

ANALYTICAL METHOD DEVELOPMENT, VALIDATION AND STABILITY STUDIES BY RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF ANDROGRAPHOLIDE AND CURCUMIN IN CO-ENCAPSULATED NANOSTRUCTURED LIPID CARRIER DRUG DELIVERY SYSTEM

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ABSTRACT

Objective: The current study aims to boost the bioavailability criteria of two natural bioactive compounds, andrographolide and curcumin by their combination in nanostructured lipid carrier (NLC) and also to develop a straightforward reverse-phase high-performance liquid chromatography (RP-HPLC) method to validate, quantify of andrographolide and curcumin simultaneously in novel NLC formulation.

Methods: The reliable chromatographic separation was executed by using a column of Phenomenex octadecylsilane (C18) at 35 °C column oven temperature using a mobile phase of 0.02 M potassium dihydrogen orthophosphate (KH₂PO₄) salt solution of pH 3.0 as a buffer and acetonitrile in 50: 50 v/v fixed ratio and 1.5 ml/min flow rate of with 20 µl injection load. The detection was carried out at 240 nm isosbestic wavelength employing a photodiode array (PDA) detector.

Results: Andrographolide and curcumin were eluted at 2.4 and 4.9 min, respectively. Quantification and linearity were achieved for both drugs at the 10-140 µg/ml range. The method is specified as the presence of excipients utilized in the formulation failed to interfere with the estimation of andrographolide and curcumin. The developed method was successfully utilized to work out the drug loading efficiency and *in vitro* drug release study of those drugs in NLC formulation and also for the estimation of those drugs from rat plasma.

Conclusion: The developed high-performance liquid chromatography (HPLC) method may be utilized in the future estimation of andrographolide and curcumin simultaneously in NLC and other nanoformulations both *in vitro* and *in vivo*.

Keywords: Andrographolide, Curcumin, Nanoformulation, RP-HPLC method development, Forced degradation

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INTRODUCTION

The bioactive compounds are gaining interest by their wide pharmacological actions like antimicrobial, antioxidants, anti-inflammatory and anticarcinogenic actions. Recently these natural compounds have been encouraged by their prospective health benefits [1-4]. These phytochemicals have important bioactivity but the main drawback related to them is their limited solubility and bioavailability [5]. To enhance the bioavailability of those compounds, the employment of nanoformulation is vital. The use of a modified drug delivery system enhanced the absorption of phytoconstituents in high blood level concentration and sometimes drug combinations result in synergistic effects [1].

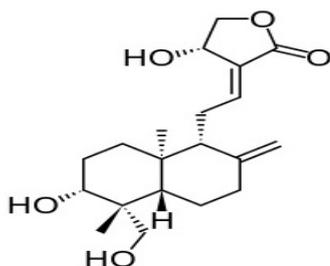


Fig. 1: Chemical structure of andrographolide

Andrographolide, a diterpenoid lactone is the major active constituent found in *Andrographis paniculata*. *Andrographis paniculata* is widely utilized in ayurvedic formulations [6]. Andrographolide has been isolated and purified in the crystalline form [7]. Andrographolide showed a wide spectrum of pharmacological activities like anti-inflammatory, neuroprotective,

antioxidant, anticancer, antimicrobial and hepatoprotective. It is also applied in the treatment of acquired immunodeficiency syndrome (AIDS) and symptoms related to immune disorders [8]. Andrographolide showed other activities like liver protection, anti-diabetic [9] and anti-malarial [10]. The chemical structure of andrographolide is shown in fig. 1.

Curcumin is the principal active ingredient present within the rhizome of turmeric (*Curcuma longa*). It is incorporated in dietary supplements and cosmetic ingredients. It has also other pharmacological actions [11-13]. The chemical structure of curcumin is shown in fig. 2.

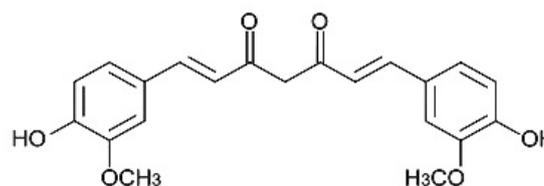


Fig. 2: Chemical structure of curcumin

Many studies were carried out to enhance the bioavailability of andrographolide and curcumin to increase their therapeutic responses [14, 15]. Antimalarial, antifertility activity of curcumin and andrographolide have shown a synergistic effect [16-18]. To overcome the bioavailability constraints of these drugs, we have incorporated andrographolide and curcumin into a nanostructured lipid carrier (NLC) system [19]. It has been demonstrated that the therapeutic activity of a drug can be correlated to its drug loading values [20]. With the increase of drug loading, the therapeutic potential also increases.

Literature survey for andrographolide and curcumin reveals high-performance thin-layer chromatography (HPTLC), high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS) methods [21-26] for singular estimation of andrographolide and curcumin. However, there is no HPLC method reported to far for the simultaneous detection and estimation of co-encapsulated andrographolide and curcumin in a nanostructured lipid carrier (NLC) system. For the development of a co-delivery system of herbal drugs, there is a requirement for an analytical method to estimate the drugs from formulations. This study aims to develop a HPLC method for the simultaneous quantification of andrographolide and curcumin in a co-encapsulated NLC system.

MATERIALS AND METHODS

Materials

Andrographolide (purity>99 %) and curcumin (purity>99 %) were procured from Sigma-Aldrich, USA. HPLC grade methanol, acetonitrile from Rankem, India were used in the experiment. All the chemicals or reagents were used of AR grade from Merck.

Compritol®888 ATO was procured from Gattefosse India Pvt Ltd (Mumbai, India). Triolein, soya lecithin, rat plasma and sodium deoxycholate was procured from Sigma-Aldrich Co (St Louis, USA). Stearylamine was procured from Sisco Research Laboratories Pvt Ltd, India. The buffer solution for the mobile phase was prepared with MilliQ water. Millex Syringe filters of 0.45 µm were used to filter the sample solutions.

Choice of wavelength

Andrographolide and curcumin primary standard stock solutions (100 µg/ml) were prepared in methanol and further diluted to a secondary standard stock solution (10 µg/ml). The solutions were scanned in an Ultraviolet-Visible (UV-Vis) Spectrophotometer in the 200-400 nm range. Andrographolide showed absorption maxima at 223 nm, while that for curcumin was found to be at 262 nm. Two standard UV spectrum crosses at a common wavelength at 240 nm, which is the isosbestic wavelength of andrographolide and curcumin and hence 240 nm was taken as a detection wavelength for HPLC analysis. The overlay UV spectrum of andrographolide and curcumin is represented in fig. 3.

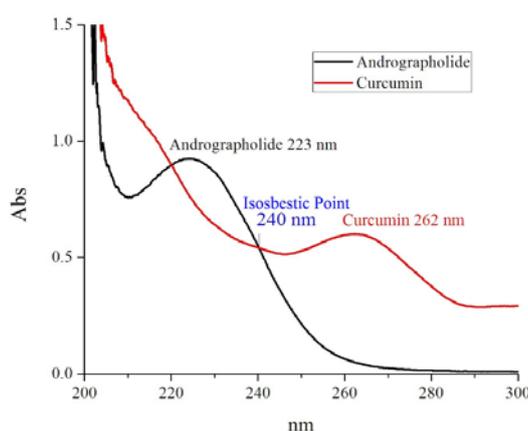


Fig. 3: UV overlay spectrum of andrographolide and curcumin

Instrumentation and chromatographic conditions

The UV 1800 UV-visible spectrophotometer of Shimadzu make equipped with UV probe software, was used for recording the UV spectrum. The HPLC system consisted of a quaternary pump (Model: Agilent 1260 series) with an autosampler, photodiode array detector (PDA) and thermostat column compartment (TCC). EZ chrome Elite software was used for data collection and analysis. Phenomenex

octadecylsilane column with 1.5 ml/min flow rate in an isocratic mode of elution was used for the separation of two drugs. The mobile phase containing 0.02 M monobasic potassium phosphate solution of pH 3.0 and acetonitrile as a ratio of 50: 50 (v/v). The mobile phase was degassed and filtered before use. All the tests were performed at a 35 °C constant column oven temperature with 240 nm UV detection and 20 µl injection load. The established chromatographic conditions are given in table 1.

Table 1: Chromatographic conditions of the validated analytical method

Parameter	Conditions
Column	Phenomenex, octadecylsilane (250 mm × 4.6 mm, 5 µm) column
Mobile phase	Buffer (0.02 M KH ₂ PO ₄ , pH 3.0): Acetonitrile = 50: 50 v/v
Solvent	First dilution in methanol and subsequent dilution in the mobile phase
Flow rate	1.5 ml/min
Column oven tempt	35 °C
Detection wavelength	240 nm
Injection volume	20 µl

Preparation of primary standard and sample solutions

A mixed standard solution of andrographolide and curcumin (500 µg/ml) was prepared in methanol as the primary stock solution. The final standard solution was obtained with an actual dilution of the standard stock solution.

Necessary quantities of nanoformulation were dissolved in methanol followed by dilution with diluent to determine the entrapment efficiency, drug loading and cumulative *in vitro* drug release study by the suggested method [27].

Method validation

Method validation was executed by ICH recommendations for system suitability, accuracy, specificity, linearity, precision, sensitivity and robustness [28].

Specificity

Specificity stipulates the proposed method's capability to separate the principal peaks from any other impurity/degradation products. To carry out the specificity analysis, blank nanoparticle (without drugs) solutions and stress degradation solutions under different conditions

were analysed into the HPLC method. The acquired peaks were compared with standard drug solutions peaks. No interference peak was observed apart from the peak of andrographolide and curcumin.

Linearity

To develop the calibration curve, andrographolide and curcumin standards were mixed to prepare 500 µg/ml of stock solution and 12.5, 25, 50, 75, 100 and 125 µg/ml concentrations were prepared for linearity from the stock solution. Each solution was injected in replicate (n = 3) and the calibration curves for andrographolide and curcumin were established by plotting peak area versus concentration.

Accuracy

Accuracy was ascertained by using the standard addition method or recovery study by spiking standard andrographolide and curcumin to the nanoformulation, followed by analysis using the proposed method. To each flask, the measured amount of andrographolide and curcumin standards (at 80 %, 100 % and 120 % levels of andrographolide and curcumin contributed from the sample) were added. Eventually, five concentration solutions were prepared with the diluent and injected into replicate (n = 3).

Precision

For estimation of method precision of the developed method, three different standard concentration levels were prepared, which contained 12.5 µg/ml (lower concentration), 50 µg/ml (middle concentration) and 125 µg/ml (higher concentration) each of andrographolide and curcumin. The intra-day precision was determined by analyzing sample solutions (n = 6) at a single batch in duplicate sets in a single day, but the inter-day precision was determined on another day. The relative standard deviations (% RSD) were determined.

LOD and LOQ

Analytical method sensitivity was ascertained by the limit of detection (LOD) to detect the lowest amount of substance and the limit of quantification (LOQ) to quantify the lowest amount of substance that can be measured within the acceptable system suitability criteria.

Robustness

To assess the robustness study of the proposed method for andrographolide and curcumin, some intentional changes in the chromatographic conditions were employed. System suitability parameters after such changes were estimated for robustness study.

System suitability

System suitability was estimated from the mixed standard solutions of andrographolide and curcumin under optimized chromatographic conditions. Retention time, theoretical plates, tailing factor, resolution and injection precision was considered for system suitability [29].

Forced degradation studies

The degradation property of andrographolide and curcumin was carried out by various forced conditions. Each 5 ml individual standard stock solution of andrographolide and curcumin was applied to various stress conditions separately like photolytic, heat, oxidative hydrolysis, UV light, acidic and alkaline hydrolysis for specified times. Finally, every 5 ml of stress-induced solution was diluted to have a final concentration of andrographolide around 51 µg/ml and curcumin around 50.4 µg/ml. Each solution was analysed against a freshly prepared mixed standard andrographolide (52 µg/ml) and curcumin (51 µg/ml) [30-31]. Optimized conditions for the forced degradation study of andrographolide and curcumin are given in table 2.

Table 2: Optimized condition for forced degradation study of andrographolide and curcumin

Stress type	Stress condition
Acidic	5 ml of 0.1 (N) HCl for 4 h at 80 °C
Alkali	5 ml of 0.1 (N) NaOH for 4 h at 80 °C
Peroxide	5 ml of 5 % v/v H ₂ O ₂ for 4 h at 80 °C
Photolytic	Kept in sunlight for 4 h
Thermal	80 °C for 4 h
UV light	254 nm UV light for 4 h

Suggested method for application

The suggested method was used for estimation of drug entrapment efficiency (DEE) of both drugs and *in vitro* drug release study of andrographolide and curcumin in the NLC system [32, 33].

Co-encapsulated drug-loaded NLC preparation

Nanoparticles have the advantage of improved bioavailability by increasing the aqueous solubility of drugs like andrographolide and curcumin. Briefly, in a glass container necessary amounts of compritol@888 ATO, triolein and stearylamine were melted until they became transparent at 75 °C to 80 °C to prepare the lipid phase and the drugs were added in this phase. The aqueous phase consisted of Milli-Q water, sodium deoxycholate and amphiphilic emulsifier soya lecithin in another glass container. Both phases were separately heated at 75 °C to 80 °C. The lipid phase and the aqueous phase were mixed slowly with continuous homogenization by a homogenizer according to the hot homogenization method. The nanoparticles were lyophilized using sorbitol as cryoprotectants and stored at 4 °C for further characterization.

Characterization of nanoparticles

The zeta potential, polydispersity index (PDI) and average particle size values of encapsulated drug-loaded nanoparticles were examined by dynamic light scattering (Zetasizer, Malvern Instruments, Malvern, UK). Sample solutions were prepared in Milli-Q water (1 mg/ml) before instrumental analysis.

Drug entrapment efficiency determination

For the estimation of drug entrapment efficiency (% DEE), a necessary amount of co-encapsulated drug-loaded NLC was dissolved in methanol followed by centrifugation (10,000 rpm) and filtration through Millex 0.45 µm syringe filter. The filtered solutions were analysed by the developed HPLC method.

In vitro cumulative drug release study

100 mg co-encapsulated drug-loaded NLC was sonicated in 10 ml of phosphate buffer, pH 6.8 (simulated intestinal fluid without enzyme) with Polysorbate 80 (1 % v/v) and placed in a dialysis bag. The bag was kept in a glass container containing 200 ml of phosphate buffer, pH 6.8 with 1 % v/v Polysorbate 80 and placed in an orbital shaker at 100 rpm constant shaking and 37 °C temperature. 1 ml sample solution was withdrawn by replacing 1 ml dissolution medium at a preplanned time interval.

Preparation of quality control (QC) sample spiked with plasma and calibration standards

Standard solutions (0.1 µg/ml) of both the drugs andrographolide and curcumin were prepared in methanol and a mixed standard solution (0.05 µg/ml for each drug) was prepared from both standard stock solutions. Calibration standard and QC samples of andrographolide and curcumin were prepared by spiking rat blank plasma (90 µl) with a mixed standard solution (10 µl) at a final concentration of 3, 5, 7, 9, 11, 15 µg/ml for calibration standard and 4, 8, 14 µg/ml for lower quality control (LQC), middle-quality control (MQC) and higher quality control (HQC) samples [34, 35].

Drug extraction recovery from plasma samples

All plasma samples were allowed to thaw at room temperature before use. An aliquot of plasma samples (100 µl) was taken in a 2 ml eppendorf tube and 100 µl methanol was added for the extraction of drugs, followed by vortexing for 2 min. Ice-cooled 100 µl acetonitrile was added to precipitate the plasma protein then vortex for 1 min. Centrifugation was done for 15 min at 4 °C. The clear solution was collected and allowed to become dry under a vacuum. 100 µl mobile phase was added to the tube, then vortex for 1 min and injected (20 µl) for the analysis [35].

RESULTS AND DISCUSSION

Development of the method

A series of trials in terms of choice of buffer in the mobile phase and its pH, composition of mobile phase, detection of wavelength, choice

of the stationary phase of column, flow rate and column oven temperature was carried out for succeeding the suggested RP-HPLC method. To select the detection wavelength, the UV spectrum in the range of 200 to 400 nm of standard andrographolide (50 µg/ml), curcumin (51 µg/ml) in methanol were recorded. Finally, the ideal

chromatographic parameters were achieved. Andrographolide and curcumin peaks were found at 2.4 and 4.9 min. System suitability parameters were found within the acceptable limit. Typical chromatograms of standard single andrographolide and curcumin and mixed drugs are in the fig. 4, 5 and 6, respectively.

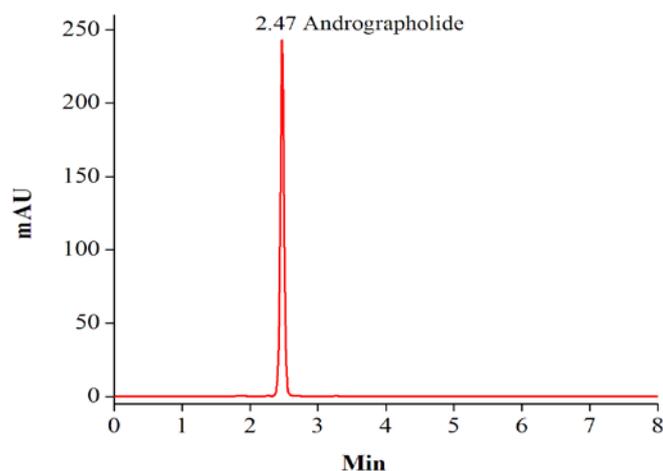


Fig. 4: Chromatogram of andrographolide standard

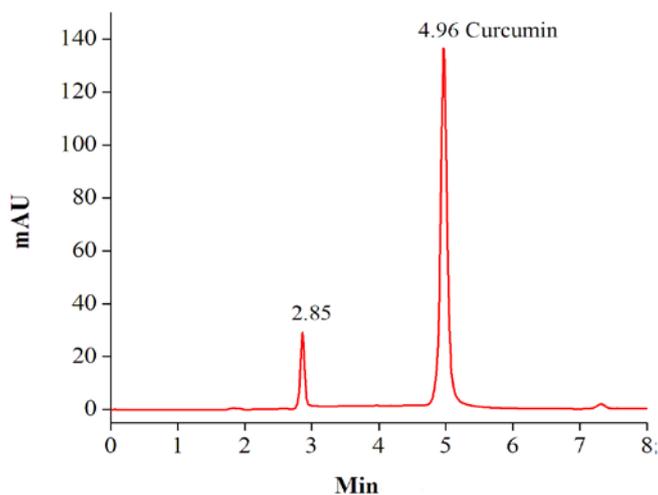


Fig. 5: Typical chromatogram of curcumin standard

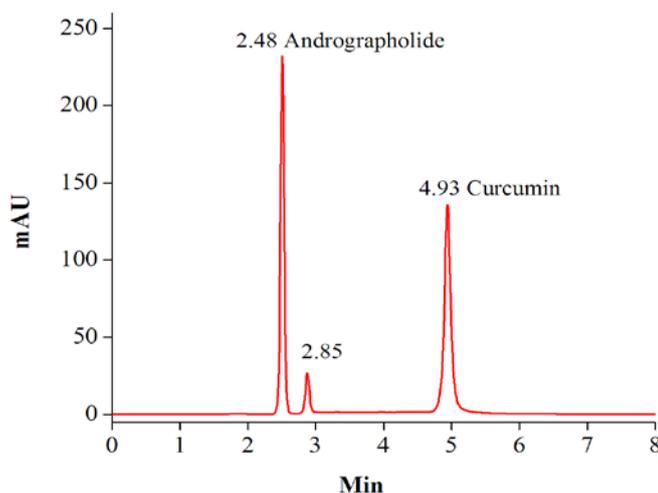


Fig. 6: Optimised chromatogram of andrographolide and curcumin in mixed standard

Method validation**Specificity**

Andrographolide and curcumin were eluted at 2.4 min and 4.9 min, respectively. The absence of interfering peaks from the NLC matrix is an evidence for method specificity. Furthermore, andrographolide and curcumin peaks were well separated from the degradation products after different stress degradation studies.

Linearity

Regression equations for andrographolide and curcumin were found to be $y = 42258x + 1170$ and $y = 44390x - 61969$, respectively. The linear concentration range was found to be 10-140 $\mu\text{g/ml}$ with a value of 0.999 for the regression coefficient for both the compounds. Calibration curves for andrographolide and curcumin are given in fig. 7.

Accuracy

The mixed standard concentration of andrographolide and curcumin was considered to be 50 $\mu\text{g/ml}$. The overall % recovery was found to be in the range of 97.83-99.67 for both andrographolide and curcumin, which indicated that the method was very accurate. Accuracy data for the andrographolide and curcumin are tabulated in table 3.

Precision

The relative standard deviation (%) of the same day (Intra) and another day (Inter) precision was found 0.085-0.520 for andrographolide and 0.128-0.625 for curcumin which was within the acceptable limit (RSD < 2 %). Precision results are summarised in table 4 and 5.

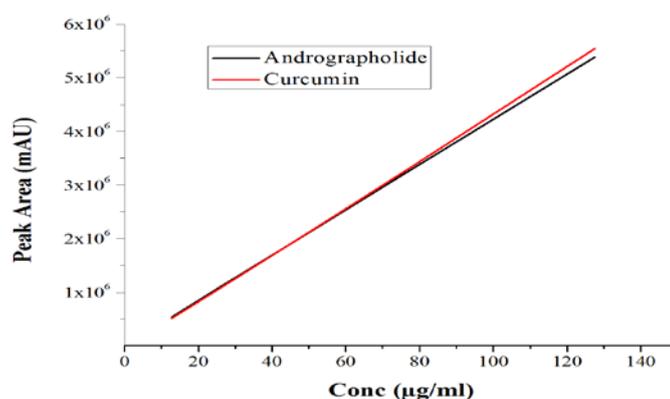


Fig. 7: Calibration curve of standard andrographolide and curcumin

Table 3: Accuracy data by the proposed HPLC method

Drugs	Spiked level (%)	Spiked amount (mg)	Recovered amount (mg)	Recovery (%)	RSD (%)
Andrographolide	80	8.3±0.15	8.25±0.12	99.39	0.82
	100	12.2±0.25	12.16±0.35	99.67	0.56
	120	15.4±0.31	15.2±0.21	98.70	0.31
Curcumin	80	8.40±0.16	8.32±0.17	99.04	0.45
	100	12.50±0.33	12.45±0.38	99.60	0.28
	120	15.50±0.23	15.30±0.25	98.70	0.61

RSD-Relative Standard Deviation; All the values are presented as mean±SD, n=3

Table 4: Intraday precision data by the proposed HPLC method

Drugs	Nominal concentration ($\mu\text{g/ml}$)	Recovered concentration ($\mu\text{g/ml}$)	Recovery (%)	Precision (Repeatability) %RSD
Andrographolide	12.5	12.12±0.83	96.96±1.02	0.120
	50	49.19±0.75	98.38±0.87	0.085
	125	124.36±0.91	99.48±0.16	0.096
Curcumin	12.5	12.21±0.68	97.68±0.92	0.312
	50	49.15±0.71	98.30±0.65	0.520
	125	124.22±0.57	99.37±0.35	0.136

RSD-Relative Standard Deviation; All the values are presented as mean±SD, n=6

Table 5: Interday precision data by the proposed HPLC method

Drugs	Nominal concentration ($\mu\text{g/ml}$)	Recovered concentration ($\mu\text{g/ml}$)	Recovery (%)	Precision (Repeatability) % RSD
Andrographolide	12.5	11.85±0.52	94.80±0.89	0.231
	50	48.92±0.67	97.84±1.03	0.128
	125	123.72±0.83	98.97±0.50	0.625
Curcumin	12.5	11.72±0.49	93.76±0.78	0.505
	50	48.73±0.78	97.46±0.69	0.212
	125	123.56±0.62	98.84±0.32	0.150

RSD-Relative Standard Deviation; All the values are presented as mean±SD, n=6

LOD and LOQ

The LOD for andrographolide and curcumin were 0.128 µg/ml and 1.008 µg/ml. The LOQ were 0.323 µg/ml for andrographolide and 3.054 µg/ml for curcumin, respectively.

Robustness

Robustness was determined by intentional modifications in method parameters like change in the rate of flow (± 0.1 ml/min), wavelength detection (± 2 nm), column oven temperature (± 2 °C), buffer: acetonitrile ratio and pH of buffer (± 0.1 unit) used in the mobile phase were done. The acquired result showed that the percent relative standard deviation (% RSD) values (< 2) remained within the acceptable limit, which indicates the developed HPLC method is highly robust. The obtained robustness results are presented in table 6.

System suitability

The resolution was found from mixed standards solution of andrographolide and curcumin to be 17.78 ± 0.06 , which specified the good separation of two peaks. The tailing factor was found to be 1.02 ± 0.02 and 1.01 ± 0.01 for andrographolide and curcumin, which represents the peaks were symmetric.

According to chromatographic peak results, the developed analytical method satisfies the criteria for system suitability [34, 35]. Parameters for system suitability are given in table 7.

Forced degradation studies

The percentage recovery and degradation study of andrographolide and curcumin after forced conditions are given in table 8.

Table 6: Robustness parameter of the proposed method

Parameters	% RSD of the area of andrographolide	% RSD of area of curcumin
A. Change in buffer pH of mobile phase		
2.9	0.061	0.052
3.0	0.081	0.521
3.1	0.056	0.610
B. Change in mobile phase composition (Buffer: Acetonitrile) (% v/v)		
48:52	0.136	0.196
50:50	0.142	0.256
52:48	0.164	0.604
C. Change in UV detector wavelength (nm)		
238	0.100	0.136
240	0.164	0.325
242	0.098	0.360
D. Change in flow rate (ml/min) of mobile phase		
1.4	0.053	0.024
1.5	0.125	0.226
1.6	0.035	0.258
E. Change in column oven tempt (°C) of mobile phase		
33	0.109	0.458
35	0.131	0.316
37	0.047	0.859

RSD-Relative Standard Deviation; All the values are presented as mean \pm SD, n=3

Table 7: System suitability parameters of the validated analytical method

Parameters	Andrographolide (mean \pm SD)	Curcumin (mean \pm SD)
Retention time	2.4 \pm 0.05	4.9 \pm 0.06
Theoretical plates/meter	9344 \pm 120	12628 \pm 114
Tailing factor	1.02 \pm 0.02	1.01 \pm 0.01
Resolution	-	17.78 \pm 0.06
Injection precision	0.089 \pm 0.03	0.07 \pm 0.04

SD-Standard Deviation; All the values are presented as mean \pm SD, n=6

Table 8: % Recovery and degradation of andrographolide and curcumin after stress conditions

Stress conditions	Recovery (%) (\pm SD)		Degradation (%) (\pm SD)	
	Andrographolide	Curcumin	Andrographolide	Curcumin
Acidic	94.99 \pm 0.59	83.42 \pm 1.21	5.01 \pm 2.1	16.58 \pm 0.95
Alkali	18.40 \pm 0.12	24.37 \pm 0.67	81.60 \pm 0.36	75.63 \pm 1.07
Peroxide	85.83 \pm 0.89	81.30 \pm 1.09	14.17 \pm 1.53	18.7 \pm 0.88
Photolytic	100.06 \pm 0.22	99.12 \pm 0.36	0 \pm 0.17	0.88 \pm 0.29
UV light	100.67 \pm 0.35	97.55 \pm 0.88	0 \pm 0.35	2.45 \pm 0.51
Thermal	99.05 \pm 0.11	99.28 \pm 0.30	0.95 \pm 0.16	0.72 \pm 0.23

SD-Standard Deviation; All the values are presented as mean \pm SD, n=3

Andrographolide was degraded by 0.1 (N) HCl (assay 94.99 %), 5 % v/v H₂O₂ (assay 85.83 %) and 0.1 (N) NaOH solution (assay 18.40 %).

Acid degradation of andrographolide produced nearly one degradation product; base degradation produced nearly two degradation products, peroxide degradation produced nearly two

degradations and thermal degradation produced about one degradation product.

Curcumin was found to be degraded by 0.1 (N) HCl (assay 83.42%), 5 % v/v H₂O₂ (assay 81.3 %) and 0.1 (N) NaOH solution (assay 24.37 %) and UV light (97.55 %) under all stressed conditions.

Acid degradation of curcumin produced about three degradation products, base degradation produced about three degradation products, peroxide degradation produced about three degradation products, photolytic degradation produced one degradation product and UV degradation produced one degradation product.

The UV absorption maxima of each degradation product of standard andrographolide and curcumin were produced at the specified retention time and determined by the HPLC with PDA detector. The retention time and UV absorption maxima for each degradation product produced are represented in table 9.

Table 9: Retention time and UV absorption maxima of degradation products of andrographolide and curcumin after stress conditions

Stress conditions	Degradation products RT (min)		Degradation products λ_{max} (nm)	
	Andrographolide	Curcumin	Andrographolide	Curcumin
Acidic	2.10	2.59	228	336
	-	2.8	-	358
	-	3.43	-	376
Alkali	1.79	1.46	220	330
	4.53	1.75	234	232
	-	1.97	-	336
Peroxide	3.51	2.26	230	322
	7.34	2.53	220	268
	-	2.83	-	350
Photolytic	-	4.07	-	302
	UV light	2.17	-	236
Thermal	3.51	-	252	-

RT-Retention time; λ_{max} -UV absorption maxima; nm-Nanometer, The HPLC chromatograms after forced degradation are represented in fig. 8-19, respectively.

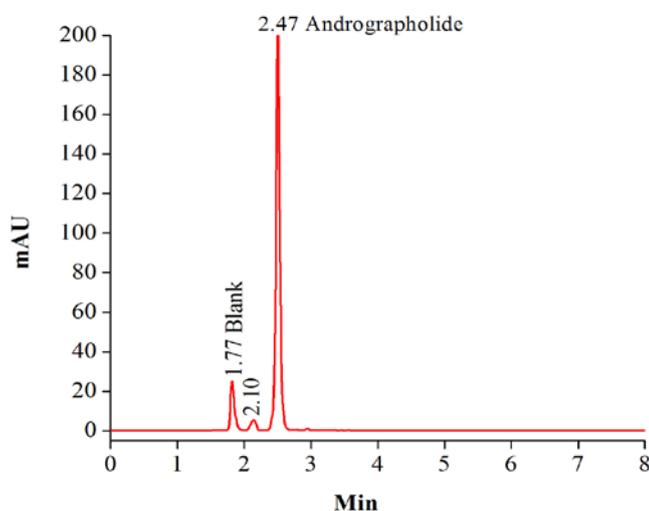


Fig. 8: Forced degradation chromatogram of standard andrographolide in 0.1(N) HCl

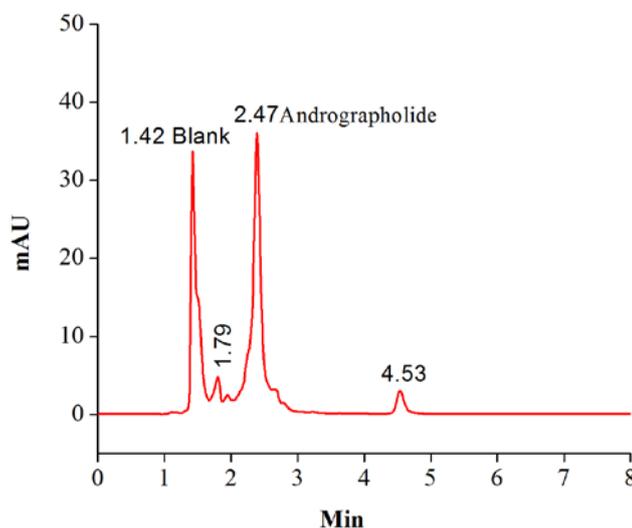


Fig. 9: Forced degradation chromatogram of standard andrographolide in 0.1(N) NaOH

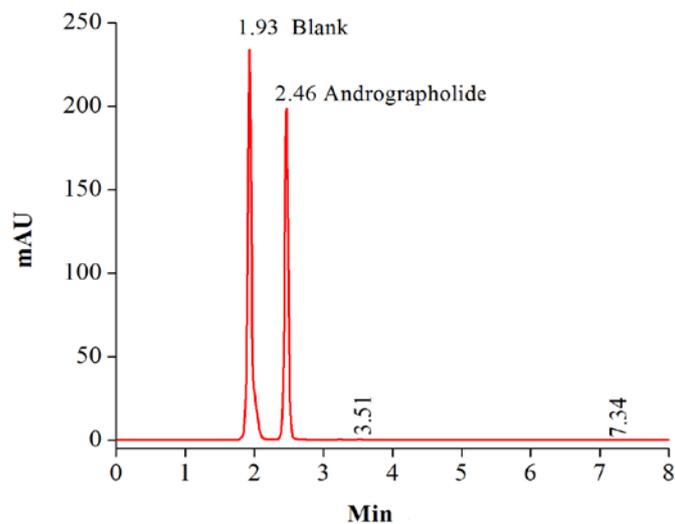


Fig. 10: Forced degradation chromatogram of standard andrographolide in 5 % v/v H₂O₂

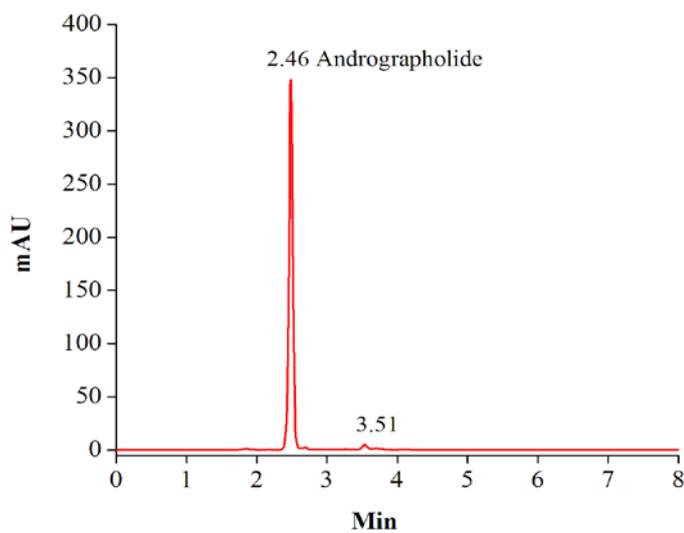


Fig. 11: Forced degradation chromatogram of standard andrographolide in heat

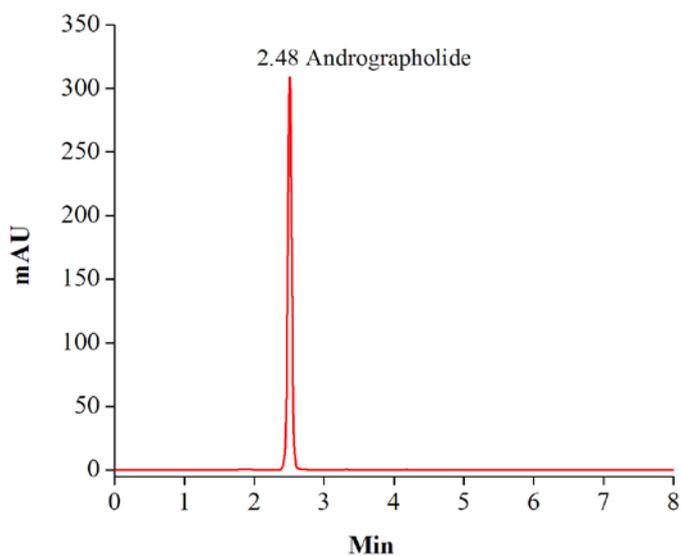


Fig. 12: Forced degradation chromatogram of standard andrographolide in photolytic

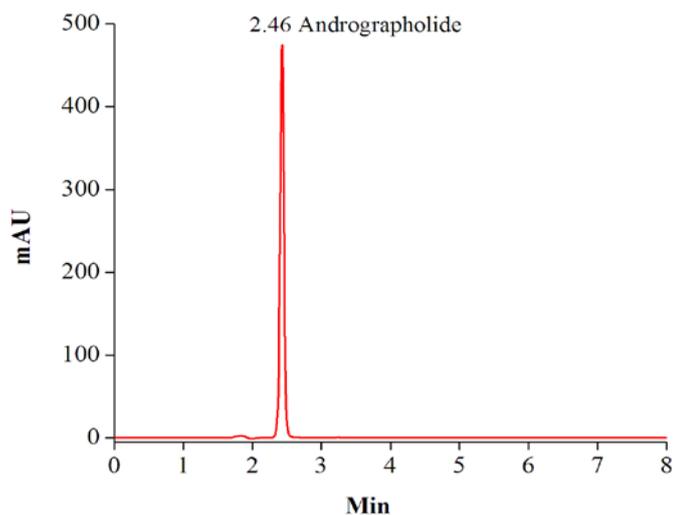


Fig. 13: Forced degradation chromatogram of standard andrographolide in UV light

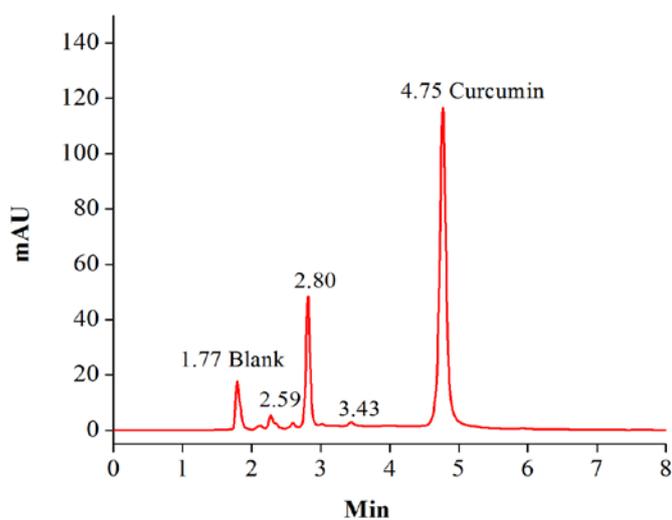


Fig. 14: Forced degradation chromatogram of standard curcumin in 0.1(N) HCl

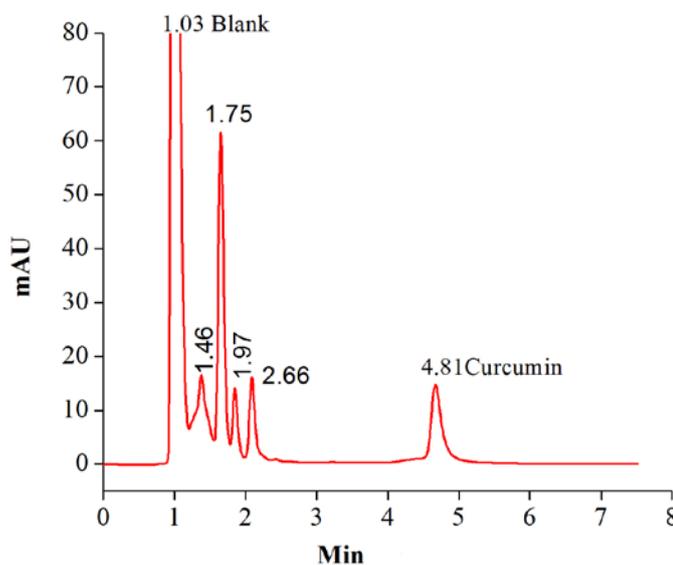


Fig. 15: Forced degradation chromatogram of standard curcumin in 0.1(N) NaOH

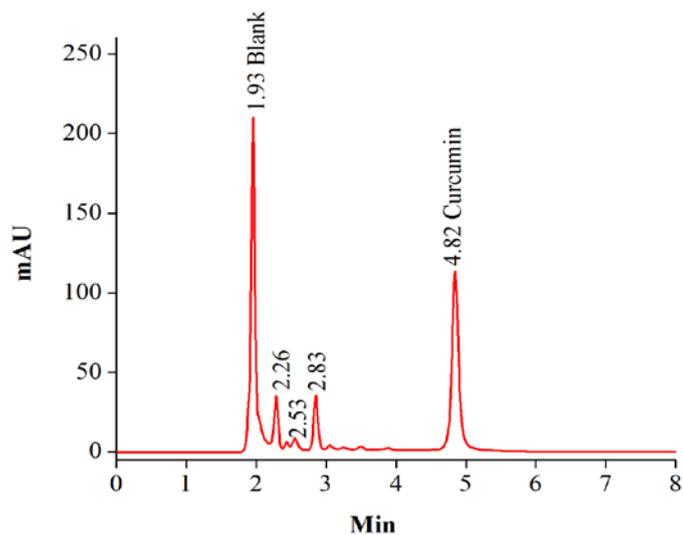


Fig. 16: Forced degradation chromatogram of standard curcumin in 5 % v/v H₂O₂

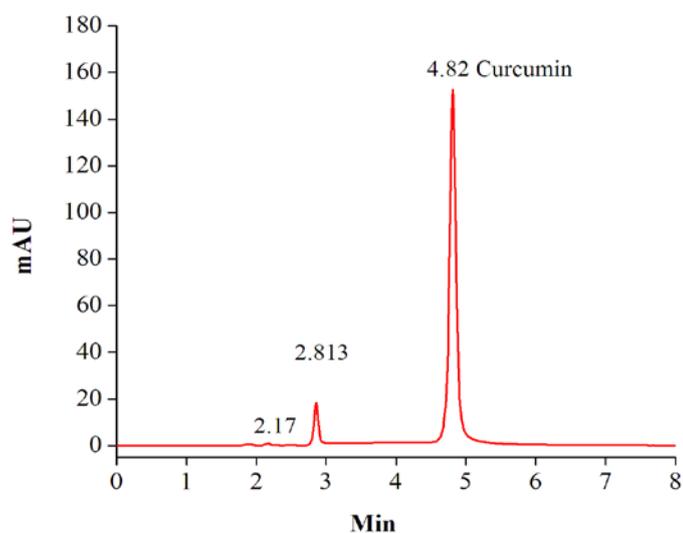


Fig. 17: Forced degradation chromatogram of standard curcumin in UV light

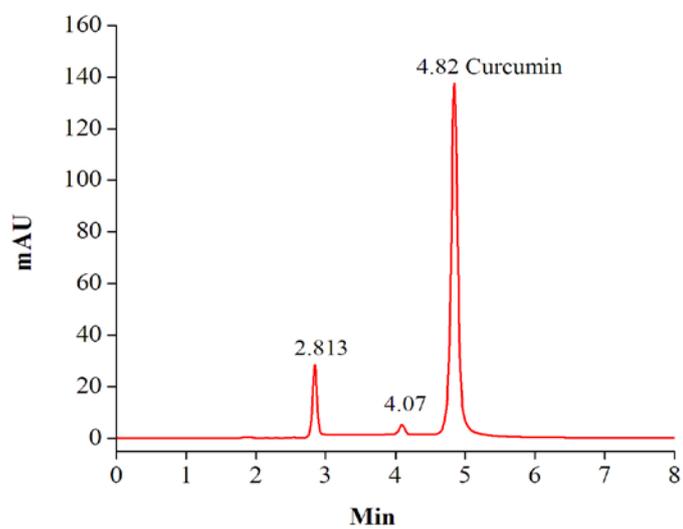


Fig. 18: Forced degradation chromatogram of standard curcumin in photolytic

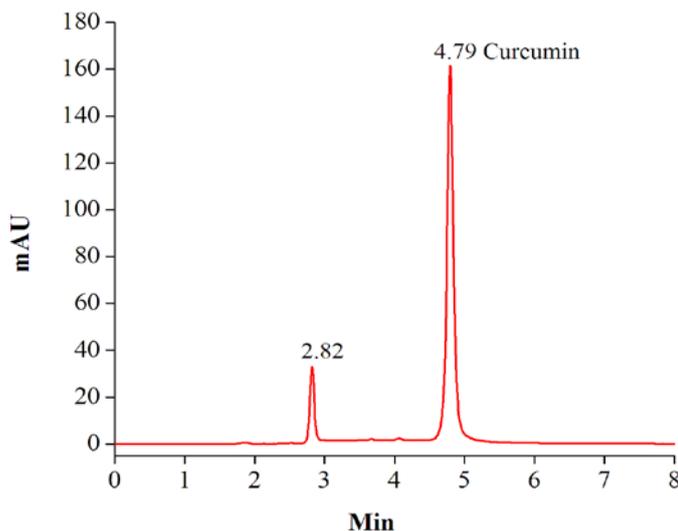


Fig. 19: Forced degradation chromatogram of standard curcumin in heat

Suggested method for application

Characterization of nanoparticles

The drug-loaded nanoformulation was shown the mean size of the particle of 145.6 nm, PDI value of 0.233 and the average zeta potential was found to be +6.64 mV, respectively. The particle size distribution of the developed nanoparticles is represented in table 10 and fig. 20.

Drug entrapment efficiency determination

The percent DEE was found to be 93.45±3.5 % for andrographolide and 94.82±2.8 % for curcumin. These results were calculated from the known concentration of a mixed standard solution of both drugs and no interference was observed from the NLC matrix. The results are represented in table 10. A typical chromatogram of andrographolide and curcumin in drug-loaded nanoparticles is shown in fig. 21.

Table 10: Characterization of andrographolide and curcumin entrapped nanoparticle

Nanoparticle formulation	Particle size diameter (nm)	PDI	Zeta potential value (mv)	%EE	
				Andrographolide	Curcumin
Andrographolide and curcumin loaded nanoparticles	145.6±5.26	0.233±0.03	+6.64±3.21	93.45±3.5	94.82±2.8

PDI-Polydispersity Index; EE-Entrapment Efficiency, All the values are presented as mean±SD, n=3

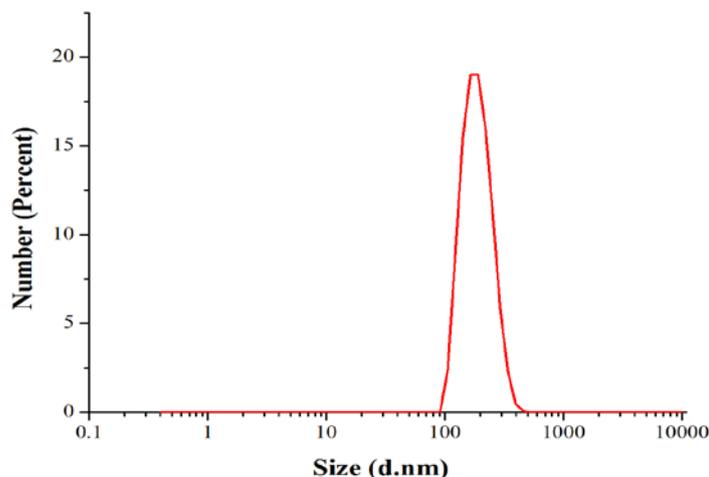


Fig. 20: Particle size distribution plot of andrographolide and curcumin co-loaded NLC

In vitro cumulative drug release study

The release profiles study of combined drug-loaded NLCs was carried out for 72 h at 37 °C. The cumulative percentage of drug release was 83.17±2.08 % for andrographolide and 81.61±2.17 %

for curcumin from drug-loaded NLCs. According to obtained results, the developed analytical method was successfully used to quantify both the drugs concurrently for *in vitro* study. Drug release profile pictures of andrographolide and curcumin from the formulated nanoparticles are presented in fig. 22.

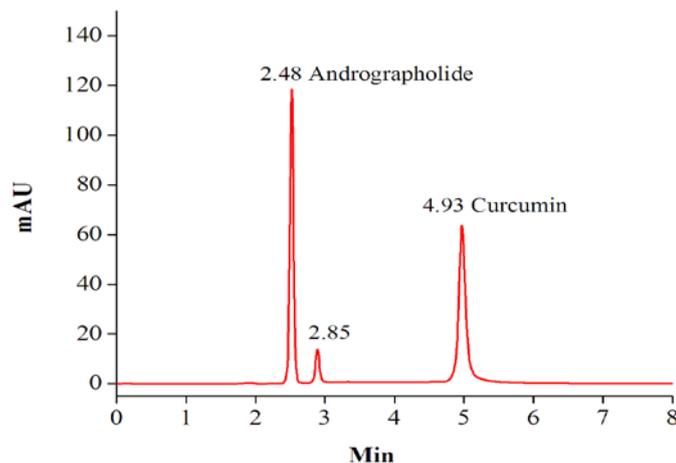


Fig. 21: Typical chromatogram of andrographolide and curcumin in drug-loaded nanoparticles

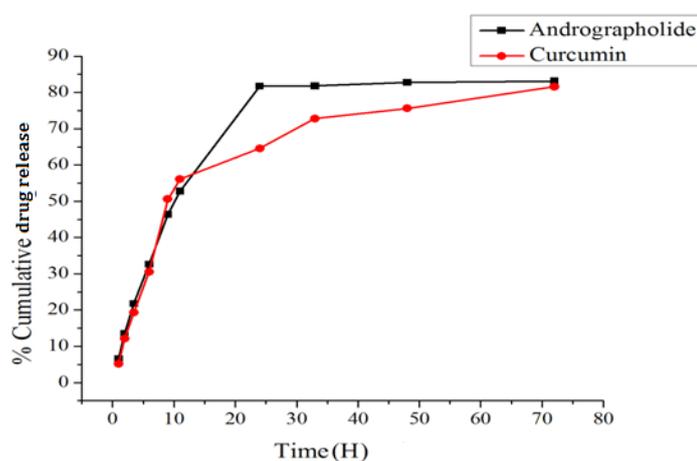


Fig. 22: Cumulative *in vitro* drug release profile of andrographolide and curcumin from co-encapsulated drug-loaded NLC (n=3)

Table 11: Recovery studies of the spiked andrographolide and curcumin from rat plasma

Drugs	LQC recovery (%)	MQC recovery (%)	HQC recovery (%)
Andrographolide	90.15±0.59	91.5±1.01	92.1±0.78
Curcumin	91.35±0.62	92.05±0.89	93.1±0.92

LQC-Lower Quality Control; MQC-Middle Quality Control; HQC-Higher Quality Control All the values are presented as mean±SD, n=3

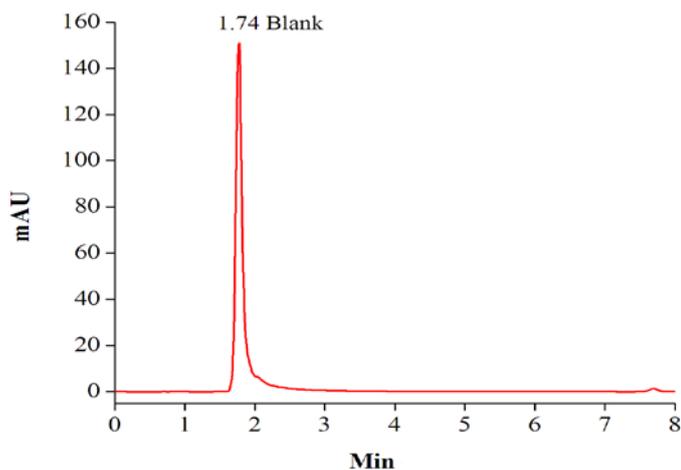


Fig. 23: Typical HPLC chromatogram of blank plasma

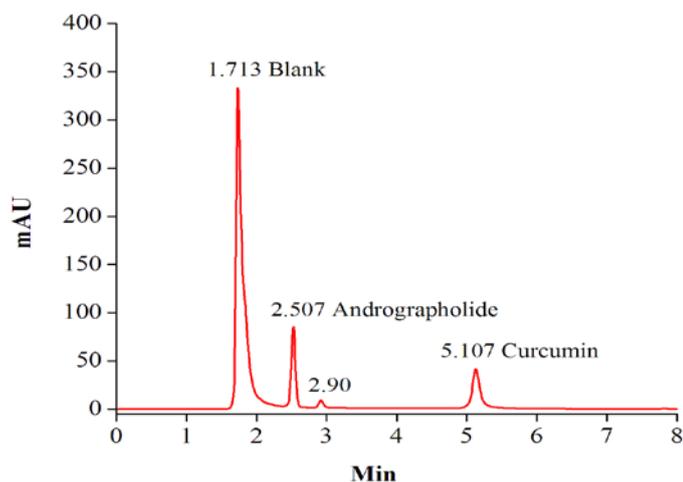


Fig. 24: Typical HPLC chromatogram of andrographolide and curcumin in plasma sample

Drug extraction recovery from plasma samples

Post extraction recovery of LQC, MQC, HQC were obtained 90.15 %, 91.5%, 92.1 % for andrographolide and 91.35 %, 92.05 %, 93.1 % for curcumin. These results indicate that this developed method can be used for a wide range of sample analyses [36]. These results are provided in table 11. The chromatogram of blank plasma and chromatogram of andrographolide and curcumin in mixed standard with plasma are presented in fig. 23 and 24.

CONCLUSION

The proposed HPLC method was unique and developed for the quantification of andrographolide and curcumin concurrently in newly developed NLCs and spiked rat plasma. The method was fully satisfied by validation parameters and specific as all degradation products produced after forced degradation study was found to be well separated from the principal peaks and all the degradation product's UV absorption was determined by HPLC PDA detector. In the robustness study, a low percent RSD value was found under varied chromatographic parameters. The method furnishes simplicity as per short retention time, isocratic mode of elution, straightforward sample preparation method, broad concentration span, low detection or quantification values and good resolution for both drugs with acceptable system suitability criteria. Moreover, the suggested HPLC method of andrographolide and curcumin is relevant for *in vivo* pharmacokinetic studies.

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AUTHORS CONTRIBUTIONS

All authors have contributed equally.

CONFLICTS OF INTERESTS

The authors have declared no conflicts of interest in publishing the paper.

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