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A Novel Reversed Phase High Performance Liquid Chromatography Method To Accurately Determine Low Concentrations Of Curcumin In Rat Plasma

Setyaningsih *D*[1,2*], Murti YB[2], Martono S[2], Hinrichs W.L.J.[3], Hertiani T[2], Fudholi A[2]

[1]*Faculty of Pharmacy, Sanata Dharma University, Yogyakarta, Indonesia*
[2]*Faculty of Pharmacy Gadjah Mada, University, Yogyakarta, Indonesia*
[3]*Department of Pharmaceutical Technology and Biopharmacy, University of Groningen, The Netherlands*

ABSTRACT

Due to its lipophilic nature, curcumin levels in plasma are very low after oral administration, and therefore hard to detect. A number of chromatographic methods, including LC/MS have been developed. Although the LC/MS method is sensitive, the matrix effect can be difficult to handle. Furthermore, LC/MS equipment is relatively expensive compared to the conventional RP-HPLC. Therefore, the aim of this study was to develop a sensitive and reliable method for the determination of curcumin concentration in plasma using an RP-HPLC. *Curcuma longa* extract was used which contains three curcuminoids. The method started by selection of mobile phase to optimally separate the curcuminoids. The best mobile phase composition was used to analyze the plasma samples. Rat plasma was spiked with curcumin and processed for protein precipitation followed by liquid-liquid extraction of curcuminoids and an internal standard, emodin. Chromatogaphic separation of curcuminoids and emodin was achieved using a Knauer C-18 column (250 x 4.6 mm; particle size: 5µm) and a gradient program of mobile phase of three solvents, methanol-acetonitrile-1% acetic acid. A gradient elution was applied with increasing the ratio of the volume percentages of acetonitrile to acetic acid from 50/45 to 53/42 during 5 minute and thereafter elution was isocratic for 15 minute. The methanol concentration was kept constant at 5 vol-% during the whole run. The method was validated according to the FDA guidelines.The method was selective, with an excellent resolution (Rs value > 2.5). The peak shape of both curcumin and emodin were symmetric with a tailing factor of 0.9-1.1. The method linearity (correlation coefficient of 0.999) was demonstrated at 6 to 200 ng/mL. The intra- and inter-day precision was 5.90-8.50% and 5.37-11.26%, respectively; the intra- and inter-day accuracy was 92.47-103.61% and 96.17-105.70%, respectively. In conclusion, the RP-HPLC method meets the validation requirements as described in the FDA guidelines and is applicable to accurately quantify curcumin concentrations as low as 6 ng/mL in rat plasma samples.

Keywords: Tumeric, *Curcuma longa*, rat plasma, RP-HPLC, validation

INTRODUCTION

Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is the active ingredient of turmeric (*Curcuma longa* L), a food spice and colorant widely used in South-eastern Asia. Since the ancient time, turmeric has been used as an important part of JAMU[1], a traditional Indonesia medicine from plants. As a part of the JAMU, in folk medicine turmeric has been used for curing and preventing many ailments like liver disease, digestion problem and dysmenorrhoea. Curcumin, the active constituent of turmeric, has been reviewed to have a variety of biological activities and pharmacological actions, such as anti-tumor, anti-inflammatory, anti-virus, anti-oxidation, anti-HIV and hepatoprotective[2]. Being granted as a Generally Recognized As Safe (GRAS) compound by the Unites State Food and Drug Administration, together with the many beneficial activities, the exploration of this compound are enormous, thus makes curcumin to be highly potential use in clinical applications. Despite the extraordinary benefits, curcumin concentration in systemic circulation after oral intake was reported to be very low. A study by Wahlstrom and Blennow in Sprague-Dawley rats reported that 75% of curcumin was found in the faeces, while negligible amount was appeared in the urine and plasma after oral administration of the dose of 1g/kg curcumin[3]. Another study revealed that 1 h after a single oral dose of 1 g/kg, the curcumin concentration in rat plasma was lower than 20 ng/ml[3]. In a human study, the maximum plasma level of curcumin was only 11 ng/mL following oral administration of 2 g curcuminoids containing 85% curcumin, and 1 hour later no curcumin levels could be detected at all[4], indicating rapid clearance from the body. The low curcumin concentration can be ascribed to its poor absorption due to its low aqueous solubility (11 ng/mL)[5] with a calculated logP of 4.12 (by a default setting of

**Author for Correspondence Email: fudholi_apt@ugm.ac.id; dewisetya03@yahoo.com*

ChemAxon 5.2.5.1). Besides being poorly absorbed, the low plasma concentration of curcumin is also attributed to the extensive biotransformation, which has been reported to occur in the intestinal tissue, liver, and kidney.

Metabolic studies in rats and mice demonstrated that curcumin glucuronate and curcumin sulfate are the major metabolites[6,7]. The low curcumin concentration found in the systemic circulation demands an assay with a sufficiently high sensitivity. It has been suggested that ability of a method to reliably quantify curcumin concentrations below 10 ng/ml is necessary for a better characterization and understanding the fate of curcumin in the body[8]. Several Reverse Phase High Performance Liquid Chromatography (RP-HPLC) methods have been developed to response to the need of a reliable and highly sensitive method for curcumin determination in biological samples. In various studies HPLC UV-Visible detection was used to analyze curcumin in the plasma samples of rats. However, in most of them a lower limit of quantification (LLOQ) below 15 ng/mL was not reached[9-11]. Although it was expected that to be more sensitive than a UV-Visible detector, the HPLC analysis using a fluorescent detector as performed by Schiborr *et al*. did not improve the method sensitivity as the detection limit was 20 ng/mL[12]. In a few studies, however, an LLOQ below the suggested value of 10 ng/mL obtained by HPLC UV-Visible detection was found. Disadvantages of these methods are that a quite high sample injection volume of 50 µL is required[13] or that it requires longer analysis time (ca 50 minutes) than the methods described above[14]. An attempt to achieve a more sensitive method, the use of an HPLC employing a mass spectrophotometer detector (LC-MS) has been explored. In two studies an LLOQ value of 0.5 ng/mL has been found[15,16]. Although the LC-MS method have been reported to have a satisfied sensitivity and selectivity with a significant reduced analysis time compared to the conventional HPLC, the equipments are relatively expensive and the effects of the matrix are not easy to handle[17]. In this study, we developed an RP-HPLC UV-Visible method using gradient system that produced reasonable separation and sensitivity and was validated according to the FDA guidelines for bioanalysis. In this developed method, curcumin concentration as low as 6 ng/mL was able to be accurately quantified with satisfactory chromatogram properties. The lower limit of detection was even 0.5 ng/mL while keeping the injection volume small (20 µL). In addition, compared to the method developed by Ireson *et al*.[14], and Li *et al*.[13], the developed method allows a substantial reduction of analysis time (ca 20 min).

MATERIALS AND METHODS

Chemicals and reagents. Curcumin as the standard compound was purchased from Nacalai, Japan. *C. longa* extract was a kind gift from PT Phytochemindo Reksa, Bogor, Indonesia. It is well known that *C. longa* extract is composed of a mixture of three curcuminoids, namely: curcumin, demethoxycurcumin, and bis-demethoxycurcumin[18]. HPLC mesurements operated using the method developed in this study indicated that the curcumin content of the extract was 67.9%. Emodin which was used as an internal standard compound was purchased from Sigma. Solid dispersion of composed of *C. longa* incorporated in a matrix of polyvinylpyrrolidone K30 (PVP K30) was prepared by drying an ethanolic solution of both components using a rotary evaporator. HPLC grade of acetonitrile and methanol were obtained from Merck, Darmstadt, Germany. Analytical grade glacial acetic acid and ethyl acetate were purchased from Merck, Darmstadt, Germany. Water was used as ultra pure water, which was prepared using a Milli-Q water purifier.

Equipment

The analysis was carried out using a 2010 HT Shimadzu HPLC (Kyoto, Japan) consisting of a serial dual plunger pump, an autosampler, and a UV detector. The HPLC was connected to a computer with LC-solution software.

Wavelength detection

Wavelength selection was carried out using a spectrophotometer (Shimadzu, Japan). A methanolic solution of curcumin was prepared at a concentration of 2.5 µg/mL. The solutions were scanned at a wavelength range of 200 – 600 nm.

Chromatographic condition

The chromatographic separation was carried out on a Knauer C18 column (250 x 4.6 mm) equipped with a precolumn (Dr. Ing. Herbert Knauer, Berlin, Germany). The packing material was Eurospher 100 with 5µm particle size. The injection volume was 20 µL and the flow rate was 1.0 mL/min. The column oven was set at a temperature of 30°C. The UV-detection was based on the wavelength scanning experiment.

Mobile phase optimization

The selection of mobile phase was based on literature[19,9,20] in which the separation of the three curcuminoids from the methanolic solution of *C. longa* extract by HPLC was reported. A volume of 20 µL was injected onto the HPLC column. The peaks resulted from elution of that mobile phase were compared and the quality of the chromatographic separation, i.e. tailing factor (T), peak resolution (Rs), and theoretical plate number (N) were determined using the LC-solution software. The recommended limits of these chromatography parameters are $T<1.5$, $Rs>2.0$, and $N>2000$[21,22]. The optimized HPLC system was tested on the chromatographic separation of extracted rat plasma sample taken at 15 minutes following oral administration of a PVP based solid dispersion formulation containing *C. longa* extract (20 mg/kg BW). This pilot animal experiment was approved by the Medical and Health Research Ethics Committee (MHREC) number: KE/FK/5/2/EC continued to KE/FK/512/EC year 2013. The plasma samples were processed according to the described method in the section "sample preparation" of this manuscript and analyzed using the optimized HPLC system. The chromatogram quality was determined by assessment of T, Rs, and N.

Stock solution, calibration samples and quality control samples

Stock solutions of curcumin and emodin as the internal standard (IS) were prepared separately in methanol at concentrations of 950 µg/mL and 1.2 mg/mL, respectively.

The stock solution of curcumin was diluted with methanol to prepare working stock solutions of 0.06, 0.125, 0.25, 0.5, 1.0, and 2.0 µg/mL. Series of concentrations of the calibration samples were prepared by spiking of 10 µL of each working stock solution into 90 µL blank plasma samples. Using a similar spiking technique to that of calibration samples, control samples as quality control (QC samples) were prepared at a concentration of 20, 50 and 100 ng/mL for precision and accuracy studies. Another set of QC samples for the stability studies were prepared at aconcentration of 20 and 100 ng/mL. The QC samples were kept at -20°C and were brought to room temperature before analysis. The stock solutions were stored at-20°C for the maximum of 3 weeks[23]. During sample preparation, the samples were properly shielded from light to prevent photodegradation of curcumin[24].

Sample preparation

IS stock solution of emodin was diluted with methanol to prepare a working stock solution at the concentration of 5.95 µg/mL. The frozen samples were thawed at room temperature. After thawed completely, the samples were vortexed for 20 sec. To 100 µL of sample, 10 µL of working stock solution of IS was added. Then, 20 µL of 2% acetic acid and 400 µL of acetonitrile was added to induce protein precipitation. The resulting mixture was vortexed for 20 sec. A volume of 1000 µL extraction medium, which consisted of 95 vol-% ethyl acetate and 5 vol-% methanol[11], was added to the sample. The mixture was vortexed for 2 minutes to extract the curcumin. After centrifugation at 1000 rpm for 20 min, the supernatant was removed quantitatively and transferred into 5 mL tubes. The solvent was then allowed to fully evaporate under nitrogen stream at the temperature of 40 °C. The dried samples were reconstituted with 100 µL methanol. To remove any undissolved substances the sample was centrifuged at 8000 rpm for 20 minutes after which the supernatant was taken. The supernatants were transferred into the inserts of the HPLC amber vials and an aliquot of 20 µL was injected onto the HPLC column. The nominal concentrations of the calibration series were 6, 12.5, 25, 50, 100, and 200 ng/mL curcumin and 0.595 µg/mL emodin.

Bioanalytical method validation

Validation was carried out to evaluate the HPLC method for the quantification of curcumin in the plasma samples. The validation procedures were according to the Food Drug Administration (FDA) guidelines for bioanalytical method validation[25]. The basic parameters for bioanalytical method validation, i.e. selectivity, accuracy, precision, sensitivity, and stability, were evaluated. Additionally, the system suitability, extraction effectivity, and robustness were also investigated.

System suitability test

System suitability was assessed by conducting six replicate injections of a 1.8 µg/mL solution of curcumin in methanol and run under the optimized chromatographic conditions. The peak areas and the retention times were determined and their RSDs were calculated. An RSD value below 2% is acceptable[21].

Selectivity

Selectivity is the ability of a method to discriminate between the analyte (curcumin) and other compounds present in the sample matrix. For the selectivity study, blank plasma samples originated from six different rats were analyzed. Plasma samples spiked with 6 ng/mL curcumin standard solution and 595 ng/mL IS solution were also analyzed. Plasma samples spiked with curcumin at the LLOQ concentration were used in the selectivity study. Selectivity was confirmed by comparing the chromatograms of spiked plasma samples with those of blank plasma samples.

Extraction effectivity

To determine extraction effectivity of the method, a set of samples with 5 replications for each concentration, low and high levels[26], were prepared by spiking curcumin working solutions (10 µL) into 90 µL blank plasma samples resulted the sample concentrations of 20 and 100 ng/mL. Each sample was supplemented with IS solution to achieve a final emodin concentration of 0.595µg/mL. Curcumin and and emodin were subsequently extracted from the samples using the method described above. In a control experiment, a set of blank plasma samples were prepared and subjected to the extraction procedure without the samples being supplemented with curcumin and IS solution. After completion of the extraction procedure, the control samples were spiked with curcumin and IS solutions at same concentration and volumes that were found in the pre-extraction spiked samples.The samples were treated as described in section “sample preparation” of this manuscript and injected into the HPLC column. Extraction effectivity for curcumin and IS was determined by calculating the ratios of the peak areas of the pre-extraction spiked samples to those of samples spiked after extraction multiplied by 100 to obtain the effectivity in %[26,27].

Calibration curve and linearity

A calibration curve was generated using curcumin solutions of six different concentrations with three replications for each concentration. The peak area ratios of curcumin to IS were plotted against the nominal concentrations of curcumin. A least-square regression analysis was used to calculate the regression equation and correlation coefficient. According to the FDA guidelines[25], the linearity of the calibration curve is justified by the value of correlation coefficient (r) of at least 0.995. Furthermore, each back calculated concentration in the calibration curve was determined and should meet the requirement of ±15% deviation from the nominal value for at least four out of six nonzero concentrations of standards. The lower limit of quantification (LLOQ) was determined by evaluation of the precision and accuracy at the lowest concentration of the calibration samples which should have a precision of less than 20% relative standard deviation (RSD) and an accuracy of 80-120%[25,26]. The carry-over was determined by analysis of the highest curcumin concentration in the calibration samples. The carry-over experiment demonstrated no signals appeared around the retention time of curcumin or emodin, indicating no carry-over was observed using the optimized HPLC system.

Precision and accuracy

Intra-day and inter-day precision and accuracy were determined using blank plasma samples spiked with curcumin solutions of three different concentrations with 5 replications of each concentration (QC samples). The concentration levels were considered as low, medium, and high, which were 20, 50, and 100 ng/mL, respectively. The curcumin concentrations were obtained by calculating the ratios of curcumin peak area to that of IS and plotting it on the calibration curve. Precision was determined as a percentage relative standard deviation (RSD), while accuracy was assessed by the percentage of the found concentration to the nominal concentration. The precision determined at each concentration level should not exceed 15% of RSD, except for the LLOQ, where it should not exceed 20% of RSD. The method is considered to be accurate when the mean value is within 15% of the actual value, except at the lower limit of quantification (LLOQ), where it should not deviate by more than 20%.

Stability experiment

The stability of curcumin in the plasma samples was investigated after three freeze thawing cycles, storage at-20°C for 10 days, and storage at ambient conditions for 4 hours. In addition, the stability of the reconstituted sample (thus a solution in methanol) was determined during the period of time the sample was placed in the autosampler of HPLC (which was at ambient conditions) before the actual measurement. Freeze-thaw stability was evaluated by thawing the plasma samples spiked with curcumin at both low and high levels (20 and 100 ng/mL) at room temperature and then refreezing them to be frozen for 24 hours at -20°C. This procedure was repeated three times after which the samples were analyzed. The 10 days stability study was conducted using plasma samples with curcumin concentrations of 20 and 100 ng/mL (QC samples). To ensure that curcumin was not degraded in the plasma sample during sample preparation including the sample extraction procedure, samples of 20 and 100 ng/mL curcumin were thawed and left unassisted for 4 hours under ambient conditions (22±2°C). After three freeze thawing cycles, or storage at -20°C for 10 days,or ambient conditions for 4 hours, the samples were prepared for and then analyzed by HPLC. The stability of the samples during the period of time they were maximally kept in the auto sampler before analysis was evaluated by reinjection of the reconstituted samples. Reconstituted samples containing 20, 50, and 200 ng/mL curcumin in methanol were reinjected after keeping them overnight in the HPLC autosampler at ambient conditions to mimic worse case conditions, i.e. to ensure the stability of the last sample in an analytical batch during the HPLC analysis (n=5 of each concentration level). The curcumin concentration of the samples subjected to the stability tests was calculated by plotting the ratios of the peak area of curcumin to the internal standard of the calibration curve, which was prepared on the day of analysis. RSD and accuracy were determined based on three replications of each concentration level. Samples are considered to be stable if the deviation of the observed concentration is within 15 % of the nominal value and the precision is below 15%[25].

Robustness

The robustness of the HPLC method was challenged by applying a deliberate change to the operational parameters of the developed method. To study the robustness, a methanolic solution of curcumin standard was used at the concentration of 0.5µg/mL at an injection volume of 20 µL. The changes applied to the parameters were the flow rate (0.6 and 0.8 mL/min), and the column oven temperature which was changed from 30°C to 26°C. The curcumin peak area resulted from the altered HPLC parameters were compared to the unaltered one. Robustness of the method was studied in triplicate. The RSDs of all collected data were calculated. The robustness is acceptable at RSD < 2%.

RESULTS AND DISCUSSION

Method development

Wavelength selection.

The wavelength detection for curcumin determination has been reported in the literature to be 420 nm [14, 13]. To be more accurate in the determination of curcumin, we conducted a wavelength scanning experiment of curcumin solution. The maximum absorption spectrum of curcumin solution in methanol was found to be 424 nm and thus, further analysis of curcumin was carried out at this wavelength. This analysis procedure involved the use of emodin as the internal standard compound, because there was an extraction method prior the determination of curcumin concentration. The use of IS is necessary as a correction factor for the loss of curcumin during sample preparation. Emodin was selected as the IS compound in this study because emodin and curcumin exhibits similar behavior with respect to extraction involving the solvents methanol, acetonitrile, and ethyl acetate, and water [13]. In addition to that, the peak of emodin was clearly observed at the wavelength used for curcumin determination (424 nm).

Selection of mobile phase.

The goal of this study was to achieve an excellent resolution in the chromatographic separation of curcuminoids. Since the source of curcumin in our research was the curcuma extract obtained from *C. longa* plant, which contains besides curcumin also demethoxycurcumin and bisdemethoxycurcumin, an acceptable peak resolution of curcumin to the neighbouring curcuminoid peaks is required to provide an accurate determination of curcumin in the plasma sample. Therefore, selecting the HPLC system e.g. the eluent composition was necessary before conducting further experiments. The selection of mobile phase was based on literature[20,19,9]. Firstly, the mobile phase composed of acetonitrile – 1% acetic acid at 54:45 v/v[19,9] was chosen to separate curcumin from the curcuminoids of the *C. longa* extract dissolved in methanol under the isocratic elution. The chromatographic condition using this eluent composition was unable to separate curcuminoids into three peaks; instead, only one peak was displayed in the chromatogram. This result indicates that the method failed to demonstrate the selectivity parameter of a method validation. Although the chromatogram showed a single

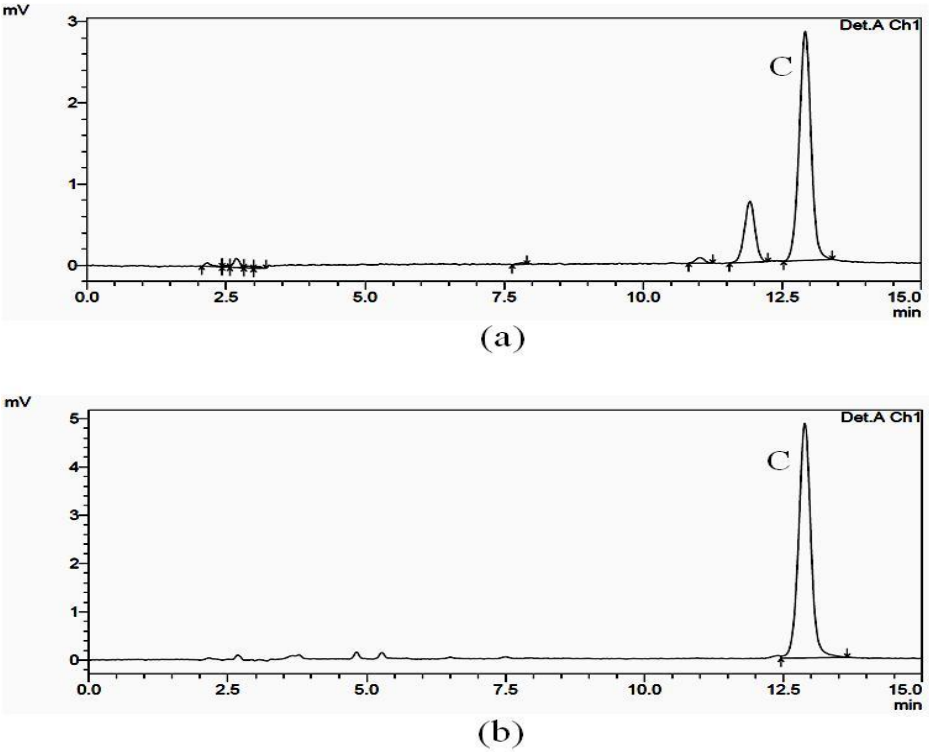

Figure 1: Chromatogram of *C.longa* extract eluted using the optimized HPLC system; (a) *C.longa* extract in methanol (9 µg/mL).; (b) curcumin stock solution 0.9 µg/mL "C" represents curcumin.

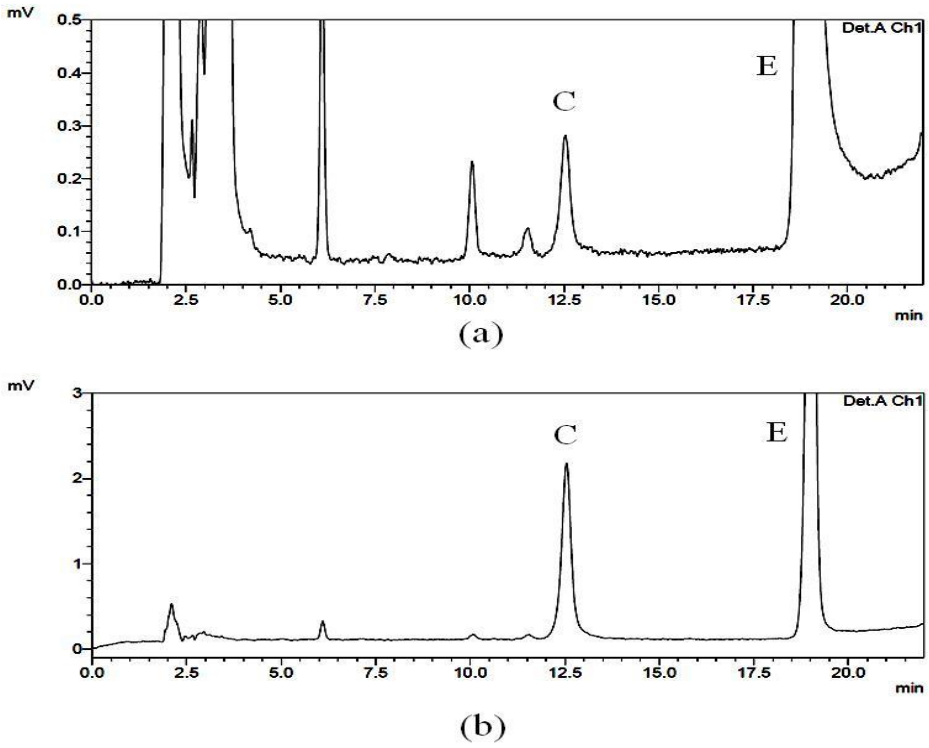

Figure 2: Chromatogram separation of curcumin in extracted plasma samples. (a) plasma sample taken at 15 minutes following oral administration of a PVP K30 based solid dispersion formulation containing *C.longa* extract. (b) curcumin standard solution (0.5 µg/mL); "C" and "E" represent curcumin and emodin, respectively.

peak with a sharp and symmetrical shape with the tailing factor (T) of 1.01 and a theoretical plate number (N) of 12449, which meets the recommendation limits (T <1.5; N ≥ 2000)[21,22] using this mobile phase composition leads to an inappropriate determination of curcumin in plasma sample when the sample contains two other curcuminoids as well. A perfect peak separation of the three curcuminoids was reported when a mixture of methanol

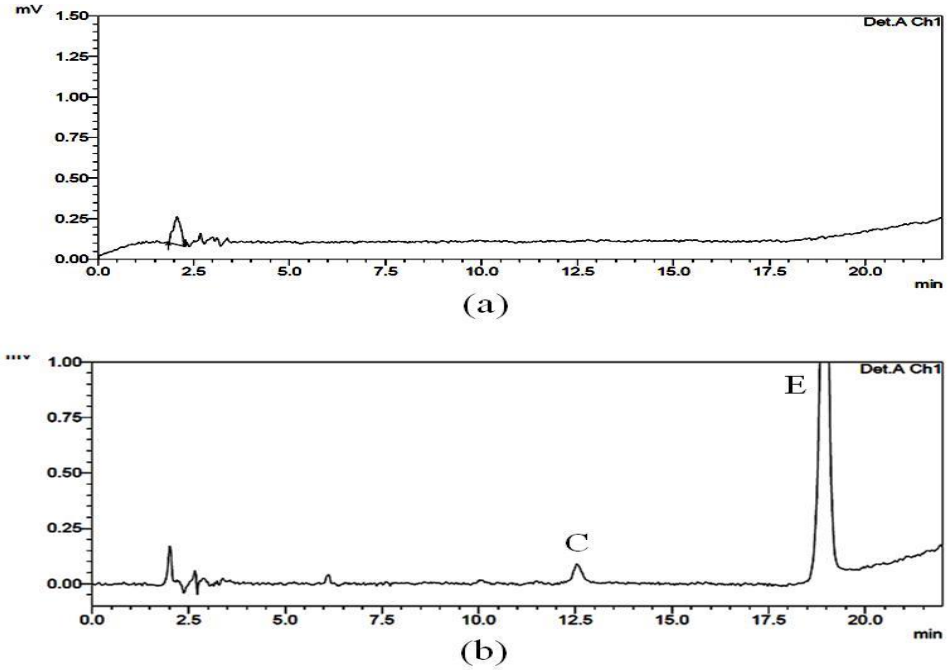

Figure 3: Representative of chromatogram of (a) blank plasma sample and (b) the plasma spiked with curcumin at the lower limit of quantification (6 ng/mL). "C" and "E" represent curcumin and emodin, respectively.

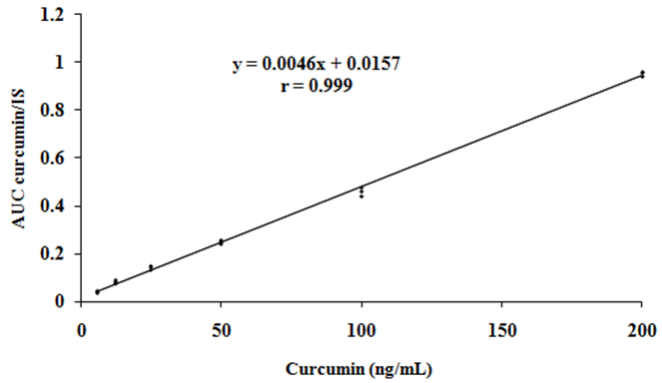

Figure 4: A calibration curve for curcumin prepared in rat plasma (n=3).

Table 1: System suitability test of curcumin

Injection	Area	Retention time (min)
1.	332774	12.994
2.	328770	12.979
3.	337665	12.980
4.	330586	12.890
5.	332107	12.880
6.	338280	12.849
Average	333363.7	12.929
SD	3831.86	0.063
RSD	1.15	0.490

and tetrahydrofuran (THF)[28] was used as mobile phase. However, THF has some disadvantages, i.e. it strongly aborbs UV light, reacts with oxygen, displays slow column equilibration[29], and causes swelling of polyether ether ketone resin, the constituent of most HPLC tubings[30]. Therefore, the use of THF in the mobile phase is not recommended in most HPLC systems[31]. An attempt to separate the three curcuminoids peaks by using a gradient elution program instead of isocratic elution program appeared to be successfull. The elution method was adapted from the work of Jayaprakasha et al.[20], in which curcuminoids from different curcuma species were analyzed. In this study, the gradient program of the method was modified in order to be able to determine curcumin in a plasma sample which also contains other two curcuminoids and emodin as the internal standard. *C. longa* extract in a methanolic solution was eluted with a mixture of methanol, acetonitrile, and 1% acetic acid aqueous solution (pH 3.5) using a gradient program. To optimize the method, different gradient programs were evaluated, i.e. the volume ratio of acetonitrile to 1% acetic acid aqeuous solution, flow rate, column oven temperature, and injection volume were varied. Chromatogram characteristics such as retention time, T, Rs, and N were determined using the LC-solution software. The optimal gradient mode was achieved by increasing the ratio of the volume percentages of acetonitrile to 1% acetic acid aqueous solution from 50/45 to 53/42 during 5 minute and thereafter isocratic elution for 15 minute. The methanol concentration was kept constant at 5 vol-% during the

Table 2: Precision and accuracy of alibration series after backcalculated from the regression equation (n=3)

Nominal concentration (ng/mL)	Mean calculated concentration (ng/mL)	Precision (%)	Accuracy (%)
6.0	5.07	17.03	84.54
12.5	14.11	10.83	112.85
25.0	26.95	6.77	107.81
50.0	49.94	3.42	99.89
100.0	95.45	4.16	95.45
200.0	201.97	1.06	100.99

whole run. Another optimized parameters of the chromatographic separation of curcuminoids were 20 µL of injection volume, 1.0 mL/min of flow rate, and 30°C. of column oven temperature. The chromatogram of *C. longa* extract solution using this method displays three peaks with a sharp and narrow shape indicating an excellent separation of the three curcuminoids (Fig. 1a). In Fig. 1a, the peak appeared at a retention time of 12.91 minutes was confirmed as curcumin peak, according to the standard curcumin solution assayed in the same analytical batch (Fig. 1b). The peak of curcumin was perfectly symmetrical in shape and separated from the neighbouring peaks with a T of 1.01 and an Rs of 2.64. The high theoretical plate number as demonstrated by a value of 17294 indicates a high efficiency in the chromatographic separation. The optimized HPLC system was tested on an extracted plasma sample containing curcuminoids. The plasma was taken at 15 minutes following oral administration a PVP K30 based solid dispersion containing *C.longa* extract. The Fig 2a presents the chromatographic separation of the extracted plasma sample. The chromatographic separation as shown in Fig. 2a displays an excellent peak separation of curcumin of the extracted plasma sample taken at 15 minutes after the rat was administered orally with the *C.longa* solid dispersion formulation. The optimized gradient system developed in this study was able to separate curcumin peak of the extracted plasma sample with a T of 0.97, Rs of 2.52, indicating a perfectly symmetrical peak and a satisfactory resolution. The retention time of the curcumin peak of the extracted plasma sample appeared consistently with that of the peak of curcumin standard solution (Fig. 2b). N of the extracted plasma sample (Fig. 2a) was 11991, which meets the recommendations limit of N > 2000. These data indicate an efficient chromatographic separation. To conclude, the developed analytical method is applicable to determine curcumin in plasma samples of rats which were administered orally with a formulation containing *C.longa* extract.

Bioanalytical method validation

System suitability test

System suitability test is an integral part in the analytical method and used to confirm the adequacy of the operating system[32]. The optimized gradient mode was used to assess the HPLC system suitability. Six repetitive injections of curcumin standard solution of the same concentration were analyzed. The data resulted in RSD values of 1.15% for the peak area and 0.49 % for the retention time (Table 1). The both RSD values were below 2% indicating the suitability of the the method.

Selectivity

The selectivity study showed that compared to plasma samples spiked with curcumin (Fig. 3b), the extracted blank plasma sample (Fig. 3a) did not show any interfering peaks at the corresponding retention times of curcumin and emodin. This result confirms the selectivity of the developed method.

Extraction effectivity

The mean extraction effectivity for the plasma spiked samples of 20 and 100 ng/mL were 97.86 ±12.58 % and 99.63±3.63 %, respectively. The extraction effectivity for the IS at the concentration of 0.595 µg/mL was 96.96±2.96%. The results indicate that the extraction method was adequate for the quantification of curcumin levels in plasma samples.

Calibration curve and linearity

The calibration standard is presented in Fig. 4. The calibration standard was obtained using six series of plasma samples spiked with curcumin standard solution. Measurements were in triplicate. The calibration curve was constructed by plotting the ratio the peak area of curcumin to that of the IS (y-axis) against the nominal curcumin concentrations (x-axis). As can be observed in Fig 4, linearity was shown over the concentration range of 6-200 ng/mL with the correlation coefficient (r) of 0.999, and the coefficient of determination (r^2) of 0.998. The least square regression analysis resulted the regression equation of y = 0.0046x + 0.0157. The back calculated concentrations based on the regression equation is presented in Table 2. Five out of six concentrations used to construct the calibration standard demonstrated below 15% deviation from the corresponding nominal values, which is within the range of the recommendation limits. The lowest concentration of the calibration series of 6 ng/mL was taken as the LLOQ value as the precision and accuracy

Table 3: Intra-day and inter-day precision and accuracy (n=5)

Nominal concentration (ng/mL)	Intra-day			Inter-day		
	Found (ng/mL)	Precision (% RSD)	Accuracy (% recovery)	Found (ng/mL)	Precision (% RSD)	Accuracy (% recovery)
20	18.49	5.90	92.47	21.14	11.26	105.70
50	44.24	7.75	88.49	52.64	8.45	105.28
100	103.61	8.50	103.61	96.17	5.37	96.17

Table 4: Stability studies of curcumin in rat plasma

Studied items	Nominal concentration (ng/mL)	Found (ng/mL)	Precision(%)	Accuracy (%)
Three freeze thawing cycles(n=3)	20	18.07 ± 1.19	6.57	90.35
	100	92.55 ± 2.09	2.25	92.55
10 days storage in -20°C(n=3)	20	20.71±1.33	6.40	103.57
	100	92.55±2.09	2.25	92.55
4 hr at room temperature(n=3)	20	19.77± 0.56	2.84	98.87
	100	97.07±4.26	4.39	97.07
Autosampler (overnight)(n=5)	20	21.20 ± 0.53	2.50	106.00
	50	46.07 ± 3.18	6.91	92.14
	200	208.59 ± 8.03	3.85	104.29

were 17.03 and 84.54%, respectively. The LLOQ value of this developed method was found to be 6 ng/mL, which corresponds to 0.12 ng in 20 µL of injection volume. Comparatively, considering the injection volumes, the LLOQs reported in literature were 1.25 ng[33] 2 ng[10], 0.3 ng[9] obtained with HPLC UV-visible detection. Using an HPLC with a fluorescence detector, Schiborr *et al*. reported a higher sensitivity with an LLOQ of 0.2 ng[12]. A recent publication reported a lower LLOQ of curcumin in plasma obtained by LC-MS/MS detector at 0.1 ng[34].

Compared to the LLOQ values found by others who used different HPLC detectors, the current LLOQ value of 0.12 ng obtained by our developed method achieves an excellent sensitivity, at which it is also comparable to the LLOQ value demonstrated by the analytical method employing an LC-MS/MS detector[34]. Apart from the LLOQ study, concentrations lower than 6 ng/mL were analyzed by the HPLC method, which were 0.5, 1.5, 3, and 4 ng/mL. At the lowest concentration of 0.5 ng/mL, the curcumin peak was still detectable. However, quantitative determination of curcumin lower than 6 ng/mL is not recommended, because no linearity was observed between the ratios of the peak area of curcumin to that of IS and the curcumin concentration at the concentrations below 6 ng/mL.

Accuracy and precision

Accuracy and precision data for intra-day and inter-day assay of spiked curcumin in plasma samples are presented in Table 3. The values of the assay for both intra- and inter-day were found to be within the acceptable limits required by the FDA guidelines (Table 3). For all concentrations used in the accuracy and precision studies, the mean concentration value was within the 15% of the intended concentration (20, 50, and 100 ng/mL) with RSD values below 15%.

Stability studies.

Stability of curcumin in plasma samples was tested at two concentration levels, low and high concentration (20 and 100 ng/mL) during (1) three freeze and thawing cycles, (2) storage at−20°C for 10 days, and (3) storage at room temperature for 4 h. In addition to that, post-preparation stability of curcumin at the concentrations of 20, 50, 200 ng/mL was evaluated, by re-injection of the reconstituted samples after overnight placement in the HPLC autosampler at room temperature. The results of the stability studies are presented in Table 4. The deviation of the observed curcumin concentration from the nominal values was below 15% in all cases, demonstrating sufficient sample stability during these challenges. Based on the stability data, it can be concluded that (1) analysis of plasma samples is possible after at least three times freeze and thawing, (2) the time between blood sampling from the animal and analysis can be at least 10 days when stored at -20°C and (3) plasma samples can be stored at room temperature for 4 hours, the time necessary for sample preparation. Furthermore, reconstituted samples in methanol can be placed in the autosampler of the HPLC at least 10-12 hours before they are analyzed.

Robustness study

The deliberate changes applied in the operation parameters of the HPLC were column oven temperature (26, 30 °C) and flow rate 0.6, 0.8, and 1.0 mL/min. The change in column oven temperature did not affect the peak area and the tailing factor as demonstrated by the RSDs< 2%, however it exerted a substantial change in the peak retention time (RSD>2%). A small variation in the flow rate resulted variation in the peak area and peak retention time with an RSD>2%, while the tailing factor was not affected (RSD< 2%). For all the variations in the operational parameters in the developed analytical method, it is indicated that in general the peak performance was influenced by the small variation introduced in the equipment; however, the small variation in the column oven temperature only shifted the peak retention time.

CONCLUSION

Considering the quality of the chromatographic separation of the tested samples and the parameters that were validated in this study, the developed analytical method based on the RP-HPLC gradient mode has demonstrated as selective, precise, and accurate. The proposed method was found to be linear in the concentration range of 6-200 ng/mL and considered to be highly selective with an LLOQ as low as 6 ng/mL. Qualitative detection of curcumin concentration in the plasma sample was possible at a concentration as low as 0.5 ng/mL. Stability studies demonstrates no significant degradation of curcumin in plasma samples during three freeze thawing cycles, storage at -20°C for 10 days, sample preparation and during storage of reconstituted samples overnight at ambient conditions. The robustness study shows that the deliberate change in the column oven temperature did not affect the

peak area and peak tailing factor. Therefore, the developed method is considered as applicable to accurately determine curcumin concentrations in plasma samples.

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