)," 2005). The method was validated in terms of linearity, sensitivity, selectivity, accuracy, and precision [28].

Determination of maximum wavelength (λ_{max})

Cinchonine solution was made with a concentration of 40 mg/L, and the maximum absorption wavelength was determined using a UV-Vis spectrophotometer from 250 to 350 nm.

Specifications/ selectivity

They compared the chromatograms of distilled water, blank-HCl, blank-NLC, and NLC Cinchonine. The analysis parameters are retention time (RF) and tailings factor (TF).

Linearity and Sensitivity

Standard solutions of cinchonine were made with various concentrations of 10, 20, 30, 40, 50, 60, 70, and 80 mg/L. Then from the different concentration variations above, analysis was carried out using HPLC. The parameters of the study are linearity (R^2), Limit of detection (LoD), and Limit of quantitation (LoQ).

$$LoD = \frac{3.SD}{b}$$

$$LoQ = \frac{10.SD}{b}$$

SD : Standart Deviasion

b : Slope

Intraday and inter days precision

Precision was determined intraday (analysis of repeatability of standard solutions on the same day) and inter-days (analysis of repeatability of standard solutions on different days/time intervals of 7 days). The precision study analyzed 6 replications of standard solutions at 3 different concentrations of 6, 30, and 50 mg/L on the same day and for the next 7 days.

Accuracy with spiked-placebo recovery method, direct method modified with liquid-liquid extraction. NLC mixture (blank): cinchonine (70:30) with various concentrations of 20, 40, and 60 mg/L then vortexed for 5 minutes (M1). 500 µL of M1, added 3 mL of chloroform, 8 mL of HCl (pH 1), and vortexed again for 5 minutes. The aqueous phase was analyzed using HPLC at the maximum wavelength. The accuracy was calculated by the follow equation:

$$\text{Accuracy} = \frac{Ca}{Ct}\ x\ 100$$

Ca: The concentration obtained from the analysis results (cinchonine from liquid-liquid extraction).

Ct: The theoretical concentration of cinchonine added to the NLC.

Entrapment Efficiency (EE) of NLC cinchonine using direct methods, modified with liquid-liquid extraction methods.

500µL of NLC cinchonine was put in centrifugal filter tubes, and centrifuged for 15 minutes at 13000 rpm, and the supernatant and filtrate were separated. The supernatant was mixed with 3 mL of chloroform, followed by 8 mL of HCl (pH 1), and vortexed for 5 minutes to form 2 immiscible liquid layers. The aqueous phase (containing cinchonine HCl) was analyzed using HPLC at the maximum wavelength. The EE was calculated by the follow equation:

$$\%EE\ direct = \frac{Ca}{Ct}\ x\ 100$$

$$\%EE\ indirect = \frac{Ct - Ca}{Ct}\ x\ 100$$

Ct: The theoretical concentration of cinchonine added to the NLC.

Ca: The concentration obtained from the results of the analysis.

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Author contributions: Concept – Hariyanti.; Design – Hariyanti.; Supervision – N.F.K., Y.C.S., R.M.; Resources – Hariyanti; Materials – Hariyanti.; Data Collection and Processing – Hariyanti.; Analysis and Interpretation – Hariyanti.; Literature Search – Hariyanti.; Writing – Hariyanti.; Critical Reviews – N.F.K., Y.C.S., R.M.

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