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A VALIDATED RP-HPLC METHOD FOR THE QUANTIFICATION OF LIQUID SELF-EMULSIFYING CURCUMIN: PHARMACOKINETIC APPROACH

(Penentusahkan Kaedah FB-KCPT Untuk Kuantifikasi Kurkumin Pememulsi Diri Cecair: Pendekatan Farmakokinetik)

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Abstract

The current study describes a validated, reliable, simple, and sensitive high-performance liquid chromatographic method with fluorescence detection (HPLC-FL) for the determination of curcumin at the *in vitro* and *in vivo* levels in pure and Self Emulsifying Drug Delivery System (SEDDS). The mean recovery of pure curcumin was 99.1%. The calibration curves were linear over the range from 31.3 to 1000 ng/mL with correlation coefficient of $R^2 \geq 0.9999$. The lower limit of quantification (LLOQ) was 31.3 ng/mL. The validation results confirmed the linearity, accuracy, and precision of the developed method. Furthermore, the curcumin-SEDDS excipients solubility were conducted, and later curcumin loading content (LC) was determined. Moreover, pharmacokinetic parameters, mean peak plasma concentration (C_{max}), time to reach peak concentration (T_{max}), and total area under the plasma concentration-time curve from time zero to the last sampling time, i.e., 1440-minute (AUC0-1440m) were successfully measured with the values of 238.81 ng/mL, 70.83 min and 11249.60 ng.min/mL, respectively. This method is suitable to detect curcumin in pure and SEDDS formulation *in vitro* and *in vivo*.

Keywords: curcumin, plasma, self-emulsifying drug delivery system, pharmacokinetic

Abstrak

Kajian semasa menerangkan kaedah kromatografi cecair (HPLC) berprestasi tinggi yang disahkan, boleh dipercayai, mudah dan sensitif dengan pengesanan pendarfluor untuk penentuan kurkumin pada tahap *in vitro* dan *in vivo* dalam Sistem Penghantaran Ubat Pengemulsi Sendiri (SEDDS) dan tulen. Purata pemulihan kurkumin tulen ialah 99.1%. Keluk penentukuran adalah linear dalam julat dari 31.3 hingga 1000 ng/mL dengan pekali korelasi $R^2 \geq 0.9999$. Had kuantifikasi yang lebih rendah (LLOQ) ialah 31.3 ng/mL. Keputusan pengesahan mengesahkan kelinearan, ketepatan dan ketepatan kaedah yang dibangunkan. Tambahan pula, keterlarutan eksipien kurkumin-SEDDS telah dijalankan, dan kandungan pemuatan kurkumin (LC) kemudiannya ditentukan.

Selain itu, parameter farmakokinetik berjaya diukur dengan kaedah ini termasuk purata kepekatan plasma puncak (C_{max}), masa untuk mencapai kepekatan puncak (T_{max}), dan jumlah kawasan di bawah keluk masa kepekatan plasma dari masa sifar hingga masa pensampelan terakhir, iaitu, nilai 1440-minit (AUC0-1440m) masing-masing 238.81 ng/mL, 70.83 min dan 11249.60 ng.min/mL Kaedah ini sesuai untuk mengesan kurkumin dalam formula tulen dan SEDDS daripada penilaian *in vitro* kepada *in vivo*.

Kata kunci: kurkumin, plasma, sistem penyampaian ubat pengemulsi sendiri, farmakokinetik

Introduction

The dried ground rhizome of the perennial herb *Curcuma longa* Linn , or also known as turmeric, has been used in Asian medicine since the second millennium BC [1]. According to the Food and Agriculture Organization of the United Nations, turmeric has been used as food preservative, coloring and flavoring agents [2]. Turmeric is traditionally used for the treatment of wounds, inflammation and tumors [3-5]. It has been linked with antioxidant, anti-inflammatory, anti-microbial, antiproliferative, angiogenesis, anticancer, antidiabetic, antirheumatic, antiviral effects and low toxicity with promising clinical application [2, 6-9]. Recently, a study showed that curcumin minimize the effect on cognitive functions, reduce fatigue as well as improving resilience to the detrimental effects of psychological stress on mood in elderly [10].

Curcuma spp. contains turmerin (a water-soluble peptide), essential oils (such as turmerones, atlantones and zingiberene) and curcuminoids which are natural polyphenols responsible for the yellow color of turmeric. There are three types of curcuminoids, namely bis-demethoxycurcumin (BDMC), demethoxycurcumin (DMC) and curcumin **[**1,7-bis(4-Hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione]. Among the three curcuminoids, curcumin which is also known as diferuloylmethane, or diferulylmethane is the most biologically active constituent.

Liu et al. reported the LLOQ of curcumin was 0.5 ng/mL through LC-MS/MS method with high sensitivity and short run time [11]. However, the curcumin extraction from rat plasma adopted by Liu et al. was complicated and time consuming [11]. May et al. used matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) to study curcumin and its metabolites in mouse serum and lung cell resulting in correlation coefficients of 0.94-0.99 with concentrations of 0.04-10.00 nmol curcumin [12]. Despite high sensitivity and short run time, LC-MS/MS and MALDI-TOF/MS are expensive instruments with hard overcome matrix effects [13].

High performance liquid chromatography (HPLC) also been employed in plasma curcumin quantification with limitation of long analysis run time and tedious extraction methods [14-16]. For instance, Heath et al. extracted curcumin with 95% ethyl acetate/5% methanol [14], and Pak et al. utilized a 3-step extraction which requires large plasma sample volume and uses of chloroform [15]. In another study, Schiborr and colleagues developed a sensitive HPLC-FD with an extremely short retention time at 1.2 minutes which may potentially be interfered by solvent and plasma front peaks [16]. Besides, the extraction step was repeated twice in their developed method [16].

Protein precipitation has been introduced as one of the most common and simple sample pre-treatment methods in removing the protein in plasma sample. It is a simpler method as compared to the extraction process. Ma et al. and Li et al. have developed simpler HPLC assay method using direct protein precipitation method for curcumin extraction with UV and PDA detection, respectively. The HPLC method developed in Li et al. had a relatively shorter run time of 3 minutes and lower LLOQ of 1 ng/mL as compared to Ma et al. [17, 18].

More recently, Anwar et al. modified and developed an analysis method for curcumin using Ultra Performance Liquid Chromatography (UPLC) with Quadrupole-time of flight-mass spectrometry (Q-TOF-MS/MS) [19]. This is a very sensitive chromatographic method which offered short sample retention time at 2.18 minutes; however, the extraction method used a toxic solvent, i.e., chloroform and required a large plasma sample volume of 500 μL. In addition, the technology requires expensive equipment that is not available in most laboratories. Therefore, the aim of this study was to develop an improved method for the quantification of curcumin in plasma using simple high performance liquid chromatography (HPLC) with rapid sample pretreatment procedure. This method is also used to quantify curcumin in self-emulsifying delivery system include solubility, loading content and formulation studies.

Materials and Methods

Chemicals and reagents

Curcumin (99.8% analytical standard) was purchased from ChromaDex (Irvine, CA). Curcuma extract was purchased from Naturalin (Bio-Resources Co. Ltd, Changsha City, China). Canola oil, corn oil, soyabean oil, sunflower oil, peanut oil, and olive oil were purchased from a local store. Oleic acid, PEG-400, Tween 20®, Tween 80®, Cremophor EL®, Cremophor RH40®, and Labrasol® were sponsored by Hovid R&D, Penang, Malaysia. HPLC grade of acetonitrile (ACN) and acetic acid (100% glacial) were obtained from Merck (Darmstadt, Germany). Water for HPLC was prepared by double glass distillation and filtered through 0.45 µm nylon membrane filter (Millipore, Bedford, MA, USA). Human plasma obtained from Hospital Pulau Pinang, Malaysia.

Method development: Instrumentation and equipment

HPLC system consisted of a Waters e2695 Separations Module auto-sampler with refrigeration unit, Waters 2475 Multi-wavelengths Fluorescence Detector. Data acquisition and analyses were performed using the Empower Software 3 (2010, Waters, US).

Analytical and chromatographic conditions

Chromatographic separation was achieved on a reversed phase Waters Symmetry® C_{18} (5µm x 4.6 mm x 250 mm) column. Column and injection temperatures were both maintained at 25°C. Isocratic solvent with flow rate of 1.2 mL/min was used. The sample was injected through a fixed sample loop of 10 µL. The excitation and emission wavelengths for curcumin were set at 365 nm and 512 nm respectively. The analysis time was 6 minutes per sample.

Preparation of mobile phase

A 1% acetic acid solution was prepared by adding 4 mL of glacial acetic acid into 400 mL of distilled water. This was then eluted together with 600 mL of acetonitrile to make up a mobile phase of acetonitrile and acetic acid 60:40 (% v/v) and adjusted to pH 3.25 with acetic acid. Liquids used for the mobile phase were filtered with 0.45µm filter paper (Millipore, Merck, Germany) and kept in amber glass bottles prior to use. Fresh mobile phase was prepared daily.

Preparation of stock solutions and calibration samples

Stock solution of curcumin was prepared in ACN at a concentration of 1 mg/mL. This curcumin stock solution was diluted 50 times with ACN to a working solution of 20 µg/mL. The calibration standards were created by mixing the appropriate amount of the working standard with human control plasma to obtain a final volume of 1 mL, resulting in six calibration standards ranging - 31.3, 62.5, 125, 250, 500 and 1000 ng/mL. Standard solutions were prepared by adding volumes of 1.565, 3.125, 6.25, 12.5, 25, and 50 µL of a working solution into 998.435, 996.875, 993.75, 987.5, 975, and 950 µL of blank plasma. All solutions were protected from light and stored at 4°C. For each validation and assay run, the calibration curve of curcumin was prepared fresh from the working solution. All the plasma samples were stored at -20°C and brought to room temperature prior to use.

Sample pre-treatment procedure

Plasma samples and working solutions were thawed to room temperature before use. An aliquot (100 µL) of plasma samples were pipetted into 1.5 mL microcentrifuge tube (Eppendorf, Germany). Direct deproteinization procedure was carried out by adding 200 µL of acetonitrile. The mixture was vortex-mixed for 20 seconds and centrifuged at 12800 x g for 15 minutes. A 100 µL aliquot of the supernatant was transferred to the HPLC vials and 10 µL of the mixture was injected into the HPLC system for further analysis.

Assay validation

In order to confirm the suitability of the method for its intended use, it was validated for specificity, linearity, precision, accuracy, limit of quantification, limit of detection and stability according to the International Conference on Harmonization guidelines [20] and Guideline on bioanalytical method validation [22].

Specificity

For a specific method, there should be no interference of impurities or other compounds in the blank plasma to the detected peak of curcumin [21]. To test specificity, 10 µL of blank plasma pre-treated according to previous section was injected for analysis. The chromatograms of spiked curcumin plasma samples were compared to the blank plasma. The specificity of the method was determined by analyzing 6 human blank plasma samples.

Linearity

Calibration standards were prepared from the curcumin stock solution at 6 concentrations ranging from 31.3 to 1000 ng/mL. Peak area of curcumin from the chromatogram was plotted against the corresponding curcumin concentrations in plasma.

Accuracy and precision

The within-day accuracy and precision of this assay were evaluated by analyzing plasma aliquots of the calibration standards in 6 replicates on the same day. The between-day accuracy and precision were determined by analyzing each calibration sample once for 6-consecutive days. According to USP [22] and FDA[21] guidelines, the acceptance criteria for within- and between-day accuracy was within ± 15% deviation (DEV) from the nominal values and precision was within 15 % relative standard deviation (RSD).

Recovery

The absolute recovery was determined at each of the calibration point. A set of drug solution was prepared at the same concentrations of curcumin and processed using the sample treatment procedure. The absolute recovery was evaluated by comparing the peak area of curcumin in the spiked plasma sample to the corresponding curcumin solution.

Stability

Due to the possibility of delayed injection or reinjection of samples in the HPLC system, the post-operative stability of treated samples in autosampler vials was assessed. In freeze-thaw stability, samples were thawed at room temperature and refrozen at -20°C over three cycles and assayed. Short-term stability was evaluated to ensure stability of curcumin in plasma to cover the sample preparation time. These samples were exposed to ambient laboratory conditions for 8 hours before curcumin assay. Whereas long-term stability of curcumin in plasma stored at -20°C was evaluated by assaying the samples at baseline, 1 month and 2 months. All stability tested samples were analyzed in 6 replicates.

Curcumin self-emulsifying formulation: Solubility study

Briefly, the solubilization capacities of various oils (Table 1) and the cosolvent, Polyethylene glycol 400, were investigated to select excipients that can solubilize the maximum amount of curcumin using the equilibrium method [23]. An excess amount of curcuma extract (2 g) was added to 10 g of the various oils, and the cosolvent separately, allowed to rotator at 10 rpm for one day at approximately 25±2°C. After that, samples were centrifuged at 12800 g for 15 min to remove the undissolved curcumin, and the supernatant was filtered through a 0.2 µm PTFE membrane filter. Later, 0.02 g of each filtrate was first dissolved in the proper volume of ACN, then further diluted with a sufficient volume of the mobile phase. Curcumin concentration in each vehicle was determined, and all measurements were in triplicate.

Evaluation of blank and loaded self-emulsifying formulation

The effectiveness of self-emulsification, also known as dispersibility assessment, is carried out based on the results of the solubility experiments, in which the selected vehicles (oil phase) were further screened with the assigned surfactants (Table 1) for their emulsification capacities by visual assessment [24]. Only formulations that were able to produce a grade A system at room temperature were chosen for curcumin-loading studies (Table 2).

Table 1. Grading system for emulsification behavior exhibited by SEDDS upon dilution

Grading	Description
Grade A	Spontaneous emulsification; demonstrating a clear appearance (60 s)
Grade B	Spontaneous emulsification; demonstrating a bluish appearance (60 s)
Grade C	Emulsion displays fine milky appearance (120 s)
Grade D	Dull, greyish white appearance with an additional oily appearance together with slow emulsification, (>120 s)
Grade E	Poor or minimal emulsification noted with large oil droplets noticed on the surface

S=Second

For the loading content (LC) evaluation; selected oil-vehicle: cosolvent with the combination of different ratios were studied. To perform the experiment, the samples were treated as per the procedure of the curcumin solubility studies, and the LC was measured via HPLC in triplicate.

Pharmacokinetic evaluation: Animals' selection

Twelve adult male Sprague-Dawley rats were selected in this study, weighing 220g to 286g. The ethical has been approved by the Animal Ethics Committee (AEC) of Universiti Sains Malaysia (USM).

Samples preparation

Similar amount of the curcumin in curcuma extracts alone, and in the loaded optimized self-emulsifying drug delivery systems (SEDDS) formulation were dissolved separately in distilled water (pH 5.6-5.8), with an administration dose of 250 mg/kg body weight of the rats through oral intubation. The formulations were well-mixed before each administration, and the dose was flushed with 2 ml of water after completion of oral administration.

In vivo study design

The study was carried out according to a 2-period, 2-sequence crossover design with a one-week washout period. The rats were randomly divided into two groups with six in each. Before administration, the rats were fasted overnight for at least 12 h with free access to water. After formulation administration, the rats were fasted for 4 h, and water was withheld for 2 h. Approximately 0.3 ml blood samples were withdrawn from the tail veins of each rat on sampling intervals at 0-minute (pre-dosing), 20, 40, 60, 90, 120, 180, 240, 360, 600, 960, and 1440 min post-dosing administration [25]. The blood samples were collected into heparinized microcentrifuge tubes and then the tubes were centrifuged at 12800 g for 15 min to separate the plasma. The plasma samples were analyzed using proposed HPLC method.

Pharmacokinetic data analysis

Using C_{max}, T_{max} and AUC0-1440m as pharmacokinetic parameters, the oral bioavailability of curcumin was compared between the optimized loaded SEDDS formula and the isolated extract. The C_{max} and T_{max} were obtained directly from the plasma concentration values, whereas the $AUC_{0\text{-}1440m}$ was calculated using the trapezoidal formula.

Statistical analysis

To test the statistical significance, a one-way analysis of variance (ANOVA) with Tukey's HSD (honest significant difference) tests was used. The difference in all the analyses was considered statistically significant when $p < 0.05$. All the statistical tests were done using (Minitab® statistical software, version 17.2.1.0, Minitab Inc., USA).

Results and Discussion

Method development: HPLC-detector Condition

The excitation and emission wavelengths were set at the characteristic wavelengths of curcumin at 365nm and 512nm [26]. In this study, acetonitrile (ACN) was chosen due to its low viscosity and strong eluting power [27]. The mobile phase was optimized by manipulating the ratio of solvent and acetic acid to achieve good resolution, short run time and symmetric peak shapes. After optimization, mobile phase made up of acetonitrile and 1% acetic acid solution (60:40, v/v; pH 3.25) with a flow rate of 1.2 mL/min and a run time of 6 minutes was used with Waters Symmetry® C_{18} column (5µm x 4.6 mm x 250 mm) in the current study.

Sample pre-treatment

A pre-treatment procedure is often needed to remove protein and potential interferences from samples prior to HPLC analysis. Various studies suggested the use of liquid-liquid/solid-liquid extraction method [15, 19] which is tedious, time-consuming and involved the use of toxic solvent. Different from liquid-liquid extraction and solid-phase extraction, protein precipitation is commonly used for fast sample clean-up and disrupting protein-drug binding [28-30]. In the current study, addition of 200 µL ACN to the plasma sample was used to deproteinize plasma protein and extract the curcumin content into solvent. This direct single deproteinization step enables us to accomplish pre-treatment step fast and easily.

Method validation

The method was validated according to the guidelines issued by the United States Pharmacopeia (USP) [22] and Food and Drug administration (FDA) [21]. The validation experiments and results obtained are described below.

Specificity

Specificity is described as the ability of a method to discriminate the analyte from all potentially interfering substances [18]. Figure 1(a) shows the sharp peak of 1000 ng/mL curcumin standard solution eluted at 4.9 minutes. Figure 1(b) shows chromatogram of blank plasma while Figure 1(c) shows plasma spiked with 1000 ng/mL curcumin standard solution, the chromatogram indicates that there is no any interference to the peak of interest. Quantitatively, the peak area of the spiked plasma and the curcumin standard solution were found to be equivalent. These results prove that the developed method is specific for curcumin analysis.

Sensitivity and linearity

In regard to the sensitivity, Signal to Noise (S/N) of the retention peak is crucial. For an acceptable detected S/N peak, the height of the corresponding peak and the difference between the largest and smallest noise values should be equal to at least five times the width at the half-height of the peak and, if possible, situated equally around the peak of interest [31]. This is also known as lower limit of detection (LLOD). In this study, the LLOD was 15.6 ng/mL.

Lower limit of quantification (LLOQ) is defined as the lowest concentration on the standard curve that can be quantified with accuracy within ±15% of nominal and precision not exceeding ±15% C.V. according to the USP [22] and FDA [21] guidelines. In the current study, even though the developed method allowed detection of as low as 15.6 ng/mL of curcumin but it was not precise as the S/N ratio is low. At concentration of 31.3 ng/mL, the accuracy and precision (C.V. %) were 95.2% and 2.1%, respectively, which met the criteria set in the guideline [21, 22]. Hence the LLOQ of the current developed method was determined to be 31.3 ng/mL.

Validation was completed by running the developed system in six independent days; hence data of all the six calibration curves was compiled for summary. Results show that all of our six calibration curves were linear in the range of 31.3-1000 ng/mL with $R^2 \geq 0.9999$.

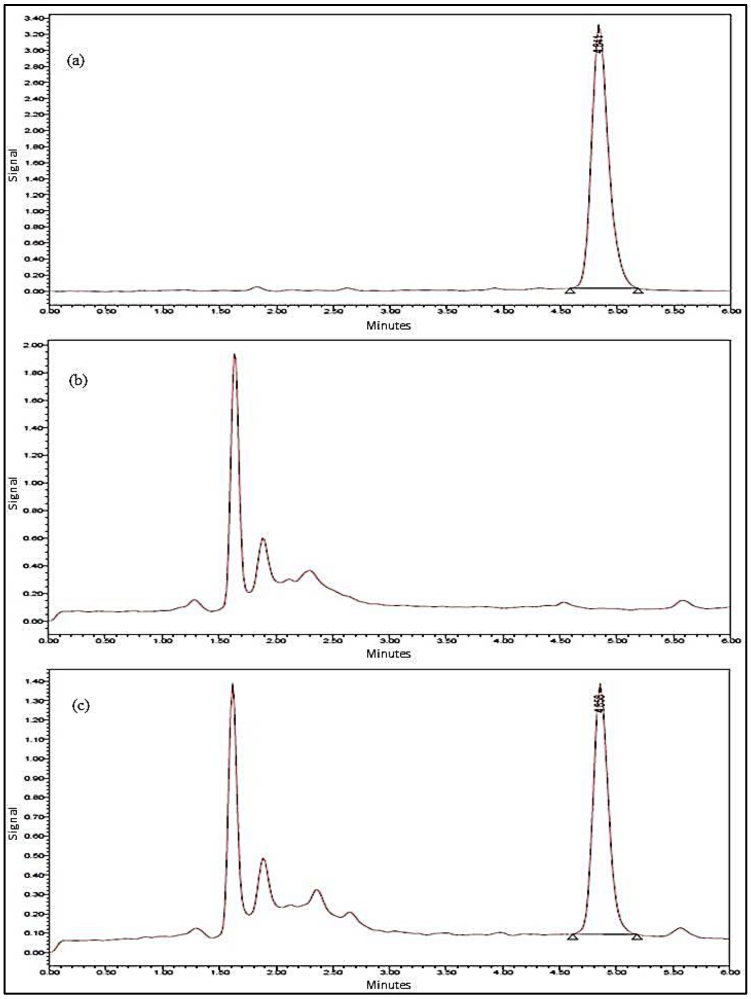

Figure 1. (a) Chromatogram of 1000 ng/mL curcumin standard solution, (b) chromatogram of blank plasma, and (c) chromatogram of plasma spiked with 1000 ng/mL curcumin standard solution. Arrow indicates curcumin retention peak.

Table 2. Regression parameters for calibration curves (n=6) of curcumin plasma assay method

Calibration Curve (Day)	**Correlation Coefficient (R^2)**	**Slope**	**Intercept**
1	0.9999	0.0086	7.2605
2	1.0000	0.0097	2.0608
3	0.9999	0.0096	-0.0870
4	1.0000	0.0096	0.2900
5	1.0000	0.0096	-2.0267
6	1.0000	0.0095	0.1690
Mean	1.0000	0.0094	1.2778
S.D.	0	0	3.2

S.D. = Standard Deviation

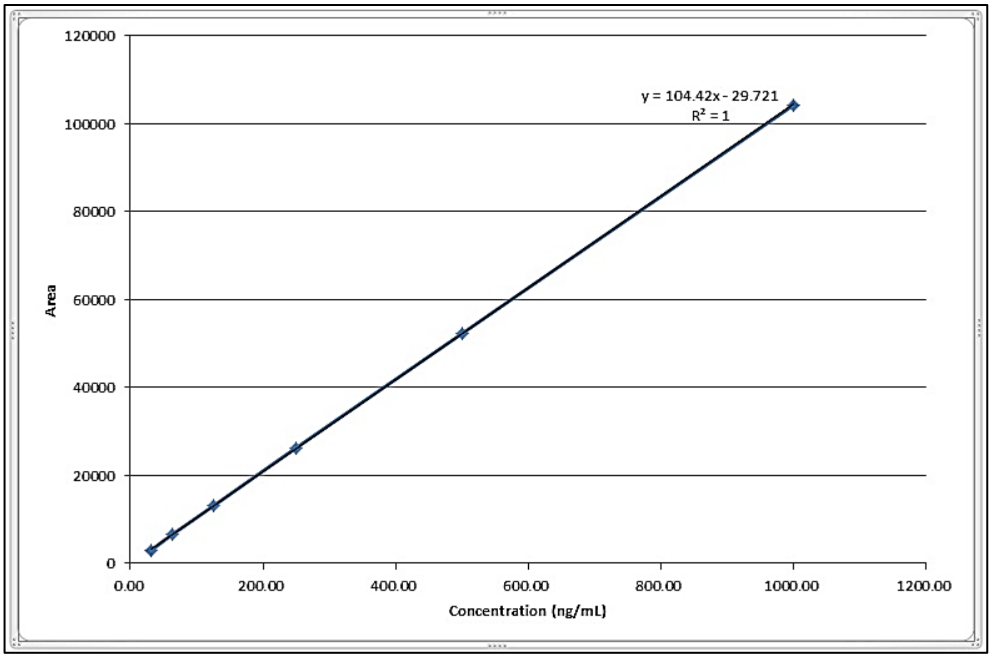

Figure 2. Example of calibration curve for curcumin plasma assay method from concentration of 31.3-1000 ng/mL

Accuracy and precision

Table 3 presents the within- and between-day accuracy and precision for each of the sample. The within-day accuracy and precision from 31.3-1000 ng/mL was in the range of 93.7-105.4 and 0.4-2.4%, respectively (Table 3 (a)). The between-day accuracy and precision from 31.3-1000 ng/mL was in the range of 96.8-100.3 and 0.4-2.3%, respectively (Table 3 (b)). In the current study, our assay successfully met the criteria for good accuracy and precision.

Recovery

A summary of the absolute recovery of curcumin was shown in Table 4. The absolute recovery of curcumin at concentrations of 31.3-1000 ng/mL was in the range of 97.9±2.1%-99.9±0.5% (Table 4). A study carried by Ma and colleagues in 2007 shown that the recovery of curcumin was 97.08% at concentration of 50 ng/mL, 95.69% at concentration of 200 ng/mL and 94.90% at concentration of 1000 ng/mL respectively [17]. Whereas the recovery of curcumin was 95.70±5.60% at concentration of 2 ng/mL, 97.70±5.80% at concentration of 20 ng/mL, 95.60±5.00% at concentration of 400 ng/mL and 96.60±8.40% at concentration of 400 ng/mL respectively in a research investigated by Li and co-workers in 2009 [18]. The absolute recovery shown in the current study was relatively high and precise as compared to the reported literature [17, 18].

Stability

Stability study is necessary in order to rule out the degradation of the investigated compound, i.e., curcumin in plasma samples. Table 5 displays the stability results of curcumin in plasma of the current study. According to Table 5, all the accuracy and precision are within 85%-115% and ≤ 15%, respectively. Post-operative stability in autosampler and short-term stability of curcumin at ambient temperature were satisfactory, with no significant degradation of curcumin being observed for up to 8 hours (Table 5(a) and (b)). Table 5(c) shows that curcumin was stable in plasma at both low and high concentration over three cycles of freeze-thaw. Although sample storage at -20°C is recommended for long term storage, the long-term stability was necessary to be evaluated at the zero-time point, 1-month and 2-months at least. Curcumin in plasma was stable for up to 2-months at -20°C (Table 5(d) and Table 5(e)). These results indicate that curcumin has an acceptable stability under all the tested conditions.

Comparison of recent method with reported method
The comparison of the recent study with the reported methods in Table 6 reveals that the optimized HPLC method developed in the recent study offers a significant advantage in terms of shorter running time. The reduced analysis time is achieved through optimized chromatographic conditions, resulting in faster elution and separation of curcumin. This not only improves the efficiency of the analysis process, but also saves valuable time in the laboratory. Despite the shorter running time, the recent method maintains comparable accuracy, and recovery in detecting curcumin levels in human plasma, making it a promising alternative for efficient and reliable curcumin analysis in clinical research and pharmaceutical applications.

Table 3. Within- and between-day accuracy and precision of curcumin plasma assay method (n=6)

QC Sample	Curcumin Concentration (ng/mL)	Mean Determined Concentration (ng/mL)	Accuracy (%)	Precision (C.V. %)
(a) Within-day	31.3	29.8	95.2	2.1
	62.5	58.6	93.7	1.7
	125	131.7	105.4	1.7
	250	247.9	99.1	2.4
	500	501.8	100.4	0.5
	1000	994.1	99.4	0.5
(b) Between-day	31.3	30.4	97.4	2.3
	62.5	60.5	96.8	1.3
	125	124.4	99.5	1.3
	250	250.1	100	1.3
	500	501.4	100.3	0.4
	1000	990.7	99.1	1

C.V. = Coefficient of Variation

Table 4. Mean recovery of curcumin in plasma (n=6)

Sample	Curcumin Concentration (ng/mL)	Absolute Recovery (%)	C.V. (%)
Curcumin	31.3	97.9	2.1
	62.5	98.9	1.7
	125	99.8	1.8
	250	98.8	2.5
	500	99.9	0.5
	1000	99	0.5

C.V. = Coefficient of Variation

Table 5. Stability of curcumin in plasma (n=6)

Sample Condition	Curcumin Concentration (ng/mL)	Mean Determined Concentration (ng/mL)	Mean Deviation (%)
(a) Short-term stability	31.3	31	-0.9
(8 hours temperature)	1000	1991.5	-0.4
(b) Post-operative stability	31.3	31.7	1.5
(8 hours in autosampler)	1000	1023.5	2.4
(c) Freeze-thaw stability	31.3	31	-0.9
(3 cycles)	1000	921.1	-7.9
(d) Long-term stability	31.3	30.9	-1.1
(1 month)	1000	952.9	-4.7
(e) Long-term stability	31.3	30.9	-1.2
(2 months)	1000	974.1	-2.6

C.V. = Coefficient of Variation

Table 6. Comparison of curcumin determination HPLC methods in human plasma

	Recent Method	Reported Method 1	Reported Method 2
Instrument	HPLC	HPLC-MS/MS	HPLC
Curcumin extraction method	Protein precipitation using acetonitrile	Liquid-liquid extraction	Protein precipitation using acetonitrile
Analysis time (min)	6	8	9
Retention time (min)	4.9	5.9	8.2
Specificity	No interference to the peak of interest	No interference to the peak of interest	No interference to the peak of interest
Sensitivity (ng/mL)	31.3	1.00	10
Linearity (R^2)	1.0000	0.998	0.999
Accuracy (Intra-Day %)	93.7-105.4	94.20-108.36	99.98-104.24
Accuracy (Inter-Day %)	96.8-100.3	94.40-108.36	100.30-102.21
Recovery (%)	≥97.9; CV=0.5-2.1	≥85.6; CV=2.44-6.86	≥101.81; CV=0.79-9.26
Stability	No significant degradation observed	No significant degradation observed	No significant degradation observed
Advantages	Short analysis time	Higher sensitivity	Higher accuracy and recovery
Reference	-	[32]	[33]

Self-emulsifying curcumin formulation: Solubility

Among the excipients studied, the solubility of curcumin in PEG-400 was significantly higher, 194.28±9.92 mg/g and the highest solubility of curcumin was observed in soyabean and corn oils at 0.71 ±4.22 mg/g and 0.71 ±5.63 mg/g ($p < 0.05$), respectively as shown in Table 7.

Table 7. Solubility study of curcumin in oil phase and co-solvent, n = 3

Potential Vehicle	Concentration of Curcumin (mg/g)	Standard Deviation
Canola oil	0.60	4.27
Corn oil	0.71	5.63
Soyabean oil	0.71	4.22
Sunflower oil	0.66	5.67
Peanut oil	0.68	2.58
Olive oil	0.61	4.67
Oleic acid	0.63	9.92
PEG-400	194.28	9.92

Evaluation of blank and loaded self-emulsifying formulation

Results revealed that the highest number of the rapidly formed (within 1 min) dispersions of "grade A" with the lowest surfactant concentrations was obtained with Cremophor EL® (50%) in soyabean oil out of the 45 formulations (from ration 1:9 to 9:1). Moreover, the curcumin loading in the curcuma extract SEDDS was the highest ($p < 0.05$) at soyabean oil: PEG-400 ratio of 3:2; thus, it was selected and combined with Cremophor EL® at a ratio of 5:5 to produce the optimized loaded SEDDS formulation (grade A).

Pre-clinical pharmacokinetic study

The concentrations of curcumin in the group of rats fed with curcuma extract loaded SEDDS formulation were significantly increased, with mean C_{max}, T_{max}, and $AUC_{0\text{-}1440m}$ values of 238.81 ng/mL, 70.83 min, and 11249.60 ng.min/mL, respectively; however, curcumin from curcuma extract alone was undetectable (below the HPLC LOQ of 31.25 ng/mL), implied that the bioavailability of this purely bioactive form was very low. Mean plasma concentrations versus time profile of curcumin are shown in Figure 3; such impact probably linked to the synergistic enhancement of curcumin absorption by PEGs and Cremophor EL® [34]. The designed SEDDS was able to offer dramatic improvements in bioavailability. Hypothetically, considering the HPLC LOQ value, the AUC was calculated for curcumin from curcuma extract alone over the time of detection for curcumin from curcuma extract loaded in SEDDS formulation. Comparing the AUC for curcumin from curcuma extract loaded in SEDDS formulation, and the hypothetical AUC for curcumin from curcuma extract alone, resulted in value of 1107.10 ng.min/mL, with 15-folds increment in the bioavailability using the developed curcuma extract-SEDD in this work. As a result, the developed curcuma extract-SEDDS formulation offered higher curcumin plasma concentration in compared to the curcumin loaded in solid lipid nanoparticles (C_{max} of 20.85 µm equal to ~7.7ng/ml) with an oral dose of 400 mg/kg [35], as well as curcumin loaded in self-micro emulsifying drug delivery system (SMEDDS) (C_{max} of 0.15 µm equal to ~0.056 ng/mL) with an oral dose of 100 mg/kg [36].

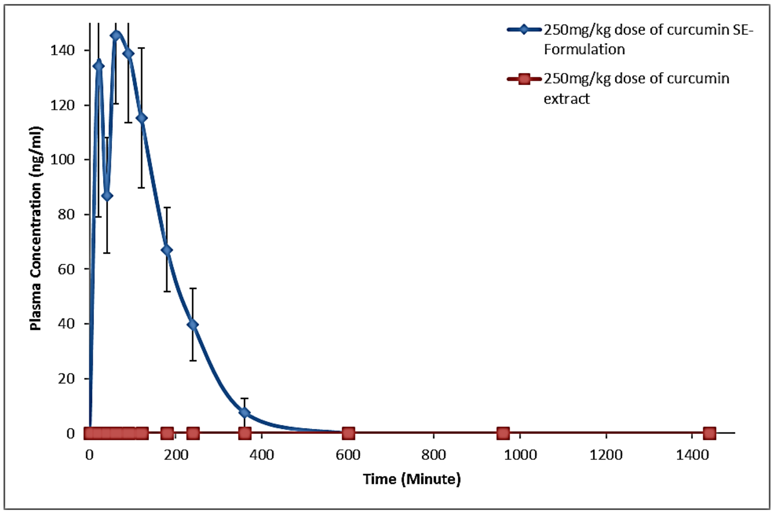

Figure 3. Mean plasma concentration-time profiles (Mean ±S.E.M., n = 6) of curcumin after oral administration of 250 mg/kg curcumin suspension and curcumin loaded in SEDDS formulation

Conclusion

A reliable, simple and sensitive high-performance liquid chromatographic (HPLC) method has been developed and validated as per the ICH guidelines for the determination of curcumin in plasma. This method offers adequate, accuracy, precision, appropriate retention time, moderate pressure and good peak shape. Curcumin remained stable for up to eight hours in the stability test, and did not degrade even after three freeze-thaw cycles. The stable long-term storage period was up to two months. The method application in quantifying the SEDDS formulation in the *in vitro* (solubility and loading content) and *in vivo* level was successfully achieved. The current method is easy and fast to perform, hence, can be applied in pharmacokinetic studies of curcumin alone, or in SEDDS formulation.

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Conflict of interest

The authors declare there are no financial/commercial conflicts of interest.

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