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Article

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A Rapid and Sensitive HPLC Method for Simultaneous Determination of Irinotecan Hydrochloride and Curcumin in Co-delivered Polymeric Nanoparticles

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Abstract

In recent years, a great deal of attention has been paid to the combined use of multiple antitumor drugs for better cancer treatment. The aims of the study are to construct a nanoparticle drug delivery system for the co-delivery of irinotecan hydrochloride and curcumin and to develop an analytical method for simultaneously quantifying these molecules, which is essential for further studies of the co-delivered nano system. The irinotecan hydrochloride and curcumin co-delivered nanoparticle (ICN) were prepared by combinatorially entrapping them into polyethylene glycol–poly lactic acid-co-glycolic acid (PEG–PLGA) polymeric nanoparticles. A simple, sensitive and rapid high-performance liquid chromatography method was developed and validated to simultaneously quantify the compounds in the co-delivered nanoparticle system. Acetonitrile and ultrapure water containing sodium dodecyl sulfate (0.08 mol/L), disodium phosphate (Na_2HPO_4, 0.002 mol/L) and acetic acid (4%, v/v) were used as the mobile phase and their ratio was set at 50:50. The flow rate was set to 1.0 mL/min, and the temperature in the column oven was maintained at 40°C. The analysis was carried out at 256 and 424 nm to assess irinotecan hydrochloride and curcumin, respectively. Detectors with only one channel can also visualize both analytes in one chromatogram at 379 nm and still demonstrate acceptable sensitivity. The retention times for irinotecan hydrochloride and curium were 3.317 and 5.560 min, respectively. The method developed was confirmed to be sensitive, accurate (recovery, 100 ± 2%), precise (relative standard deviation, RSD ≤ 1%), robust and linear ($R^2 \geq 0.9996$) in the range from 2.05 to 1050 μg/mL. The presented method has been used to quantify irinotecan hydrochloride and curcumin in the co-delivered ICN nano system to assess the drug delivery quality of the nanoparticles and can also be used for routine analysis because of its simplicity and accuracy.

Introduction

Combining multiple antitumor drugs in the treatment of cancer has been demonstrated as one of the most effective ways to reduce the side effects of drugs, overcome multiple drug resistance (MDR) and improve the therapeutic efficacy of the same (1, 2). Many novel pharmaceutical systems have been designed to simultaneously deliver a number of active ingredients to achieve synergistic effects in cancer therapy (3–5).

Irinotecan hydrochloride is a potent inhibitor of topoisomerase I, which has been utilized to treat various cancers, especially colorectal and lung conditions (6). Irinotecan is prone to interconversion

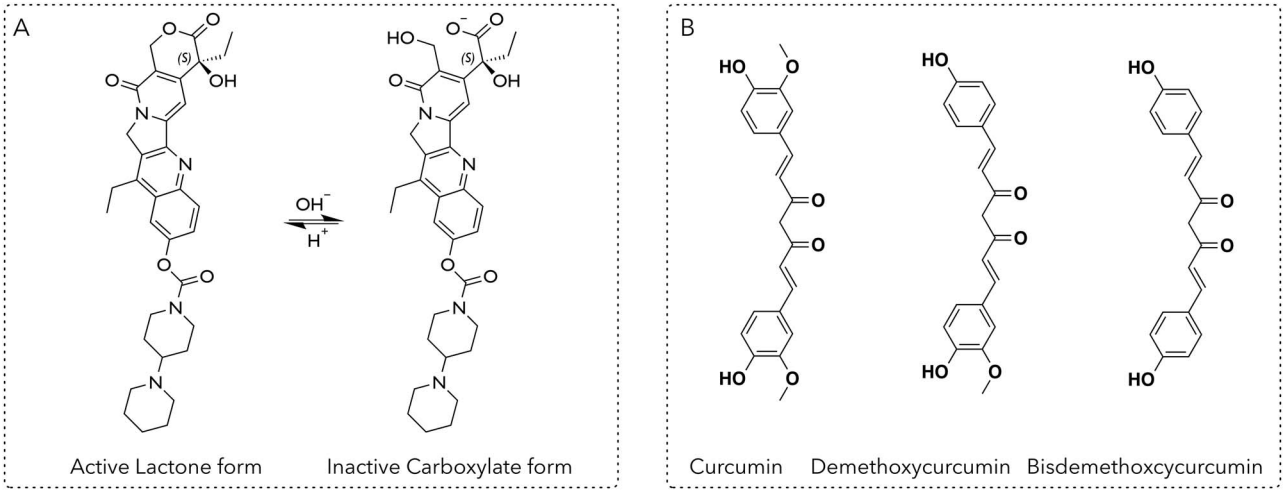

Figure 1. Chemical structures of irinotecan and curcuminoids. (A) Irinotecan undergoes pH-dependent hydrolysis to form the pharmacologically inactive carboxylate form; (B) Three representative components of curcuminoids.

between its carboxylate and lactone forms (Figure 1A), exhibited through changes in the pH of the molecular environment; notably, only the closed lactone form is pharmacologically active (7).

Curcuminoids are naturally hydrophobic polyphenols found in the extract of *Curcuma longa*, a medicinal plant widely used in Asian countries (8). As shown in Figure 1B, they contain three main components, namely curcumin, demethoxycurcumin and bisdemethoxycurcumin. These molecules evince an intrinsic anticancer effect and other pharmacological properties, such as anti-inflammatory, antioxidant and antimicrobial qualities (9). But their clinical use has been very limited due to the extremely low water solubility (10).

When combined with curcuminoids, the anticancer effect of irinotecan hydrochloride can be heightened by regulating relevant pathways inside cancer cells (11, 12), or by furthering the apoptosis of cancer cells (13). Co-delivering irinotecan hydrochloride and curcuminoids via nanotechnologies could represent a good strategy to overcome the drawbacks of the two molecular species as well as to achieve an enhanced cancer treatment. The authors, however, are unaware of any studies in the literature on the co-delivery and simultaneous determination of these compounds despite that many separate nano systems (14, 15) and analytical methods for both molecular species (16–18) have been reported.

The present study prepares a nanoparticle drug delivery system for the co-delivery of irinotecan hydrochloride and curcuminoids and provides a high-performance liquid chromatography (HPLC) method for simultaneously separating and analyzing these molecules. Chromatographic conditions are optimized to separate and quantify the compounds by adjusting the composition of the mobile phase and selecting UV-Vis wavelengths suitable for detection. The analysis was carried out at 256 and 424 nm to assess irinotecan hydrochloride and curcumin, respectively. Detectors with only one channel can also visualize both analytes in one chromatogram at 379 nm and still demonstrate acceptable sensitivity. The method has proven to be sensitive, accurate (recovery, 100 ± 2%), precise (relative standard deviation, RSD ≤ 1%), robust and linear ($R^2 \geq 0.9996$) in the range from 2.0 to 1050 μg/mL. The presented method has been used to quantify irinotecan hydrochloride and curcumin in the co-delivered irinotecan hydrochloride and curcumin co-delivered nanoparticle (ICN) nano system to assess the drug delivery quality of the nanoparticles and can also be used for routine analysis with related analytes.

Materials and Methods

Materials and instrumentation

Curcumin (curcuminoid content ≥94%, curcumin ≥80%), irinotecan hydrochloride (≥ 98% HPLC, powder), acetonitrile (ACN, HPLC grade), methanol (MeOH, HPLC grade), dimethyl sulfoxide (DMSO, HPLC grade), acetic acid (HPLC grade), hydrochloride, sodium dodecyl sulfate (SDS, ≥ 98% GC, powder) and polyethylene glycol–poly lactic acid-co-glycolic acid ($PEG_{Mn\ 2000}$–$PLGA_{Mn\ 4500}$,) were purchased from Sigma-Aldrich (Czech Republic) and utilized as received. Disodium phosphate (DSP, Na_2HPO_4) was provided by PENTA s.r.o. (Czech Republic). Ultrapure water was prepared in laboratory using a Milli-Q integral water purification system. Spectroscopic analyses were performed on a DIONEX UltiMate 3000 HPLC system, equipped with an LPG-3400SD Standard Quaternary Pump and a DAD-3000 Diode Array Detector (DAD).

Nanoparticle preparation

The ICN were prepared via an antisolvent method using PEG–PLGA as drug carriers. Briefly, curcumin (1.0 mg), irinotecan hydrochloride (1.0 mg) and PLGA-PEG (10 mg) were dissolved in DMSO (1.0 mL), which was then added dropwise into ultrapure water (10 mL) under stirring (400 RPM, 10 min). Nanoparticle water suspension was obtained after removing organic solvent by dialysis against ultrapure water (Molecular Weight Cut-Off of dialysis membrane, 3000). The hydrodynamic sizes and surface charges of nanoparticles were recorded by dynamic light scattering (DLS).

Assessment of drug delivery quality

To assess the drug delivery quality of this nanoparticle co-delivery system, ICN nanoparticle suspensions with or without centrifugation (10,000 RPM, 10 min) were diluted with ACN before being injected into HPLC to determine any difference in drug content prior to and following centrifugation. The drug-loading capacity (DL %), drug entrapment efficiency (EE %) and drug molar ratio (DR) were calculated according to the following equations:

$$\text{DL (\%, irinotecan hydrochloride)} = (I_{1\ or\ 2})/(I_0 + P_0) \times 100\% \quad (1).$$

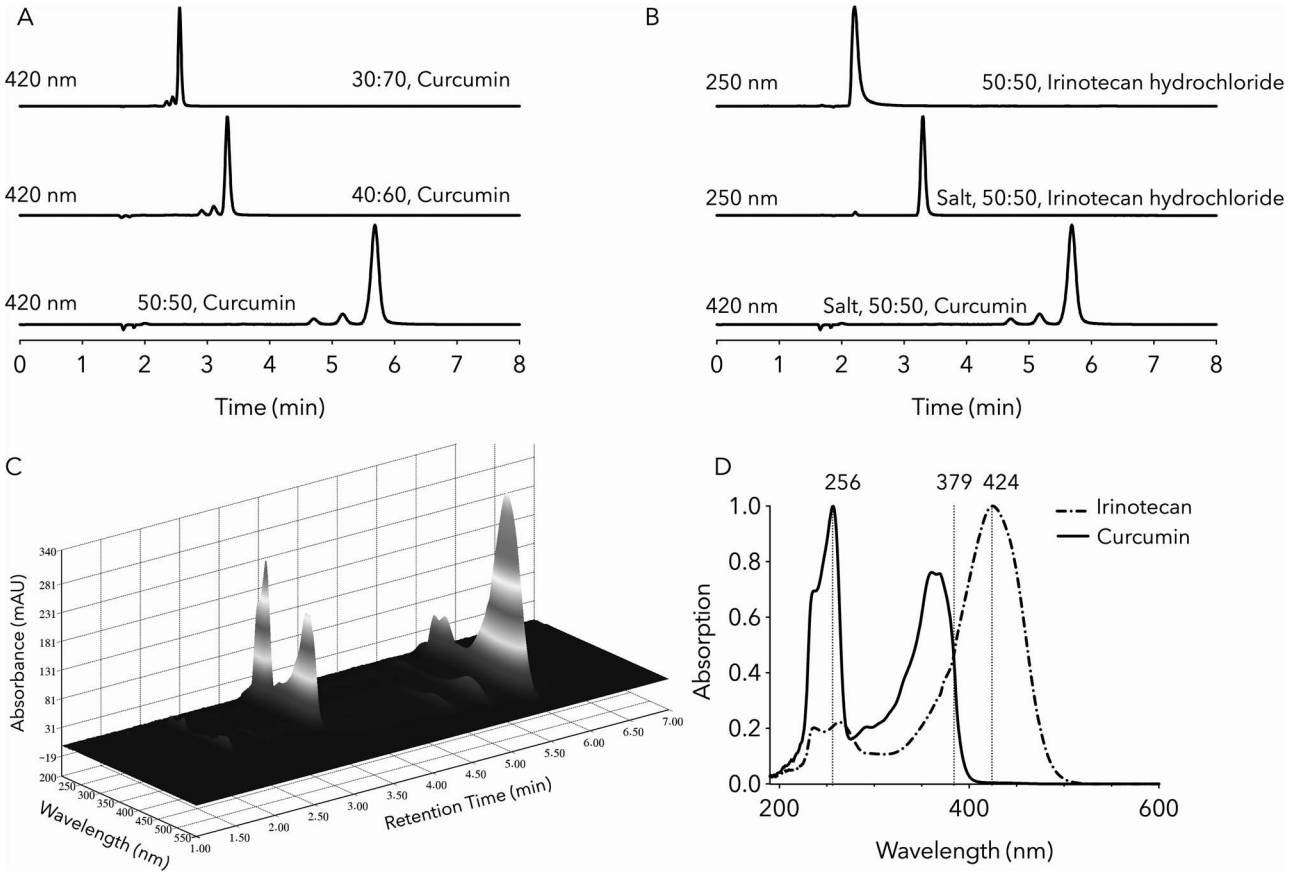

Figure 2. The optimization of chromatographic conditions. (A) Curcuminoids were eluted with acetonitrile and water (containing 4% acetic acid). Three curcuminoids can be well separated by decreasing the amount of acetonitrile from 70 to 50%; (B) Adding salts (sodium phosphate and sodium dodecyl sulfate) overcomes the peak tailing of irinotecan and does not affect the elution of curcuminoids; (C) 3D UV-Vis absorption of irinotecan and curcuminoids; (D) Overlap of UV-Vis absorption of irinotecan and curcumin.

DL (%, curcumin) = $(C_{1\text{ or }2})/(C_0 + P_0) \times 100\%$ (2).

EE (%, irinotecan hydrochloride) = $I_{1\text{ or }2}/I_0 \times 100\%$ (3).

EE (%, curcumin) = $C_{1\text{ or }2}/C_0 \times 100\%$ (4).

DR = molar amount of C_2/molar amount of I_2 (5).

Above, DL represents the drug-loading capacity, EE represents the drug entrapment efficiency and DR means drug molar ratio. *C* stands for the weight of curcumin, *I* represents the weight of irinotecan hydrochloride and *P* represents the weight of polymer PEG–PLGA. The right subscript 0 represents the amount of relevant drug molecule initially added; the right subscripts 1 and 2 represent the amount of relevant drug molecule before and after centrifugation, respectively.

Preparation of stock solution

Curcumin stock solution (1.05 mg/mL) was prepared by accurately weighing 10.5 mg of curcumin and dissolving it into 10 mL of ACN. Irinotecan hydrochloride stock solution (1.05 mg/mL) was prepared by weighing 10.5 mg of irinotecan hydrochloride and dissolving it into 10 mL of ACN. Additionally, to prepare a combined stock solution containing both curcumin and irinotecan hydrochloride, 10.5 mg of curcumin and 10.5 mg of irinotecan hydrochloride were accurately weighed and dissolved into 10 mL of ACN. All the prepared stock solutions were stored at 4°C in darkness for further use.

Development of analytical method

Chromatographic separation was carried out on a C_{18} column (Kinetex 2.6 μ C_{18} 100A, 150 mm × 4.6 mm) fitted with a pre-column (WATREX 50 mm × 4 mm, ReproSil 100 C_{18}, 5 μm). ACN and ultrapure water containing DSP (0.002 mol/L), SDS (0.08 mol/L) or acetic acid (4%, v/v) were used as the mobile phase. The flow rate was set to 1.0 mL/min, and the temperature in the column oven was maintained at 40°C. The volume injected for all injections equaled 0.5 μL. The UV-Vis absorption of curcumin or irinotecan hydrochloride was determined by HPLC, on the aforementioned system equipped with a DAD detector.

Validation of analytical method

Validation of the method was carried out according to current guidelines issued by the International Conference on Harmonization (ICH), ref. Q2 (R1) (ICH, 2017) (19).

Specificity. Blank ACN, standard curcumin solution, standard irinotecan hydrochloride solution, mixed irinotecan hydrochloride curcumin solution and ICN nanoparticles diluted with ACN were injected into HPLC so as to determine the specificity of the analytical method.

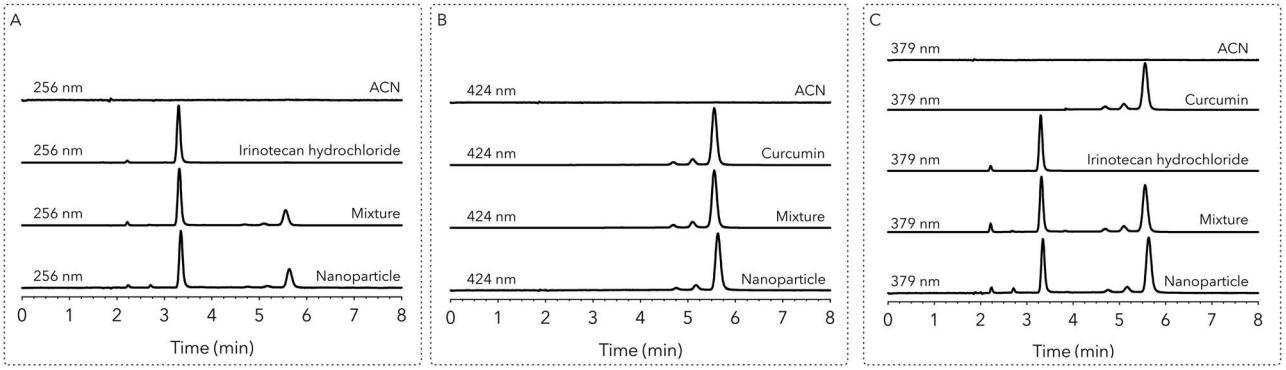

Figure 3. Specificity for detecting irinotecan and/or curcuminoids in different forms under various wavelengths. (A) Specificity for detecting irinotecan at 256 nm; (B) Specificity for detecting curcuminoids at 424 nm; (C) Specificity for detecting both irinotecan and curcuminoids at 379 nm.

Linearity and range. Serial dilutions were prepared of a mixture of curcumin and irinotecan hydrochloride in ACN at concentrations ranging from 2.05 μg/mL to 1.05 mg/mL. The calibration curve was plotted in triplicate, displaying peak area versus concentration. Analysis of least square regression was conducted on the data obtained and analysis of variance (ANOVA) test ($\alpha = 0.05$) was used to assess the regression significance.

Sensitivity. The sensitivity of the analysis method was observed with respect to the limit of detection (LOD) and limit of quantitation (LOQ). The LOQ was determined as the first concentration of calibration curves. The LOD was estimated based on the standard deviation (σ) of y-intercepts and the slopes (s) of the regression lines. The equations are represented below:

$$\text{LOD} = 3.3\,\sigma/\text{s} \quad (6).$$

Accuracy. To evaluate the accuracy of the method, a recovery experiment was performed. Three different levels (30, 200 and 500 μg/mL) of curcumin and irinotecan hydrochloride were added to the matrix samples: ACN and nanoparticle ACN solution. Their responses were estimated from the relevant calibration curve. Accuracy was determined by recovery with a relative error based upon actual and estimated concentrations.

Precision. Repeatability was analyzed by injecting three different levels (30, 200 and 500 μg/mL) of curcumin and irinotecan hydrochloride solution into the HPLC system in three replicates within a single day. Intermediate precision was also determined by analyzing the same three concentrations of curcumin and irinotecan hydrochloride solutions on 2 days in five replicates. RSD (%) with a confidence interval pertaining to the estimated concentrations was calculated for each set of data according to the calibration curve.

Robustness. The robustness of the proposed method was determined by changing a chromatographic condition, such as the mobile phase ratio (±0.5%), flow rate (±0.1 mL/min) and column temperature (±3°C). Figures for mean recovery (± % confidence interval) as well as relative error were reported.

Results

Development of analytical method

Selection of solvents

Curcuminoids eluted with ACN and water containing 4% acetic acid are shown in Figure 2A. The detective wavelength of curcuminoids used during solvent selection was set at 420 nm according to the literature report (20). When the ratio of ACN in the mobile phase decreases from 70 to 50%, three curcuminoids can be well separated and the retention time for bisdemethoxycurcumin, demethoxycurcumin and curcumin were 4.790, 5.100 and 5.560 min, respectively. Besides, the resolutions of two adjacent peaks stand at 2.30 and 2.41, respectively. In addition, the asymmetry factor of 1.00 ± 0.10 demonstrates good symmetry of the chromatographic peaks.

Irinotecan hydrochloride was also eluted with the same mobile phase and detected at 250 nm (21). However, as shown in Figure 2B, a tailing peak with an asymmetric factor of 2.01 is observed. Adding DSP (0.002 mol/L) and SDS (0.08 mol/L) into water increases the retention time of irinotecan hydrochloride from 2.187 to 3.317 min, and the asymmetric factor decreases to an acceptable value of 1.16 (22). The addition of DSP and SDS in the mobile phase keeps irinotecan hydrochloride in its neutral form and gets rid of the peak tailing. Besides, adding salts does not affect the elution of curcuminoids, hence the mobile phase comprising 50% ACN and 50% water containing DSP (0.002 mol/L), SDS (0.08 mol/L) and acetic acid (4%, v/v) was chosen as the mobile phase for the simultaneous separation and quantification of irinotecan hydrochloride and three curcuminoids.

Selection of wavelength. Figure 2C shows 3D UV-Vis absorbance profile of irinotecan hydrochloride and curcuminoids, within which the representative chromatographic peaks are well separated from each other. In order to obtain far more sensitivity and a high degree of precision, the maximum UV-Vis absorption at 256 and 424 nm (Figure 2D) were chosen to detect irinotecan hydrochloride and curcumin, respectively. Since overlap exists in the UV-Vis absorption spectra of irinotecan hydrochloride and curcumin, 379 nm was selected for detecting both irinotecan hydrochloride and curcumin in one chromatogram concurrently.

Validation of analytical method

System suitability test. An assay was performed in duplicate six times, the detection wavelength having been set to 379 nm. The statistical results of the suitability study for the given system are displayed in Table I.

Specificity. The chromatograms of the blank solution, irinotecan hydrochloride, curcumin and the mixture at 256, 424 and 379 nm are given in Figures 3A, B and C, respectively. Within these, each compound is verified as not interfering with one another.

Table I. System Suitability Results (379 nm, $n = 6$)

Parameters	Acceptable criterion	Irinotecan	Bisdemethoxycucumin	Demethoxycucumin	Curcumin
Retention time (min)	N.A.	3.317 ± 0.001	4.795 ± 0.002	5.100 ± 0.001	5.560 ± 0.001
Precision of retention time	RSD (%) ≤ 1%	0.03	0.00	0.02	0.02
Precision of peak area	RSD (%) ≤ 2%	0.63	0.62	0.96	0.68
Asymmetry factor (European pharmacopoeia)	0.95–1.05	1.02 ± 0.01	1.03 ± 0.01	1.03 ± 0.01	1.01 ± 0.01
Plate number (N)	N ≥ 2000	9785 ± 67	11943 ± 102	11673 ± 83	12197 ± 67
Resolution (European pharmacopoeia)	≥ 2.0	9.12	2.33	2.35	N.A.

Linearity and range. The assay for each standard was performed in duplicate three times and the representative chromatograms of irinotecan hydrochloride and curcuminoids detected at different wavelengths are shown in Figure 4. The linearity of the detector response for the standards was analyzed by least-square regression method and the regression equations for the calibration curves are displayed below:

For irinotecan hydrochloride detected at 256 nm:

$Y = 0.02061 * X + 0.1129$, $R^2 = 0.9997$ and $F = 24238$.

For curcumin detected at 424 nm:

$Y = 0.03954 * X + 0.007464$, $R^2 = 0.9996$ and $F = 18881$

For irinotecan detected at 379 nm:

$Y = 0.01275 * X + 0.08087$, $R^2 = 0.9997$ and $F = 21810$

For curcumin detected at 379 nm:

$Y = 0.01619 * X + 0.01796$, $R^2 = 0.9996$ and $F = 19268$

The results showed that the squares of the linear correlation coefficients (R^2) were above 0.999, indicating the good linearity of the calibration curves. ANOVA of regression showed that obtained F values ($F_{obtained}$) are far superior to the critical value ($F_{critical} = 5.5914$) ($F_{critical} < < F_{obtained}$), which demonstrates that the linear regression is significant and the method is linear over the whole tested concentration range (23). The validity of the assay was verified by means of ANOVA, which showed that there is linear regression with no deviation from linearity ($P < 0.001$) and can be used for the quantification of irinotecan hydrochloride and curcumin in the tested range.

In addition, the regression coefficients of equations at 256 and 424 nm, representing the rate of change of one variable (Y) as a function of change in the other (X), are larger than those at 379 nm, demonstrating the greater sensitivity of signal response when altering the concentration of the analytes.

Sensitivity. The calculated method LODs for irinotecan hydrochloride at 256 nm and curcumin at 424 nm equal 2.12 and 4.94 ng/mL, respectively, hence are lower than those at 379 nm (6.26 ng/mL for irinotecan hydrochloride and 8.71 ng/mL for curcumin), indicating higher sensitivity. The LOQ value was taken as the lowest concentration of calibration curves that could be quantitatively measured, namely 2.05 μg/mL.

Accuracy. Table II details results on the accuracy of the analytical method. The overall recovery (%) determined by multiple analysis is within 101.07 ± 0.54, and the RSD values for the experimental data are less than 1%.

Precision. The precision of this analytical method describes the degree of accord between a series of data from an identical sample (24). Such precision has been evaluated at three different levels, and the results of inter- and intra-day precision are expressed as RSD (in percent) of a statistically monumental number of experimental samples, as shown in Tables III and IV, respectively.

The values of RSD for validation of precision were found to be less than 1%, which demonstrates good agreement between the experimental data obtained from multiple analysis of the same sample.

Robustness. Table V details results for robustness under differing chromatographic conditions. The variations in column temperature, flow rate and mobile phase ratio within given limits that were exhibited engendered mean recoveries (%) ranging between 98.0 and 102.0, while the maximum RSD (%) equaled 1.50, indicating it to be a sufficiently robust method.

Application of analytical method

As shown in Figure 5, the average hydrodynamic size of ICN nanoparticles in water is 210.7 ± 0.9074 nm, with a polydispersity index (PDI) of 0.206 ± 0.013; hence sufficient to instigate enhanced permeability and retention (EPR) for the treatment of solid cancers. The surface Zeta Potential of ICN nanoparticles in water suspension stood at −24.19 ± 0.954 mV, indicating good stability under normal conditions.

Regarding HPLC analysis, as shown in Figure 3, no unexpected peaks or variations in the chromatograms were found, this being an analytical method considered specific to and practicable for simultaneously quantifying both curcumin and irinotecan hydrochloride in the given ICN nanoparticle water suspensions. Variations in drug content in the nanoparticles preceding and subsequent to centrifugation are presented as DL (%) and EE (%), as displayed in Figure 5. DL reflects the amount of drug delivered per amount encapsulated and EE is the percentage of drug that is successfully entrapped into the nanoparticles. No significant differences were discerned between either group, indicating that the prepared ICN nanoparticles were uniformly distributed and suspended in water and that the irinotecan hydrochloride and curcumin molecules are not adsorbed on the nanoparticle surface. The loss of drugs should be because of the operation during preparation and harvest.

Discussion

In the present study, a drug co-delivery polymeric nanoparticle system based on irinotecan hydrochloride and curcumin was constructed. Their drug loading and entrapment quality was assessed based on the established HPLC method. Optimization of HPLC conditions was performed by adjusting the mobile phase and selecting

Table II. Accuracy of the Analytical Method

Spike level (μg/mL)	Measured concentration (μg/mL) from ACN solution				Measured concentration (μg/mL) from Nanoparticle ACN solution			
	Irinotecan (256 nm)	Curcumin (424 nm)	Irinotecan (379 nm)	Curcumin (379 nm)	Irinotecan (256 nm)	Curcumin (424 nm)	Irinotecan (379 nm)	Curcumin (379 nm)
30.00	30.18	30.97	30.16	30.22	30.48	30.47	30.26	30.22
	30.11	30.75	30.51	30.71	30.41	30.35	30.41	30.31
	30.19	30.72	30.39	30.44	30.59	30.52	30.69	30.54
RSD (%)	0.14	0.64	0.59	0.81	0.30	0.29	0.72	0.54
Recovery (%)	100.53 ± 0.15	101.82 ± 0.62	101.18 ± 0.59	101.52 ± 0.82	101.64 ± 0.30	101.49 ± 0.29	101.51 ± 0.73	101.19 ± 0.55
200.00	200.35	202.79	203.52	204.19	201.37	203.31	205.52	204.19
	201.53	202.54	203.98	203.53	202.4	204.35	203.98	203.53
	199.86	200.42	201.52	201.23	203.66	201.72	202.52	201.23
RSD (%)	0.43	0.64	0.64	0.77	0.57	0.65	0.74	0.77
Recovery (%)	100.29 ± 0.43	100.96 ± 0.65	101.50 ± 0.65	101.49 ± 0.78	101.24 ± 0.57	101.56 ± 0.66	102.00 ± 0.75	101.49 ± 0.78
500.00	501.52	502.18	503.33	500.14	502.42	501.34	503.26	501.74
	501.76	503.26	505.88	501.37	504.13	503.42	505.71	502.94
	502.49	501.68	500.12	503.34	504.71	501.59	499.95	505.43
RSD (%)	0.10	0.16	0.57	0.32	0.24	0.23	0.57	0.37
Recovery (%)	100.38 ± 0.10	100.47 ± 0.16	100.62 ± 0.58	100.32 ± 0.32	100.75 ± 0.24	100.42 ± 0.23	100.59 ± 0.58	100.67 ± 0.38

Accuracy acceptance criteria, recovery (%), 100 ± 2

Table III. Intra-day Precision of the Analytical Method in Matrices (ACN solution and Nanoparticle solution)

Spike level (μg/mL)	Measured concentration (μg/mL) from ACN solution				Measured concentration (μg/mL) from Nanoparticle ACN solution			
	Irinotecan (256 nm)	Curcumin (424 nm)	Irinotecan (379 nm)	Curcumin (379 nm)	Irinotecan (256 nm)	Curcumin (424 nm)	Irinotecan (379 nm)	Curcumin (379 nm)
30.00	30.16 ± 0.04	30.81 ± 0.14	30.35 ± 0.18	30.46 ± 0.25	30.49 ± 0.09	30.45 ± 0.09	30.45 ± 0.22	30.36 ± 0.17
RSD (%) [a]	0.14	0.61	0.59	0.81	0.30	0.29	0.72	0.54
Recovery (%)	100.53 ± 0.15	101.82 ± 0.62	101.18 ± 0.59	101.52 ± 0.82	101.64 ± 0.30	101.49 ± 0.29	101.51 ± 0.73	101.19 ± 0.55
200.00	200.58 ± 0.86	201.92 ± 1.30	203.01 ± 1.31	202.98 ± 1.55	202.48 ± 1.15	203.13 ± 1.32	204.01 ± 1.50	202.98 ± 1.55
RSD (%) [a]	0.43	0.64	0.64	0.77	0.57	0.65	0.74	0.77
Recovery (%)	100.29 ± 0.43	100.96 ± 0.65	101.50 ± 0.65	101.49 ± 0.78	101.24 ± 0.57	101.56 ± 0.66	102.00 ± 0.75	101.49 ± 0.78
500.00	501.92 ± 0.51	502.37 ± 0.81	503.11 ± 2.89	501.62 ± 1.61	503.75 ± 1.19	502.12 ± 1.14	502.97 ± 2.89	503.37 ± 1.88
RSD (%) [a]	0.10	0.16	0.57	0.32	0.24	0.23	0.57	0.37
Recovery (%)	100.38 ± 0.10	100.47 ± 0.16	100.62 ± 0.58	100.32 ± 0.32	100.75 ± 0.24	100.42 ± 0.23	100.59 ± 0.58	100.67 ± 0.38

[a] Triplicate for each injection; precision acceptance criteria, RSD less than 1% within each level.

Table IV. Inter-day Precision of the Analytical Method in Matrices (ACN solution and Nanoparticle solution)

Spike level (μg/mL)	Measured concentration (μg/mL) from *ACN solution*				Measured concentration (μg/mL) from Nanoparticle ACN solution			
	Irinotecan (256 nm)	Curcumin (424 nm)	Irinotecan (379 nm)	Curcumin (379 nm)	Irinotecan (256 nm)	Curcumin (424 nm)	Irinotecan (379 nm)	Curcumin (379 nm)
30.00	30.26 ± 0.04	30.21 ± 0.14	30.35 ± 0.18	30.25 ± 0.25	30.19 ± 0.09	30.25 ± 0.09	30.05 ± 0.22	30.26 ± 0.17
RSD (%) [a]	0.15	0.45	0.58	0.62	0.30	0.29	0.72	0.54
Recovery (%)	100.87 ± 0.15	100.70 ± 0.46	101.17 ± 0.57	100.83 ± 0.60	100.63 ± 0.31	100.83 ± 0.30	100.17 ± 0.72	100.87 ± 0.55
P [b] value	0.5471	0.3926	0.2937	0.2578	0.2598	0.3658	0.5478	0.5891
200.00	200.28 ± 0.85	201.72 ± 1.35	202.84 ± 2.42	203.98 ± 1.54	203.45 ± 1.15	202.76 ± 1.19	202.34 ± 2.45	203.47 ± 3.27
RSD (%) [a]	0.42	0.63	1.20	0.79	0.57	0.58	0.72	0.58
Recovery (%)	100.14 ± 0.43	100.86 ± 0.65	101.42 ± 1.23	101.99 ± 0.78	101.73 ± 0.57	101.38 ± 0.61	101.17 ± 1.24	101.74 ± 1.62
P [b] value	0.3651	0.3545	0.5642	0.4201	0.3564	0.6412	0.3574	0.2565
500.00	504.72 ± 0.50	503.57 ± 0.80	505.51 ± 2.90	503.52 ± 1.63	504.35 ± 1.19	504.27 ± 1.19	501.57 ± 2.80	505.57 ± 1.89
RSD (%) [a]	0.12	0.15	0.54	0.36	0.24	0.23	0.57	0.38
Recovery (%)	100.94 ± 0.12	100.71 ± 0.15	101.10 ± 0.58	100.70 ± 0.36	100.87 ± 0.25	100.85 ± 0.24	100.31 ± 0.59	101.11 ± 0.38
P [b] value	0.1471	0.2145	0.2345	0.2634	0.2547	0.1950	0.2314	0.2456

Precision acceptance criteria, RSD less than 1% within each level. [a]Triplicate in two different days. [b]Student's t-test with significance level of 95% for 2 days.

Table V. Robustness Results under Different Chromatographic Conditions

Parameter	Modification	Mean recovery (Triplicate for each injection)			
		Irinotecan (256 nm)	Curcumin (424 nm)	Irinotecan (379 nm)	Curcumin (379 nm)
Column temperature	37°C	99.55 ± 1.33	98.54 ± 1.12	99.32 ± 1.45	98.31 ± 1.71
	40°C	99.76 ± 0.73	101.01 ± 0.53	101.54 ± 0.73	100.43 ± 0.63
	43°C	99.26 ± 0.33	99.01 ± 0.53	99.54 ± 0.73	101.20 ± 0.63
	RSD (%)	0.25	1.32	1.22	1.50
Flow rate	0.9 mL/min	99.47 ± 0.43	99.91 ± 0.67	99.84 ± 0.79	101.20 ± 0.13
	1.0 mL/min	99.76 ± 0.73	101.01 ± 0.53	101.54 ± 0.73	100.43 ± 0.63
	1.1 mL/min	99.86 ± 0.95	99.51 ± 0.43	99.58 ± 0.53	99.20 ± 0.93
	RSD (%)	0.20	0.78	1.06	1.01
Mobile phase ratio	50.5:49.5	98.86 ± 0.75	100.51 ± 0.63	101.58 ± 0.93	99.81 ± 0.43
	50:50	99.76 ± 0.73	101.01 ± 0.53	101.54 ± 0.73	100.43 ± 0.63
	49.5:50.5	99.36 ± 0.95	98.51 ± 0.43	99.78 ± 0.83	99.20 ± 0.74
	RSD (%)	0.45	1.32	1.02	0.62

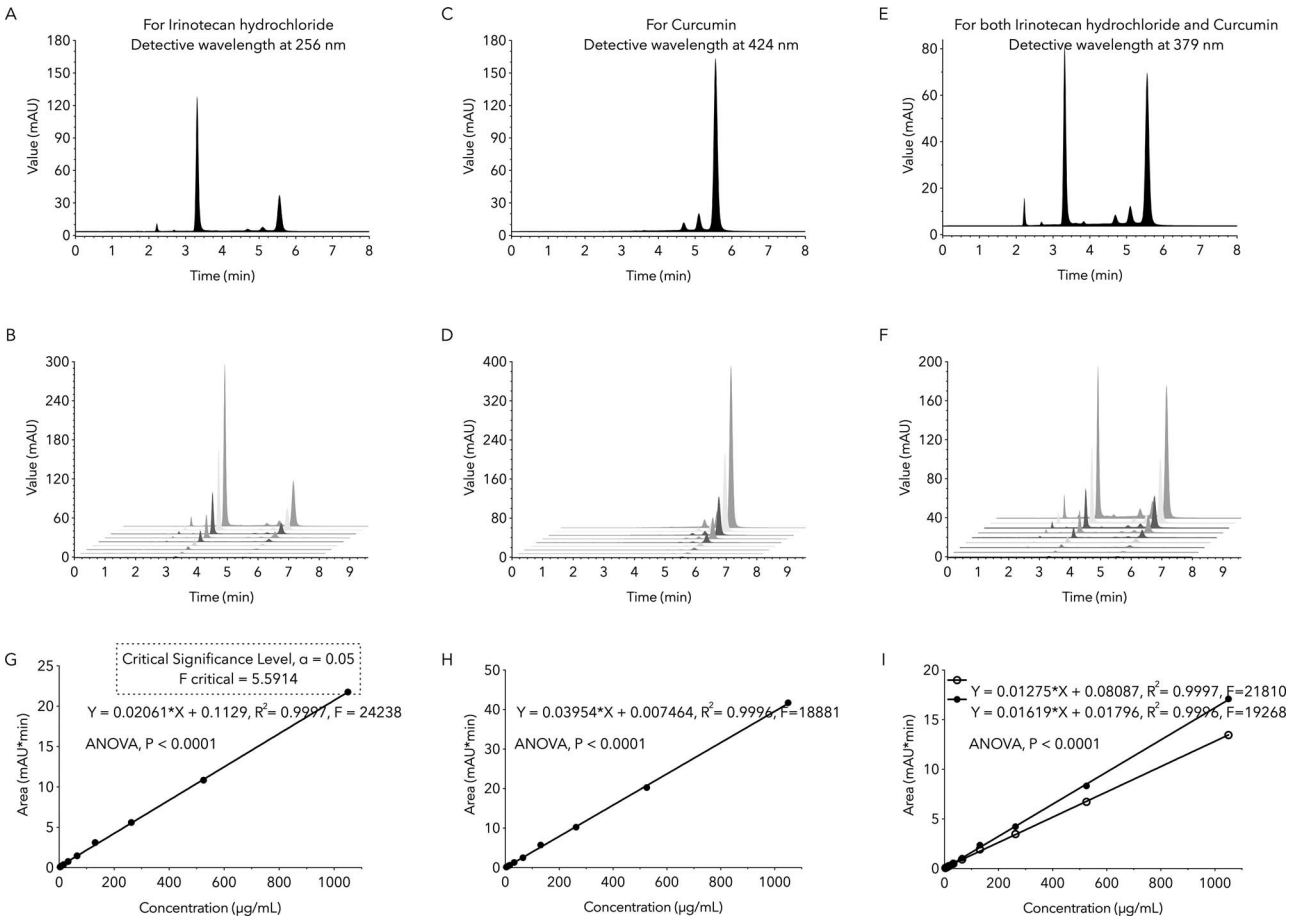

Figure 4. Chromatogram at 256 nm for detection of irinotecan (A) with different concentrations (B); Chromatogram at 424 nm for detection of curcuminoids (C) with different concentrations (D); Chromatogram at 379 nm for detection of both irinotecan and curcuminoids (E) with different concentrations (F); Regressed calibration curve at 256 nm (G), 424 nm (H) and 379 nm (I).

the detective wavelengths. Acetonitrile and ultrapure water containing sodium dodecyl sulfate (0.08 mol/L), disodium phosphate (Na_2HPO_4, 0.002 mol/L) and acetic acid (4%, v/v) were used as the mobile phase and their ratio was set at 50:50. DSP and SDS were used in the mobile phase to keep irinotecan hydrochloride in its neutral form and get rid of the peak tailing. The analysis can be carried out to simultaneously quantify irinotecan hydrochloride and curcumin at two different detective wavelengths, namely 256 and 424 nm. Detectors with only one channel can also visualize both analytes in one chromatogram at 379 nm and still demonstrate acceptable sensitivity. The method developed was validated to be sensitive, accurate (recovery, $100 \pm 2\%$), precise (RSD $\leq 1\%$), robust and linear ($R^2 \geq 0.9996$) in the tested range.

The molar ratio of curcumin to irinotecan hydrochloride in the co-delivered nanoparticles was approximately 2:1, indicating more hydrophobic curcumin molecules entrapped inside the nanoparticles than amphiphilic irinotecan hydrochloride. The authors surmise that during formation of the nanoparticles the extremely hydrophobic curcumin molecules aggregate together with the hydrophobic part of PLGA–PEG polymer, forming hard inner cores, and the hydrophobic portion of the irinotecan molecules are embedded inside the inner cores; this is due to hydrophobic interactions between the molecules themselves. The water-soluble part of PLGA–PEG and hydrophilic piperidine rings of irinotecan (25) stretch out and decrease the contact area between the nanoparticles, thereby boosting the stability of the nanoparticles owing to steric repulsion. During the formation of nanoparticles, the functions of curcumin and irinotecan hydrochloride differ. The hydrophobic curcumin molecules form an inner core with the hydrophobic part of PLGA–PEG. Meanwhile, the entire amphiphilic irinotecan hydrochloride molecule serves as a surfactant and heightens particle stability (26, 27).

Based on the current assessment of the drug loading and entrapment quality of this co-delivered nanoparticle system, further work such as the *in vitro* and *in vivo* therapeutic efficacy of both molecules will be carried out on relevant cancer models.

Conclusions

In this research, development has focused on innovative pharmaceutical systems for co-delivering irinotecan hydrochloride and curcumin, giving rise to the simple, sensitive and rapid analytical method described herein with its basis on HPLC. The analytical method developed was confirmed to be sensitive, accurate (recovery, $100 \pm 2\%$), precise (RSD $\leq 1\%$), robust and linear ($R^2 \geq 0.9996$) in the range from 2.0 to 1050 μg/mL, which has been used for the simultaneous quantification of irinotecan hydrochloride and curcumin in the co-delivered pharmaceutical systems to assess their drug delivery quality

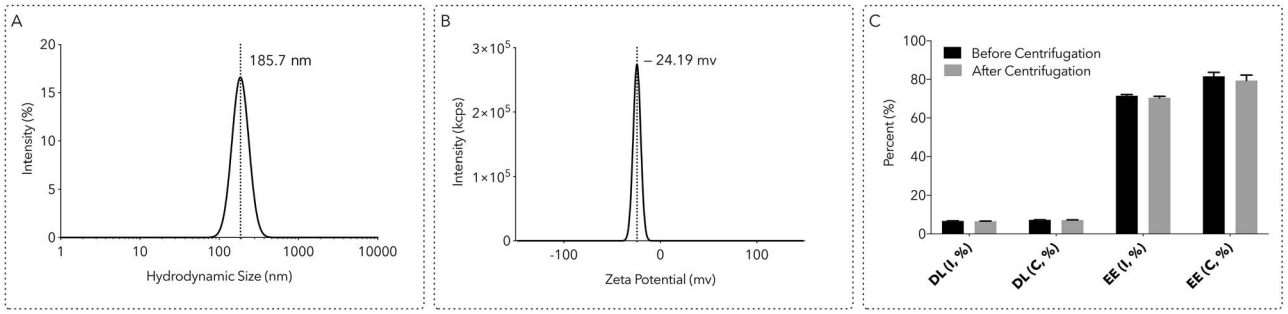

Figure 5. Typical hydrodynamic size (A) and zeta potential (B) of irinotecan and curcuminoids co-delivered nanoparticles. The drug-loading capacity and drug entrapment efficiency prior to and following centrifugation process (C).
Abbreviation: DL (I, %), drug-loading capacity of irinotecan; DL (C, %), drug- loading capacity of curcumin; EE (I, %), drug Entrapment Efficiency of irinotecan; EE (C, %), drug Entrapment Efficiency of curcumin.

and can also be used for routine analysis with related analytes because of its simplicity and accuracy.

Declaration

The authors state no conflicts of interest exist to their knowledge.

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