Heliyon 6 (2020) e04561

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

A stability-indicating UPLC method for the determination of curcumin diethyl disuccinate, an ester prodrug of curcumin, in raw materials

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ARTICLE INFO

Keywords:
Curcumin diethyl disuccinate
Stability-indicating method
UPLC
Validation
Prodrug
Analytical chemistry
Optical spectroscopy
Chromatography
Pharmaceutical chemistry
Pharmaceutical science

ABSTRACT

A stability-indicating reversed-phase ultra-performance liquid chromatographic (UPLC) method for quantitative analysis of curcumin diethyl disuccinate (CDD) in raw materials was developed for applications in product development and quality control. Chromatographic separation was performed using the Waters ACQUITY UPLC® H-Class system consisting of an ACQUITY UPLC® BEH C18 (1.7 μm, 2.1 × 50 mm) column and a photodiode array detector set at a wavelength of 400 nm. The mobile phase consisting of 2%v/v acetic acid in water and acetonitrile was delivered at a flow rate of 0.3 mL/min under gradient elution program. The method was validated according to the ICH Q2(R1) guideline for the validation of analytical procedures. The method was found to be linear over the concentration range of 8–12 μg/mL with the coefficient of determination >0.995. The accuracy of the method established as %recovery ranged from 98.3 – 100.8%. The precision of the method expressed as %CV was found to be <1%. The coelution of degradation products from six stress test conditions was not observed. The method was robust under the variation of chromatographic parameters. The method was successfully applied in the determination of CDD content in raw materials.

1. Introduction

Curcumin diethyl disuccinate (CDD, Figure 1A) is an ester prodrug of curcumin (Figure 1B) with improved chemical stability in phosphate buffer (pH 7.4) and cytotoxicity against colorectal adenocarcinoma (Caco-2) cells in comparison with curcumin [1]. It also demonstrated superior anti-HepG2 hepatocellular carcinoma activities in the *in vitro* and *in vivo* models [2, 3]. Encapsulation of CDD in various types of nanoparticles increased cellular uptake and cytotoxicity of CDD against several cancer cell lines [4, 5, 6]. Furthermore, CDD at doses of 25 and 200 mg/kg exhibited analgesic activities and anti-inflammatory effects in mouse models [7]. Pharmacokinetic studies of CDD in rats showed that CDD was rapidly converted to curcumin and increased plasma concentrations of curcumin after intravenous administration [8, 9]. The *in vitro* plasma metabolism study indicates that plasma carboxylesterase is an enzyme responsible for the hydrolysis of CDD in rats [10]. Therefore, CDD has the potential to be further developed as a prodrug of curcumin for various therapeutic applications.

Quality control of drug substances is one of the key factors in the development of a new molecular entity for pre-clinical and clinical studies. An analytical method for quality control of active pharmaceutical ingredients (APIs) and drug products should be a validated stability-indicating method (SIM) [11, 12]. The SIM is usually a chromatography-based method that can quantify target analytes without interferences from impurities generated during the manufacturing process and storage. The development of SIM requires forced-degradation samples that can be prepared by exposing APIs or drug products to different stress conditions, including acid, base, moisture, heat, light, and oxidizing agents [12]. The SIM can be achieved by assessment of the ability of the method to efficiently separate target analytes from its degradation products using the forced degradation samples.

To date, a number of liquid chromatography-based methods for CDD analysis have been reported. The first reported method is the HPLC-UV method with an application in a chemical kinetic study for curcuminoid prodrugs [1]. The method is time-consuming and lacks specificity, which limits its application for the quality control of CDD raw materials. Other HPLC-UV methods were developed to determine CDD and

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https://doi.org/10.1016/j.heliyon.2020.e04561
Received 20 May 2020; Received in revised form 5 July 2020; Accepted 22 July 2020

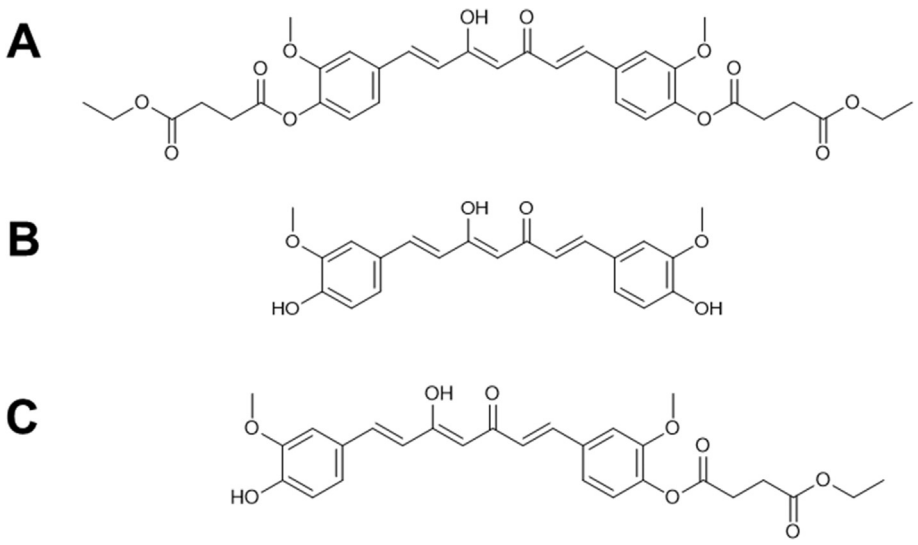

Figure 1. Chemical structures of (A) curcumin diethyl disuccinate, CDD (B) curcumin, and (C) monoethylsuccinyl curcumin, MEC.

curcumin in plasma [10] or in Caco-2 permeates [2]. However, the methods have not yet been validated and proved to be SIM. Recently, we reported the validated LC-MS/MS method for the pharmacokinetic study of CDD in rats [8]. Although the LC-MS/MS is a powerful tool for analysis of target analytes due to its high sensitivity and specificity, the method is a high-cost technique and may be unnecessary for the quantitative analysis of target analytes in APIs. Up to date, the validated SIM for the quantitative determination of CDD raw materials based on liquid chromatography coupled with UV-vis detection for the analysis of CDD has not been reported.

In the present study, we developed and validated a UPLC method for the quantitative determination of CDD content in raw materials. Forced degradation studies of CDD under different stress conditions, including acid, base, moisture, heat, light, and oxidation, were conducted to facilitate the development of the SIM that could analyze CDD with acceptable specificity. The method was found to be efficient for the separation of CDD from its degradation products and successfully validated according to the ICH Q2(R1) guideline [13] in terms of specificity, accuracy, precision, and sensitivity.

2. Experimental

2.1. Materials and reagents

CDD, monoethylsuccinyl curcumin (MEC, Figure 1C), and curcumin (purity >98%, by HPLC) were synthesized using the previously published methods [1, 10] and used as the reference standards in the validation and assay. One pilot batch of CDD raw material was prepared in our laboratory [3] while a manufacturing batch was obtained from Chengdu BK Biotech Co., Ltd. (Sichuan, China). Reagent-grade glacial acetic acid was obtained from Scharlab (Barcelona, Spain). HPLC-grade acetonitrile was procured from Thomas Baker (Mumbai, India). Reagent-grade dimethyl sulfoxide (DMSO) was purchased from Carlo Erba (Val de Reuil, France). High purity water was prepared in-house using UHQ Water System (Milli-Q, USA). Quinine monohydrochloride dihydrate USP standard (Lot no. R071S0, purity 100%) was purchased from USP.

2.2. Instrumentation

The Waters ACQUITY UPLC® H-Class system (Waters Corporation, Milford, MA, USA) comprised of quaternary pump, column oven, autosampler, and photodiode array detector was used for method development, forced degradation studies, and method validation. The Empower 3 software was used for controlling instrument operation and processing data. Chromatographic separation was achieved on an ACQUITY UPLC® BEH C18 1.7 µm, 2.1 × 50 mm column (Waters Chromatography Ireland Limited, Dublin, Ireland). The mobile phase consisting of 2 %v/v acetic acid in water (A) and acetonitrile (B) was delivered at a flow rate of 0.3 mL/min. The gradient elution program was optimized as follows: initial A-B of 55:45 at 0.0 min; linear-gradient A-B of 20:80 from 0.0−2.7 min; isocratic A-B of 20:80 from 2.7−4.5 min; linear-gradient A-B of 55:45 from 4.5−5.0 min; isocratic A-B of 55:45 from 5.0−7.0 min. The column oven temperature and detection wavelength were set at 33 °C and 400 nm, respectively. The injection volume was 2 µL.

2.3. Preparation of standard and system suitability solutions

Standard stock solutions of CDD, curcumin, and MEC (100 µg/mL) were separately prepared by dissolving 5 mg of each compound with DMSO in 50-mL volumetric flasks. A 5 mL of the standard stock solution of CDD was transferred to another 50-mL volumetric flask and diluted to volume with a diluent (20:80 of 2 %v/v acetic acid in water:acetonitrile) to obtain a working standard solution of CDD at the concentration of 10 µg/mL. Standard solutions of curcumin and MEC at the concentration of 10 µg/mL were prepared similarly to the working standard solution of CDD. Then, the standard solutions were filtered through 0.45 µm nylon membrane filters before analysis.

A system suitability solution was prepared by diluting the standard stock solutions of CDD, curcumin, and MEC (100 µg/mL) with the diluent to obtain a solution containing 10 µg/mL of each compound. The system suitability solution was filtered through a 0.45 µm nylon membrane filter before analysis.

2.4. Forced degradation studies

Forced degradation studies of CDD under six stress conditions were performed as recommended by the ICH Q1A(R2) guideline [11]. The samples were prepared at the nominal concentration of 10 µg/mL and filtered through a 0.45 µm nylon membrane filter before analysis.

2.4.1. Procedure for acid hydrolysis

A 5 mg of CDD was added into a 15-mL glass-stopper test tube followed by the addition of 200 µL of 0.1 N HCl. The mixture was vortexed until homogeneously dispersed. The sample was kept at 80 °C for 3 h. Then, 200 µL of 0.1 N NaOH was added to neutralize the residual acid. After that, the sample was further dissolved with 10 mL of DMSO. The resulting solution was transferred to a 50-mL volumetric flask followed by diluting to volume with DMSO. Subsequently, 1 mL of this solution

was pipetted into a 10-mL volumetric flask and diluted to volume with the diluent.

2.4.2. Procedure for basic hydrolysis

A 5 mg of CDD was dispersed in 200 μL of 0.1 N NaOH in a 15-mL glass-stopper test tube. The mixture was vortexed until homogeneously dispersed. The sample was incubated at 80 °C for 3 h. Then, 200 μL of 0.1 N HCl was added to neutralize the residual hydroxide. After that, the sample was further dissolved with 10 mL of DMSO. The resulting solution was transferred to a 50-mL of volumetric flask and made up to the volume with DMSO. Subsequently, 1 mL of this solution was pipetted into a 10-mL volumetric flask and diluted to volume with the diluent.

2.4.3. Procedure for moisture hydrolysis

A 5 mg of CDD was dispersed in 200 μL water in a 15-mL glass-stopper test tube. The mixture was vortexed until homogeneously dispersed. The sample was heated at 80 °C for 3 h. After that, the remained sample was dissolved with 10 mL of DMSO. The resulting solution was transferred to a 50-mL volumetric flask followed by diluting to volume with DMSO. Afterward, 1 mL of this solution was transferred into a 10-mL volumetric flask and diluted to volume with the diluent.

2.4.4. Procedure for oxidative degradation

A 5 mg of CDD was mixed with 200 μL of 3% H_2O_2 in a 15-mL glass-stopper test tube and incubated at 80 °C. After 3 h, the residual sample was dissolved with 10 mL of DMSO and subsequently transferred to a 50-mL of volumetric flask. The transferred solution was diluted to volume with DMSO. Then, 1 mL of the solution was pipetted into a 10-mL volumetric flask and diluted to volume with the diluent.

2.4.5. Procedure for thermal degradation

A 5 mg of CDD was heated at 80 °C in a 15-mL glass-stopper test tube. After that, the sample was dissolved with 10 mL of DMSO, transferred to a 50-mL volumetric flask, and adjusted to volume with DMSO. Then, 1 mL of the solution was transferred to a 10-mL volumetric flask and diluted to volume with the diluent.

2.4.6. Procedure for photolysis

The photostability of CDD was investigated in both solid substance and solution according to the ICH Q1B guideline [14]. A 2 %w/v aqueous solution of quinine monohydrochloride dihydrate in a 1-cm quartz cell was used as light intensity indicator. The quinine solution was placed beside the test sample during the study. The experiment is appropriately valid when the change in UV absorbance at 400 nm of at least 0.5 is obtained. Based on the ICH Q1B, the overall illumination on a sample is greater than 1.2 million lux hours with an integrated near ultraviolet energy of greater than 200-watt h/m^2. For the photostability of CDD as the solid substance, 5 mg of CDD was placed in a photostability chamber with direct exposure to the fluorescent and UV light at room temperature until the change in UV absorbance of the quinine solution at 400 nm of at least 0.5 was obtained. Then, the tested sample was dissolved in 10 mL of DMSO and diluted with the diluent to obtain the final concentration of 10 μg/mL. For the photostability of CDD in solution, the standard stock solution of CDD in a 1-cm quartz cell was placed in a photostability chamber with direct exposure to the fluorescent and UV light at room temperature. The experiment was terminated under the same criteria as the solid state after the 5-day period. Subsequently, the irradiated solution was diluted with the diluent to obtain a sample solution at the concentration of 10 μg/mL.

2.5. Method validation

The method was validated according to the ICH Q2(R1) guideline for the validation of analytical procedures in the aspect of assay procedures [13].

2.5.1. System suitability

The system suitability test was performed to verify the reproducibility and performance of the chromatographic system prior to analysis. For the system reproducibility, the working standard solution of CDD at the concentration of 10 μg/mL was injected in five replicates and the variation of retention time and peak area representing the precision of the injection was evaluated.

The system suitability solution containing a mixture of CDD, curcumin, and MEC at the concentration of 10 μg/mL for each compound was freshly prepared to verify the system performance. The resolutions between curcumin and MEC, and between MEC and CDD were determined. In addition, the tailing factor and the theoretical plate count of CDD were calculated to evaluate the performance of the analytical system.

2.5.2. Specificity

Curcumin and MEC are potential impurities in CDD raw materials. Specificity of the method was evaluated by separately injecting the diluent, curcumin standard solution (10 μg/mL), CDD standard solution (10 μg/mL), MEC standard solution (10 μg/mL), system suitability solution, and forced degradation samples.

2.5.3. Linearity and range

Linearity demonstrates the ability of the analytical method to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range. Range of the method describes the concentration interval from the upper to the lower levels. A series of CDD standard solutions at the concentrations of 8, 9, 10, 11, and 12 μg/mL were prepared by diluting appropriate volumes of the standard stock solution of CDD (100 μg/mL) with the diluent. Linearity and range were established by constructing a calibration curve between the CDD standard solutions versus the peak areas. The slope, intercept, and coefficient of determination (r^2) of the calibration curve were calculated.

2.5.4. Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD is the lowest amount of an analyte in a sample which can be detected by an analytical method. The concentration at LOD should give a signal-to-noise ratio (S/N) of $\geq$3 with the precision of five injections (% CV) $\leq$ 15%. The standard stock solution of CDD was serially diluted with the diluent to obtain an LOD solution at the nominal concentration of 0.050 μg/mL for LOD determination. The LOD solution was injected in five replicates. The S/N and %CV at LOD were determined.

The LOQ is defined as the lowest amount of an analyte in a sample that can be quantitatively determined with acceptable accuracy and precision and have the S/N $\geq$ 10. The accuracy and precision were accepted at %recovery of 80–110% and %CV of $\leq$11%, respectively. The standard stock solution of CDD was serially diluted with the diluent to obtain an LOQ solution at the nominal concentration of 0.075 μg/mL for LOQ determination. The LOQ solution was prepared for five replicates and each replicate was injected in triplicate. The S/N, %recovery and % CV at LOQ were calculated.

2.5.5. Accuracy

Accuracy of CDD analysis was determined using the CDD standard solutions prepared by diluting appropriate volumes of the standard stock solution of CDD with the diluent to obtain the final concentrations at 8, 10, and 12 μg/mL, which are equivalent to 80, 100, and 120% nominal concentrations, respectively. Each standard solution was prepared for three replicates and each replicate was injected in triplicate. Accuracy was performed by the spiked standard method and expressed as %recovery calculated using the following equation:

$$\%\text{recovery} = [\text{Amount found } (\mu g/mL)/\text{Amount added } (\mu g/mL)] \times 100$$

Table 1. System suitability parameters (n = 5).

Injection no.	Retention time (min)	Peak response	USP Tailing factor	USP plate count
1	3.721	340560	1.11	109777
2	3.712	339977	1.11	108260
3	3.709	340580	1.10	106986
4	3.707	340448	1.08	102171
5	3.710	339689	1.07	101671
Mean	3.712	340251	1.09	105773
%CV	0.15	0.12		

2.5.6. Precision

Precision of the analytical procedure was evaluated by determining repeatability (intra-day precision) and intermediate precision of the method. Repeatability was assessed by triplicate injections of three replicates of the CDD standard solutions at 8, 10, and, 12 μg/mL prepared as described in section 2.5.5. For intermediate precision, three replicates of the CDD standard solutions at 8, 10, and 12 μg/mL were assayed in triplicate on two different days by two different analysts.

2.5.7. Robustness

Robustness of the developed method was investigated under small variations in method parameters, including the detection wavelength and column temperature. Suitability of system including retention time and peak response of CDD was observed when method parameters were varied from regular method condition. Furthermore, the sample solution stability was also observed in comparison with the freshly prepared standard solution.

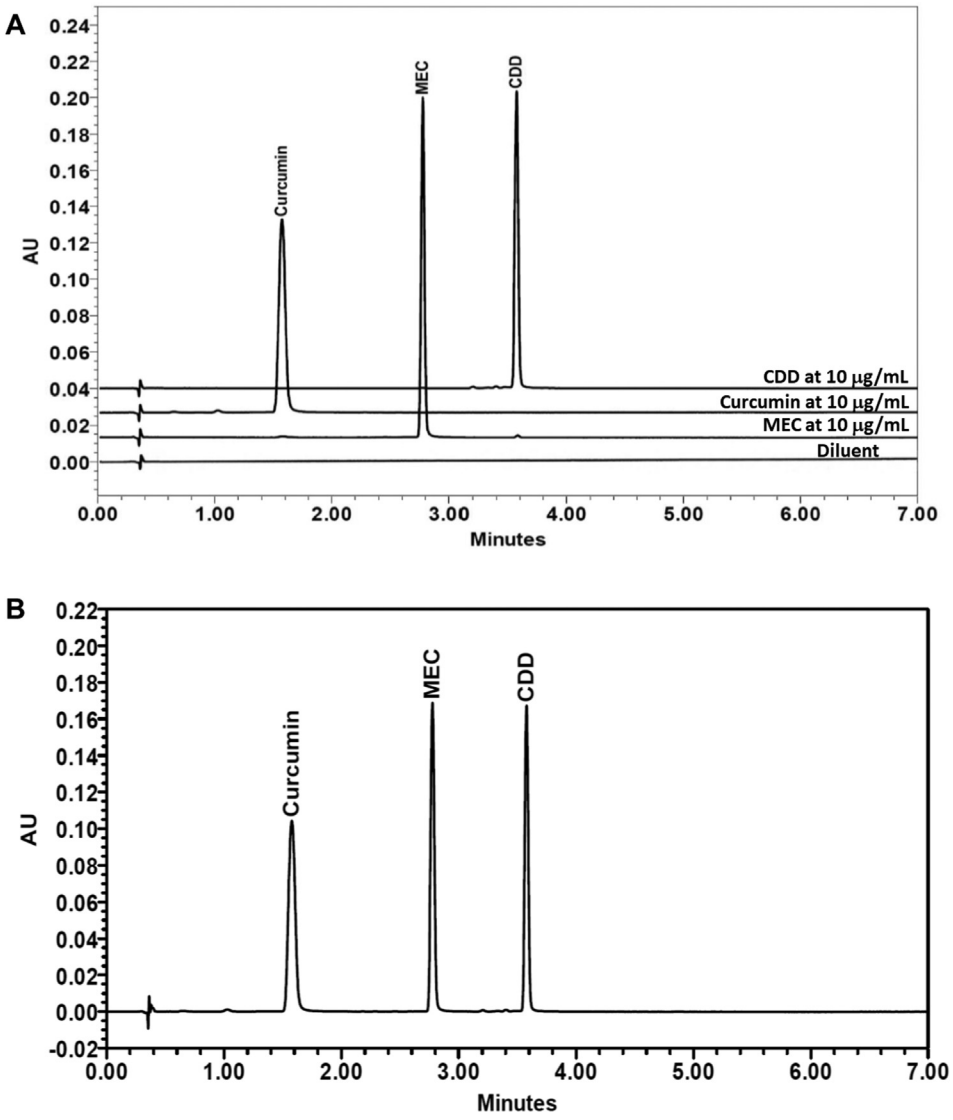

Figure 2. (A) Overlaid chromatograms of diluent, curcumin standard solution, CDD standard solution, and MEC standard solution. (B) A chromatogram of the mixture solution of curcumin, MEC and CDD.

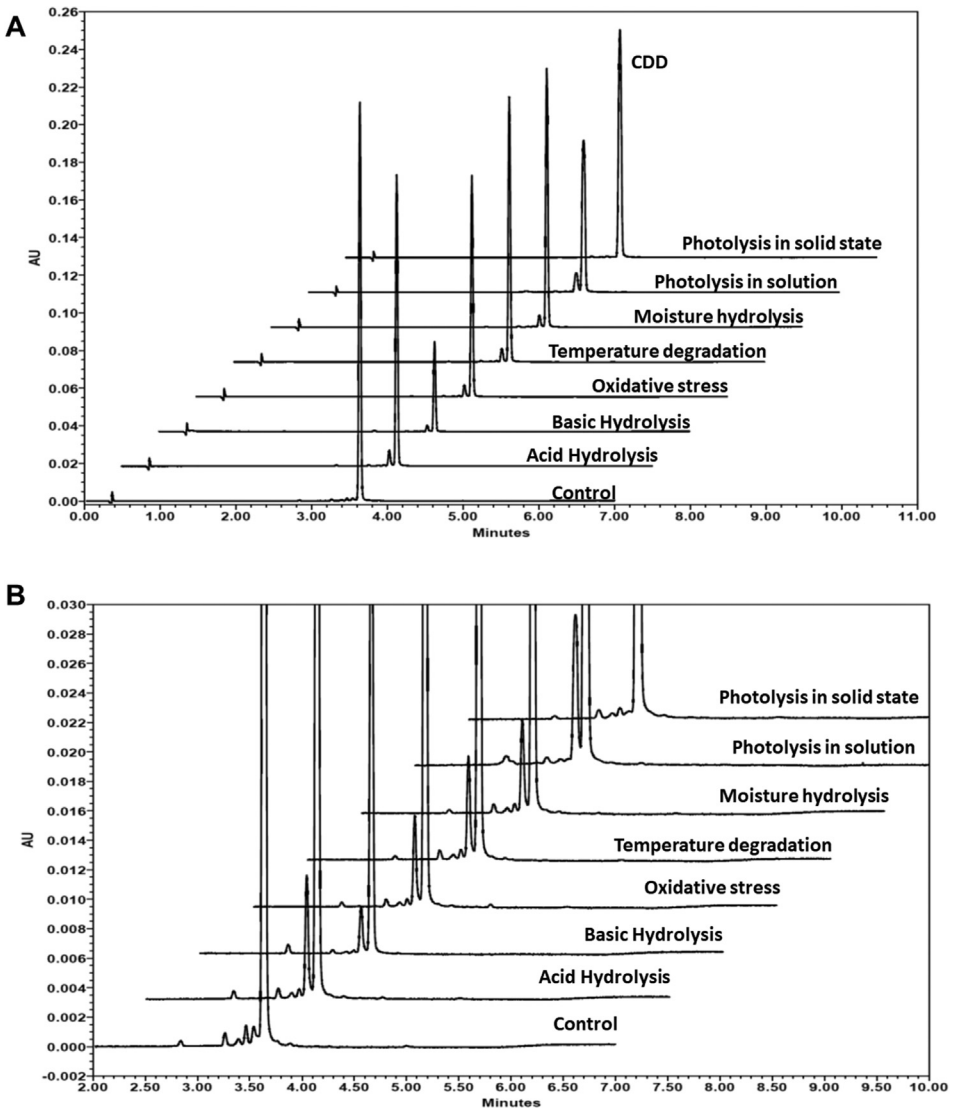

Figure 3. (A) Overlaid chromatograms of forced degradation samples of CDD under various conditions. (B) The zoomed overlaid chromatograms of forced degradation samples of CDD.

2.5.8. Method application for quality control of CDD raw materials

The methodology was applied in the quantitative analysis of 2 batches of CDD raw materials. The working standard and system suitability solutions were prepared as described in section 2.3. CDD samples from different batches were separately prepared at the final concentration of 10 μg/mL in the diluent. Then, these solutions were filtered through a 0.45 μm nylon membrane filter prior to analysis. The percentages of weight (%w/w) of CDD in raw materials were determined by comparing the peak responses of the CDD samples with that of the working standard solution.

3. Results and discussion

3.1. Method development

Two potential impurities in CDD raw materials are curcumin and MEC. Curcumin is a starting material for CDD synthesis, while MEC is an intermediate occurred during an esterification reaction. In addition, both curcumin and MEC can be generated via the ester hydrolysis of CDD during the storage of CDD raw materials. Therefore, the analytical method developed for the quantitative determination of CDD in raw materials required efficient separation of CDD from curcumin and MEC. The chromatographic separation of CDD from its degradation products was achieved with the gradient elution program varying the ratio of 2% v/v acetic acid in water (A) and acetonitrile (B) at the flow rate of 0.3 mL/min. Initially, acetonitrile was ramped up from 45% to 80% in a linear fashion for 2.7 min (from 0-2.7 min) to ensure that the analytes can be separated on the column. The UPLC chromatograms of the diluent, the curcumin standard solution, the MEC standard solution, the CDD standard solution, and the system suitability solution containing curcumin, MEC, and CDD are shown in Figure 2. Curcumin was eluted at 1.6 min, corresponding to the elution of curcumin at about 66% acetonitrile. Subsequently, acetonitrile was maintained at 80% for 1.8 min (from 2.7−4.5 min) for elution of CDD and other impurities. At 80% acetonitrile, MEC and CDD were eluted at 2.8 and 3.6 min, respectively. Finally, acetonitrile was decreased from 80% to 45% in 0.5 min and maintained for another 2 min prior to subsequent injection. The use of acetic acid at 2 %v/v in water is adequately acted as a mobile phase additive for suppressing tautomerization of curcumin. The lower amount of acetic acid is insufficient to make this suppression while the higher amount of acetic acid is not appropriate due to its unpleasant odor which is not environment-friendly during instrument operation. Formic acid is more

Table 2. Peak purity values of curcumin diethyl disuccinate under various stress conditions.

Treatment	Purity angle	Purity Threshold	Peak purity*
Control	0.437	3.156	Passed
Acid Hydrolysis (0.1 N HCl) at 80 °C for 3 h.	0.536	3.782	Passed
Basic Hydrolysis (0.1 N NaOH) at 80 °C for 3 h.	1.874	12.131	Passed
Oxidative stress (3% H_2O_2) at 80 °C 3 h.	0.655	4.296	Passed
Temperature degradation at 80 °C for 3 h.	0.575	4.060	Passed
Moisture hydrolysis at 80 °C for 3h.	0.584	4.149	Passed
Photolysis in solid state, 1.2 million lux h.	0.546	3.522	Passed
Photolysis in solution,1.2 million lux h.	0.651	3.693	Passed

* The peak purity value is valid when the purity threshold value is greater than the purity angle value.

eco-friendly as it has less odor, but it is insufficient to suppress the tautomerization of curcumin. The C-18 reversed-phase column was selected due to the low polarity of the analytes. The detection wavelength was chosen at 400 nm according to the maximum absorption characteristic of CDD. The photodiode array was employed to ensure peak purity under several stress conditions. The developed chromatographic condition was found to provide sufficient separation between CDD and its degradation products including curcumin, MEC, and other degradation products generated during forced degradation.

3.2. System suitability

Suitability of system was verified to ensure the validity of chromatographic condition prior to performing experiments. The resolutions between curcumin and MEC, and between MEC and CDD were 14.7 and 14.1, respectively. The average USP tailing factor of CDD was 1.09. These results indicate that the method provides sufficient chromatographic separation with symmetrical peak shape. Deviations of the peak area and retention time in terms of %CV were used to demonstrate the system reproducibility. The %CV values of peak area and retention time

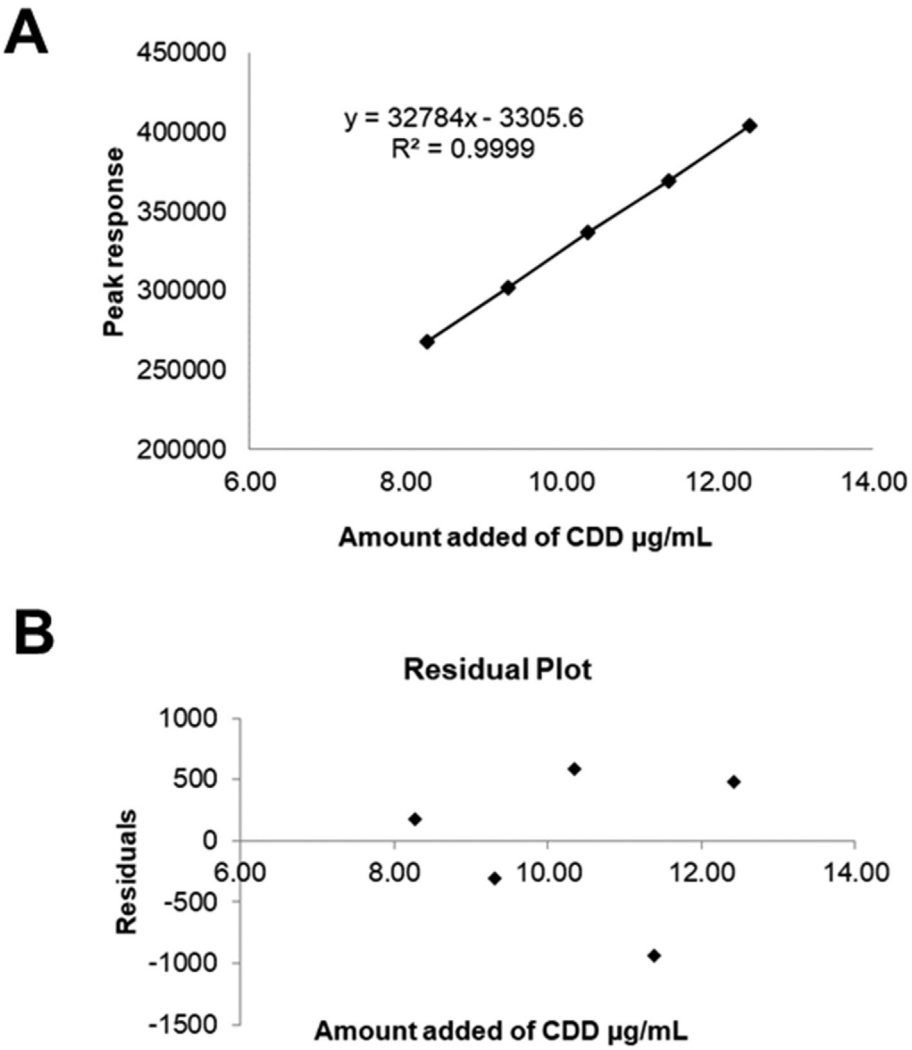

Figure 4. Linearity of the method. (A) Calibration curve of CDD and (B) Residual plot of linearity.

Table 3. Range of the method.

Level (%)	Amount added (μg/mL)	Amount found (μg/mL)
80	8.07	7.99
90	9.08	8.98
100	10.09	10.02
110	11.10	11.00
120	12.11	12.08
Slope/Intercept		1.0097/0.1732
Coefficient of determination (r^2)		0.9998

Table 4. Limit of detection (LOD) and limit of quantitation (LOQ) (n = 5).

Injection no.	LOD		Sample no.	LOQ			
	Peak response	S/N		Added (μg/mL)	Found (μg/mL)	%Recovery	S/N
1	1760	12	1	0.0752	0.0724	96.3	14
2	1580	10	2	0.0752	0.0708	94.1	14
3	1548	11	3	0.0752	0.0752	100.0	14
4	1574	11	4	0.0752	0.0720	95.7	14
5	1583	11	5	0.0752	0.0692	92.0	14
Mean	1609	11			0.0719	95.6	14
%CV	5.3%					3.1%	

of CDD were found to be 0.12 and 0.15 %, respectively. The USP plate count was greater than 20,000. The system suitability results shown in Figure 2 and summarized in Table 1 suggest the suitability of the chromatographic system for CDD analysis.

3.3. Specificity and forced degradation studies

Specificity is the ability of the analytical method to discriminate an analyte from matrix components, impurities, and degradation products. As shown in Figure 2, there is no interference observed at the retention time of CDD. The chromatograms show no coelution peaks of the known degradation products (MEC and curcumin) at the retention time of CDD. According to Figure 3, the forced degradation results showed that CDD degraded under the stress conditions with different extents. CDD was stable under the photodegradation in the powder form but degraded into several degradation products under other stress conditions. Although the chromatograms of the forced degradation samples had a small peak of the unknown degradation product at 3.5 min prior to the CDD peak, the resolution between such peak and CDD is greater than 2, indicating the sufficient separation. It is noteworthy that each degradation product was observed with the small chromatographic peak area although CDD underwent substantial degradation, indicating the poor mass balance in the forced degradation studies of CDD. In addition, curcumin as the expected impurity was not observed on the chromatograms. It is evident that curcumin degrades to other degradation products such as ferulic acid, feruloyl methane, vanillin, spiroepoxides, and bicyclopentadiones, which do not contain chromophores that absorb the wavelength used in this method [15, 16]. MEC presented as a small chromatographic peak on the chromatograms under the stress conditions possibly due to its rapid degradation into curcumin and other degradation products similar to the degradation pathways of curcumin. The occurrence of the degradation products with the absence of chromophores that can absorb the wavelengths used in this method possibly leads to the poor mass balance in the forced degradation studies of CDD. The purity indices determined using the Empower 3 software for the CDD peak on the chromatograms of all stress test samples were found to be higher than the purity angle as shown in Table 2. The peak purity analysis results indicated that the CDD peak had no co-eluting compounds. The overall forced degradation results indicated that the method had sufficient specificity and was subsequently validated as the SIM for the quantitative determination of CDD in raw materials.

3.4. Linearity and range

System linearity was determined in the range of 8–12 μg/mL of the CDD standard solutions in triplicate. A plot between the concentrations versus the peak responses of CDD, linear regression equation and coefficient of determination (r^2) are shown in Figure 4A. Linear regression analysis showed that the method was linear over the concentration range of 8–12 μg/mL. The residual plot showed that residuals of response were

Table 5. Accuracy of UPLC analysis of CDD (n = 3).

Target Level	Amount added (μg/mL)	Amount found (μg/mL)	%Recovery
80%	8.17	8.03	98.4
	8.17	8.03	98.3
	8.17	8.07	98.8
100%	10.25	10.17	99.2
	10.25	10.32	100.8
	10.25	10.22	99.8
120%	12.11	12.12	100.1
	12.11	12.14	100.3
	12.11	12.11	100.0

Table 6. Repeatability and intermediate precision of CDD analysis (n = 3).

Type of precision	%Recovery of CDD solutions at target levels		
	80%	100%	120%
Repeatability			
1st replicate	98.4	99.2	100.1
2nd replicate	98.3	100.8	100.3
3rd replicate	98.8	99.8	100.0
Mean (n = 3)	98.5	99.9	100.1
%CV	0.2	0.8	0.1
Mean (n = 9)	99.5		
%CV	0.9		
Intermediate precision			
Analyst 1			
1st replicate	98.4	99.2	100.1
2nd replicate	98.3	100.8	100.3
3rd replicate	98.8	99.8	100.0
Analyst 2			
1st replicate	98.2	98.6	98.2
2nd replicate	98.1	98.7	98.5
3rd replicate	99.5	99.6	99.1
Mean (n = 18)	99.1		
%CV	0.8		

Table 7. Robustness of the developed method (n = 5).

Parameters	Modification	Retention time (min)		Peak response	
		Mean	%CV	Mean	%CV
Detection wavelength (nm)	400	3.549	0.1	332327	0.4
	398	3.555	0.1	332299	0.5
	402	3.553	0.0	329458	0.5
Column temperature (°C)	33	3.549	0.1	332327	0.4
	31	3.570	0.2	334159	0.2
	35	3.529	0.1	332470	0.4

independent on the established concentrations (Figure 4B). Range of the method was also evaluated over the CDD concentration range of 8–12 μg/mL. The amounts of CDD added and found and regression analysis for the range were summarized in Table 3.

3.5. LOD and LOQ

The LOD and LOQ for CDD analysis were experimentally determined and the results showed that the concentration for LOD was 0.050 μg/mL (%CV = 5.3) with the S/N of 11, while the concentration for LOQ was 0.075 μg/mL with the S/N of 14. The %recovery was within 92–100% with the %CV of 3.1. The LOD and LOQ results summarized in Table 4 indicated sufficient sensitivity for the assay of CDD in raw materials.

3.6. Accuracy and precision

Accuracy of the analytical method of the assay of CDD was expressed as %recovery. The %recovery values range from 98.3 – 100.8%. The % recovery values at each concentration level are presented in Table 5. Precision of the analytical method is expressed as %CV. The %CV for repeatability was 0.9%. The intermediate precision was demonstrated by analysis of CDD in the different day by different analyst. The %CV for intermediate precision was 0.8%. The results of repeatability and intermediate precision are summarized in Table 6.

3.7. Robustness

The system suitability solution was prepared and injected in six replicates with variations of detection wavelength and column temperature to assess robustness of the method. The retention times of CDD were relatively similar when the chromatographic system was carried out using different detection wavelengths and column temperatures. In addition, the small variations of detection wavelength and column had no impact on the %CV of retention time and peak response (%CV < 2%). The results of robustness are summarized in Table 7. Furthermore, stability of the CDD standard solution at 10 μg/mL was also evaluated to ensure the bench top shelf-life (25 ± 5 °C) of the solution during the experiment for 6 h as shown in Table 8. The results implied that the

Table 8. Stability of the CDD standard solution at 10 μg/mL (n = 1).

Time (h)	% remaining
0	100.0
4	98.0
6	99.2

Table 9. The percentage amount (%w/w) of two batches of CDD raw materials.

Lot no.	Batch size	Mfg. date	%w/w
B#20161110	85 G	10-11-16	98.4
B#20180717	1 KG	17-07-18	96.9

chromatographic condition and sample preparation for CDD are practically robust.

3.8. Method application for quality control of CDD raw materials

The validated method was applied in quality control of CDD raw materials. Two batches of CDD were analyzed. The CDD raw material from the pilot scale (lot no. B#20161110) has higher CDD purity than the batch from the 1-kg manufacturing batch (lot no. B#20180717). These results suggest that the large-scale CDD synthesis procedure may need additional optimization to attain CDD raw materials with higher purity. The assay results reporting as on dried basis are summarized in Table 9.

4. Conclusion

A fast, specific, precise, accurate, and robust stability-indicating reversed-phase UPLC method for CDD analysis was developed. Forced degradation studies showed that CDD was degraded under acid hydrolysis, oxidation, moisture hydrolysis and solution-state photolysis conditions. The developed method is capable to efficiently separate CDD from its degradation products. The proposed method here can be employed in the quality control of CDD raw materials.

Declarations

Author contribution statement

P. Rojsitthisak, P. Ratnatilaka Na Bhuket and W. Wichitnithad: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

O. Sudtanon: Performed the experiments; Analyzed and interpreted the data.

Funding statement

This work is supported by Chulalongkorn University (Grant No. CU_GR_62_97_33_06).

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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