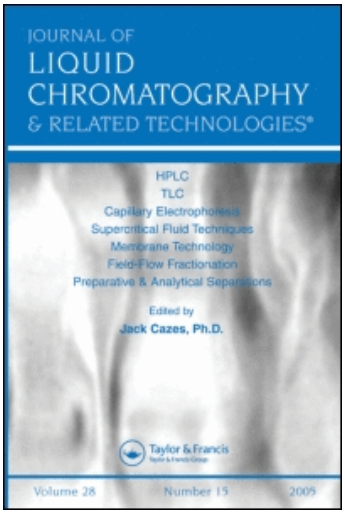

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Development and Validation of a Reversed Phase HPLC Method for Simultaneous Determination of Curcumin and Piperine in Human Plasma for Application in Clinical Pharmacological Studies

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Abstract: A sensitive and specific HPLC method was developed for the simultaneous determination of curcumin and piperine in human plasma in view of the potential therapeutic application of curcumin and piperine in various diseases. The HPLC method consisted of isocratic elution with acetonitrile-methanol-trifluoroacetic acid-water (17.6:35.3:0.1:47.0, v/v/v/v) with a flow rate of 2.5 mL min^{-1} on a Chromolith® SpeedROD RP-18 (50 × 4.6 mm) column at an ambient temperature. Ultraviolet detection was performed in programe mode at 415 nm for curcumin, 335 nm for piperine, and 280 nm for β-17-estradiol acetate (internal standard). Curcumin, piperine, and internal standard were extracted from plasma using ethyl acetate-propanol (9:1 v/v). Mean extraction recoveries for curcumin, piperine, and internal standard were 91.3, 91.4, and 92.9%, respectively. The assay was linear over the therapeutic concentration range (10–500 ng mL^{-1}) for both drugs with correlation coefficients of $r^2 > 0.99$. Limit of detection and limit of quantification were 1 ng mL^{-1} and 10 ng mL^{-1} for both curcumin and piperine. The method was applied to the determination of the

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concentrations of curcumin and piperine in healthy volunteers after treatment with 1500 mg curcumin and 500 mg piperine, and should find an applications in pharmacokinetic studies of these compounds.

Keywords: Curcumin, Healthy volunteers, HPLC, Piperine

INTRODUCTION

Curcumin [1,7-bis (4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3,5-dione] is the major yellow pigment present in turmeric, a commonly used spice, derived from the roots of the plant *Curcuma longa*.[1] It has been reported to have anti-tumorigenic, anti-oxidant, anti-cancer, anti-inflammatory, anti-microbial, and anti-malarial effects.[2] Recently, curcumin assumed greater significance because of its possible role in combination therapy with artemisinin derivatives for the treatment of malaria.[2,3] It is also under clinical evaluation as a potential cancer chemopreventive agent.[4,5] When curcumin is administered orally, it is poorly absorbed in the intestine.[6–9] Several studies have reported low bioavailability of curcumin in both rodents and humans despite the wide range of physiological and pharmacological properties.[4,10,11] Rapid intestinal metabolism of curcumin, especially sulfation, glucuronidation, and reduction are responsible for its relatively low blood concentrations.[1] Piperine, an active alkaloid of *Piper longum* and *Piper nigrum* has been reported to enhance the bioavailability of several drugs through inhibition of drug metabolism.[12,13]

Several HPLC methods have been developed to quantify curcumin[1,8,9,14,15] and piperine[16] in biological samples. Recently, Liu et al.[9] reported a validated LC/MS/MS assay for the determination of curcumin in rat plasma while Ma et al.[15] developed an HPLC method with 4-hydroxybenzophenone as an internal standard in the same matrix. A radiometric determination of curcumin has also been reported.[6] Bajad et al.[16] used a HPLC method for the determination of piperine in rat plasma. Verma and Joshi[17] reported a HPTLC method for identification and quantification of curcumin and piperine in an ayurvedic formulation. Shoba et al.[7] stated that a low dose of piperine can enhance curcumin bioavailability by 2,000% in humans, and the combination is well tolerated.

In view of the potential pharmacological effects of curcumin and the possible role of piperine in the enhancement of the bioavailability of curcumin, we describe a sensitive and specific reverse phase HPLC method for the simultaneous determination of curcumin and piperine in human plasma. The method has been applied to monitor the plasma concentrations of both the compounds in human volunteers.

EXPERIMENTAL

Reagents

Acetonitrile and methanol (HPLC grade) were purchased from Ranbaxy Fine Chemicals, Delhi, India while trifluoroacetic acid was purchased from Merck, Germany. Water was deionized and triple distilled. All other chemicals were of analytical grade and were used without further purification. Curcumin, piperine, and β-17-estradiol acetate (internal standard) were supplied by Sigma Chemicals, USA. Their structures are given in Figure 1. A phosphate buffer solution (12 mM; pH 3.40) was prepared by adding 0.1 mL of acetic acid to 9.9 mL of $K_2HPO_4 \cdot 3H_2O$ solution (0.273 g of di-potassium hydrogen phosphate-3 hydrate dissolved in 100 mL distilled water).

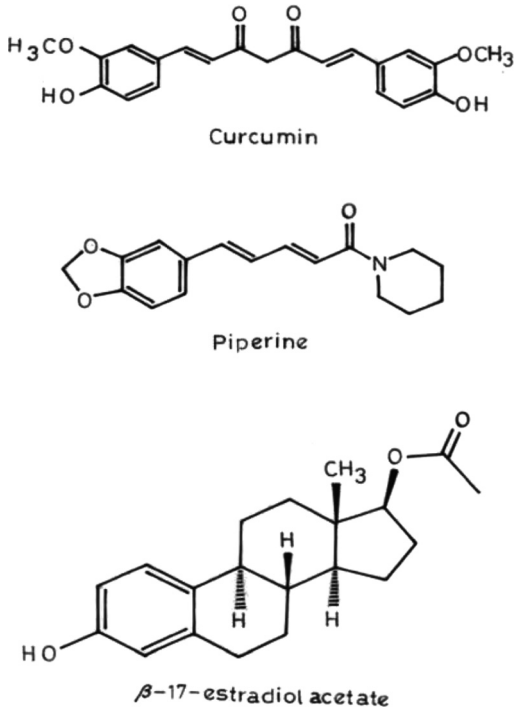

Figure 1. Structures of curcumin, piperine and β-17-estradiol acetate (internal standard).

Apparatus and HPLC Conditions

A Hitachi's HPLC system included a Rheodyne^{TM} injector (Model 7125, Cotati, California, USA) equipped with programmable variable wavelength UV-VIS detector (Model L-2420), pump (Model L-2130), and software D2000 Elite HPLC System Manager. The mobile phase consisting of acetonitrile–methanol-trifluoroacetic acid-water (17.6:35.3: 0.1:47.0, v/v/v/v) was delivered in isocratic mode at a flow rate of 2.5 mL min^{-1} on a Chromolith® SpeedROD RP-18 (50 × 4.6 mm) supplied by Merck, Germany at an ambient temperature. To obtain maximal sensitivity, a programmable variable wavelength UV-VIS detector was operated at λ_{max} for curcumin (415 nm), piperine (335 nm), and β-17-estradiol acetate (280 nm) at their respective retention times for data acquisition.

Stock Solutions

Stock solutions of curcumin, piperine, and β-17-estradiol acetate (internal standard) were prepared by dissolving 10 mg of each in 10 mL of methanol to give 1 mg mL^{-1}. All solutions were prepared in amber glassware as curcumin and piperine have a poor light stability.[16,18]

Calibration Standards, Quality Control Samples, and Internal Standard Working Solution Preparation

Different working solutions containing curcumin, piperine, and β-17-estradiol acetate were obtained by dilution of stock solutions with methanol. Calibration standards were prepared daily by fortification of blank plasma (0.5 mL) with 100 µL of the appropriate working solution, giving concentrations of 10, 50, 100, 250, and 500 ng mL^{-1} of curcumin[7] and piperine[16] of plasma. β-17-estradiol acetate (internal standard) concentration was 1500 ng mL^{-1}. Quality control samples of drug free plasma spiked with 10 (lower limit of quantification), 50 (low), 100, 250 (medium), and 500 ng mL^{-1} (high) of curcumin and piperine and 1500 ng mL^{-1} internal standard were prepared and stored at −20°C. These samples were analyzed during the study period.

Extraction

For the extraction of the drug from plasma, 100 µL of β-17-estradiol acetate (IS) (1500 ng mL^{-1}), 0.5 mL of distilled water, 100 µL of phosphate buffer

(pH 3.40), and 6 mL of extraction reagent comprised of ethyl acetate and propanol (9:1,v/v) were added to 0.5 mL aliquot of the sample (standard or analyte). The amber coloured test tube was vortexed (Scientific Industries Inc., Bohemia, USA) for 30 sec and then shaken well in a rotating mixer for about 15 min. The mixture was then centrifuged (IEC Centra-7-International Equipment Company, Needham Heights, USA) at 1,000 g for 15 min to separate the phases. The upper organic phase was transferred to a clean amber coloured glass tube and evaporated to dryness at 40°C in a vortex evaporator (Hake Bucher, NJ, USA). The residue was reconstituted with 100 µL of methanol and 40 µL of solution was injected on to the HPLC for analysis. During all operations, samples were protected from light to minimize decomposition of curcumin and piperine.[16,18]

Method Validation

Calibration and Linearity

Calibration levels were selected on the basis of expected plasma concentration.[7,16] β-17-estradiol acetate (internal standard) concentration was 1500 ng mL^{-1}. Unweighted linear least squares regression was used to analyze the calibration curves and determine the correlation coefficient. Peak height ratio (drug: internal standard) was the dependent variable. Software D 2000 Elite HPLC System Manager was used to generate calibration curves. The calibration equation relating y (drug/IS; peak height ratio) to x (concentration ng mL^{-1}) was fitted and correlation coefficient (r^2) and % error were calculated.

Lower Limit of Detection (LLD) and Quantification (LLQ)

The lower limit of detection for each compound was stated to be the smallest concentration detectable by UV-VIS at their λ max with signal to noise ratio of 5:1. The limit of quantification was defined as the lowest concentration on the calibration curve, which could be measured with an intra-assay precision and accuracy $<20\%$.[19]

Recovery

Extraction recovery of curcumin and piperine was determined by comparison of five preparations at each of the spiked concentrations of both the drugs in plasma to the response of reference standards added to blank plasma extract. The reference standards were prepared by extracting plasma and reconstituting the evaporated extracts with working solutions of curcumin, piperine, and internal standard. The recovery for curcumin

and piperine was performed at five concentrations (10, 50, 100, 250, and 500 ng mL^{-1}) in human plasma. Recovery of β-17-estradiol acetate (internal standard) was determined at 1500 ng mL^{-1}.

Precision and Accuracy

In order to assess the intra- and inter-day precision and accuracy of the assay, quality control (QC) samples were prepared as described above. The intra-assay data were obtained by replicate analysis of QC plasma samples (n = 5). The inter-assay data were obtained by analyzing the same QC samples over a period of 10 days. Intra-assay and inter-assay precision for β-17-estradiol acetate was assessed at 1500 ng mL^{-1}. Precision was evaluated by determining the relative standard deviations (RSD's) of the estimated concentrations, while accuracy was determined as the percentage difference between the expected and measured drug concentrations.

Stability

The short and long term stability of curcumin and piperine was assessed from three quality control samples, low (10 ng mL^{-1}), medium (100 ng mL^{-1}), and high (500 ng mL^{-1}), along with β-17-estradiol acetate (1500 ng mL^{-1}), stored under different conditions: at room temperature for 8 h and at -20°C for 45 days. Stock solution stability was determined at 4°C and at room temperature for 8 h. Freeze-thaw stability was assessed over three cycles. QC samples at three different concentrations were thawed at room temperature and refrozen at -20°C over three cycles and assayed. The compounds were considered stable if assay variation was <10%. During all the operations, samples were protected from light due to the susceptibility of curcumin and piperine to decompose.[16,18]

Re-Injection Reproducibility

Re-injection reproducibility of the method was assessed by injecting 50 µL of β-17-estradiol acetate (internal standard; 1500 ng mL^{-1}). Thirty plasma extracted samples were injected to compare the β-17-estradiol acetate peak height of each injection with the mean value. Re-injection reproducibility of 5 samples each of curcumin (250 ng mL^{-1}) and piperine (250 ng mL^{-1}) was also evaluated.

Method Performance during a Clinical Study

The developed method was used to analyze the concentrations of curcumin and piperine in plasma samples of 5 healthy volunteers administered

with an oral dose of 1.5 gm of curcumin powder (3 capsules of 500 mg each) combined with 500 mg of piperine powder. Intravenous blood (2.0 mL) was drawn from each healthy volunteer at 0, 30, 45, and 60 min post drug administration into a sterilized glass tube and centrifuged at 1000 × g for 15 min to separate plasma and blood cells. Heparin was used as an anticoagulant. Plasma was stored at −20°C. The study was approved by the Ethical Committee of the ISPAT General Hospital, Rourkela, Orissa and informed consent was obtained from all subjects.

RESULTS

Various proportions of acetonitrile–methanol–trifluoroacetic acid and water as mobile phase were used to achieve the separation of curcumin, piperine, and internal standard. It was found that separation was best achieved using acetonitrile–methanol–trifluoroacetic acid-water (17.6:35.3:0.1:47.0, v/v/v/v) as mobile phase, delivered in isocratic mode at a flow rate of 2.5 mL min^{-1} at an ambient temperature. The chromatographic behavior of curcumin, piperine, and internal standard with the change in the mobile phase composition of acetonitrile, methanol, trifluoroacetic acid and water indicated that an increase in the proportion of acetonitrile and methanol decreases retention, while an increase in the proportion of trifluoroacetic acid and water increases the retention of all the compounds following reverse phase mechanism. Curcumin, piperine, and β-17-estradiol acetate (internal standard) were detected initially at 262 nm[14] but the sensitivity was low. Therefore, HPLC separation of curcumin, piperine, and β-17-estradiol acetate (internal standard) in plasma was accomplished using a variable wavelength UV-VIS detector in programmed mode, where curcumin, piperine, and β-17-estradiol acetate were detected at their respective retention times with their λ max of 415, 335, and 280 nm, respectively. Such a modification enhanced the sensitivity of the method between 3 to 4.4 fold as well as helping in reducing the interference from endogenous plasma components. Figure 2a represents the chromatographic separation of curcumin, piperine, and β-17-estradiol acetate extracted from spiked plasma and detected at 262 nm. Separation of curcumin, piperine, and β-17-estradiol acetate detected at their λ max of 415, 335, and 280 nm, respectively, in programme mode is shown in Figure 2b. The chromatographic behavior of a blank plasma extract from a healthy volunteer before administration of the drug is given in Figure 3a. Six plasma samples from drug free volunteers were extracted and analyzed. Interference due to endogenous plasma peaks was not detected. Figure 3b represents the HPLC chromatogram of a plasma extract obtained from a healthy volunteer treated with 1500 mg of curcumin and 500 mg of piperine at 30 min.

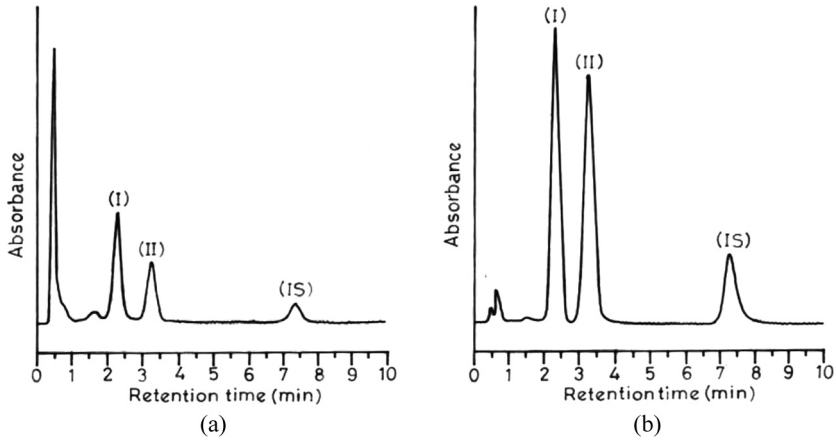

Figure 2. (a) Chromatographic behaviour of piperine, curcumin and β-17-estradiol acetate (internal standard) extracted in plasma at 262 nm. Peak I: Piperine, Peak II:Curcumin, IS: β-17-estradiol acetate (internal standard). (b) The chromatographic behaviour of piperine, curcumin and β-17-estradiol acetate (internal standard) extracted in plasma at their respective λ max. Peak I: Piperine, Peak II: Curcumin, IS: β-17-estradiol acetate (internal standard).

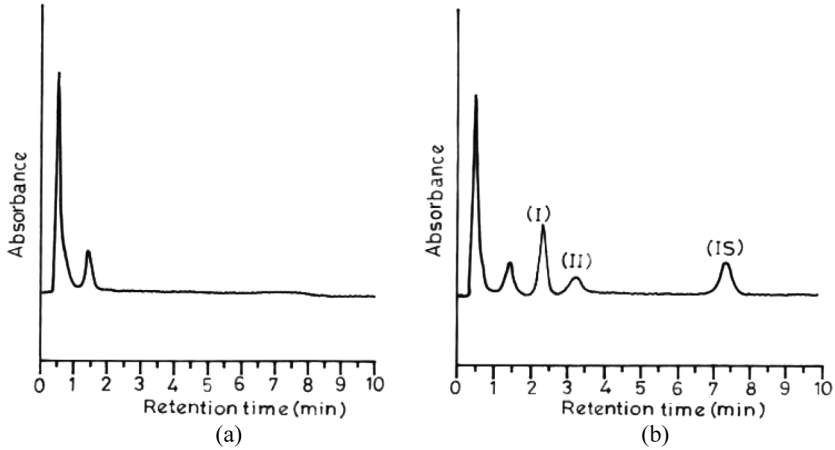

Figure 3. (a) HPLC profile of an extracted blank plasma sample. (b) HPLC chromatogram of an extract of plasma sample obtained from blood taken at 30 min from a healthy volunteer treated with 1500 mg of curcumin and 500 mg of piperine. Peak I: Piperine(242.5 ng ml^{-1}), Peak II: Curcumin(62.6 ng ml^{-1}), IS: β-17-estradiol acetate (internal standard).

The average calibration equation relating y (drug/IS; peak height ratio) to x (concentration ng mL^{-1}) calculated from ten analytical runs at five concentrations cited above were as follows: (i) curcumin: $y = 0.0093 \times -0.0121$; (ii) piperine: $y = 0.0087 \times -0.0095$. The calibration curve was linear over the range of 10–500 ng mL^{-1} of curcumin and piperine in human plasma with the mean correlation coefficients $r^2 = 0.9998$ and 0.9997, respectively. The lower limit of detection for curcumin and piperine was 1 ng mL^{-1} and the limit of quantification was 10 ng mL^{-1} with a mean intra-assay precision (RSD) of 8.7% and 9.0%, with an accuracy of 92.0% and 105.0% for curcumin and piperine, respectively.

Intra-day mean relative standard deviations (RSD's) for curcumin and piperine were 5.7 and 5.4%, respectively, while inter-day mean RSD's for curcumin and piperine were 5.9 and 6.1%, respectively (Table1). Intra-day assay RSD's for curcumin were $\leq$8.7% and for piperine $\leq$9.0%, while inter-day assay RSD's were $\leq$9.3% for curcumin and $\leq$9.8% for piperine. Mean accuracy for curcumin and piperine was

Table 1. Accuracy and precision of the HPLC method for the determination of curcumin and piperine in spiked plasma samples (n = 5)

	Curcumin			Piperine		
Concentration added (ng mL^{-1})	Measured (ng mL^{-1})	Accuracy (%)	Precision R.S.D. (%)	Measured (ng mL^{-1})	Accuracy (%)	Precision R.S.D. (%)
Intra-day						
10 (LLQ)	9.2	92.0	8.7	10.5	105.0	9.0
50	46.6	93.2	6.5	47.0	94.0	6.9
100	94.5	94.5	5.9	96.1	96.1	5.1
250	240.1	96.0	4.1	241.6	96.6	3.7
500	448.4	96.9	3.5	491.4	98.3	2.2
Mean ± S.D.		94.5 ± 2.0	5.7 ± 2.1		98.0 ± 4.8	5.4 ± 2.7
Internal standard 1500 (ng mL^{-1})	1428	95.2	2.3			
Inter-day						
10 (LLQ)	9.1	91.0	9.3	10.6	106.0	9.8
50	46.1	92.2	7.2	46.6	93.2	7.9
100	94.1	94.1	6.6	94.2	94.2	6.4
250	238.3	95.3	4.1	239.5	95.8	4.1
500	487.1	97.4	2.3	484.6	96.9	2.3
Mean ± S.D.		94.0 ± 2.5	5.9 ± 2.7		97.2 ± 5.1	6.1 ± 3.0
Internal standard 1500 (ng mL^{-1})	1439	95.9	1.6			

Table 2. Percentage recovery of the HPLC method for curcumin and piperine in human plasma with different extraction reagents

Extraction reagent	Percentage Recovery	
	100 ng mL^{-1} curcumin	100 ng mL^{-1} piperine
Methanol[7]*	79.0	67.0
Ethyl Acetate[8,9,16]	81.4	77.0
Ethyl Acetate:Methanol (10:1)[14]	87.6	88.2
Ethyl Acetate:Propanol (9:1)[1]	92.8	92.9

*Numbers in the [ ] represents reference.

$\geq$91% (Table 1). Intra-day assay RSD for β-17-estradiol acetate (IS) was 2.3%, while inter-assay RSD was 1.6%. Intra and inter-assay accuracy for internal standard was 95.2% and 95.9%, respectively.

Different extracting reagents, as reported earlier,[1,7–9,14,16] were tried to get maximum extraction recovery of curcumin and piperine from human plasma to achieve the lowest possible quantifiable concentration with consistent reproducibility and the results are given in Table 2. The recoveries for curcumin and piperine from 10 to 500 ng mL^{-1} were highest with ethyl acetate:propanol (9:1, v/v). The average extraction recovery of curcumin, piperine, and β-17-estradiol acetate in human plasma was 91.3, 91.4, and 92.9%, respectively (Table 3).

The short and long term stability of curcumin, piperine, and β-17-estradiol acetate (IS) stored in plasma and methanol was tested. There was no significant reduction in the concentrations of curcumin, piperine, or internal standard. Plasma samples were stable over three freeze-thaw

Table 3. Extraction recovery of the HPLC method for curcumin and piperine in human plasma with ethyl acetate: propanol (9:1)

Concentration (ng mL^{-1})	Recovery(%) mean $\pm$ SD (n = 5)	
	Curcumin	Piperine
10 (LLQ)	90.0 $\pm$ 4.2	86.7 $\pm$ 5.1
50	87.5 $\pm$ 2.2	90.7 $\pm$ 4.2
100	93.3 $\pm$ 2.5	92.2 $\pm$ 4.1
250	91.7 $\pm$ 3.3	92.3 $\pm$ 3.8
500	93.8 $\pm$ 1.8	95.3 $\pm$ 3.2
Mean $\pm$ SD	91.3 $\pm$ 2.6	91.4 $\pm$ 3.1

Table 4. Freeze-thaw stability of curcumin and piperine in human plasma*

Freeze-thaw cycle number	Concentration ($ng\,mL^{-1}$)											
	Curcumin						Piperine					
	10		100		500		10		100		500	
	Obs.	Dev.	Obs.	Dev.	Obs.	Dev.	Obs.	Dev.	Obs.	Dev.	Obs.	Dev.
0	8.7	13	88.9	11.1	469.7	6.1	8.8	12	89.7	10.3	470.8	5.8
1	9.6	4	94.8	5.2	476.2	4.8	9.1	9	90.2	9.8	468.5	6.3
2	9.1	9	90.7	9.3	485.1	2.9	8.9	11	95.4	4.6	481.2	3.8
3	9.2	8	95.9	4.1	478.4	4.3	9.8	2	93.8	6.2	476.3	4.7

*All QC samples were analysed in triplicate. Mean values are reported.
Obs. = observed; Dev. = deviation.

cycles and the variation was within acceptable limits (Table 4). Liu and coworkers[9] reported no significant loss of curcumin after storage in plasma at room temperature for 4 h, while Heath and colleagues[14] found that the curcumin samples in plasma after three freeze-thaw cycles were within 91% of their baseline values. No appreciable change in the peak height of β-17-estradiol acetate (internal standard; $1500\,ng\,mL^{-1}$) was observed after 30 plasma extracted sample re-injections and the values were within $\pm 8.1\%$ of the mean value. Five samples each of plasma extracted samples of curcumin ($250\,ng\,mL^{-1}$) and piperine ($250\,ng\,mL^{-1}$) were also evaluated and the values were within $\pm 4.0\%$ of the mean value.

The method was applied to determine the concentrations of curcumin and piperine in plasma obtained from 5 healthy human volunteers at 0, 30, 45, and 60 min after the oral dose of 1.5 gm of curcumin powder (3 capsules of 500 mg each) combined with 500 mg of piperine powder. Mean concentrations of curcumin at 30, 45, and 60 min were 54.9, 114.3, and $30.9\,ng\,mL^{-1}$, respectively, whereas mean concentrations of piperine were 226.7, 184.9, $157.5\,ng\,mL^{-1}$, respectively (Table 5).

Table 5. Curcumin and piperine concentration in plasma of healthy human volunteers

Time (min)	Mean Concentration ± S.D. (n = 5)	
	Curcumin ($ng\,mL^{-1}$)	Piperine ($ng\,mL^{-1}$)
0	0.0	0.0
30	54.9 ± 4.9	226.7 ± 12.7
45	114.3 ± 10.6	184.9 ± 9.2
60	30.9 ± 6.6	157.5 ± 7.5

DISCUSSION

A precise and rapid reversed phase HPLC method has been developed for simultaneous determination of curcumin and piperine in human plasma for the first time due to numerous therapeutic activities of curcumin[2] and the potential role of piperine as a bioavailability enhancer of several drugs.[12]

The method has been validated according to FDA guidelines.[20] The method is robust, specific, and sensitive for the determination of curcumin and piperine in short run time (less than 8 min). Sensitivity was increased up to 3–4 times by detecting all compounds at their respective λ max. β-17-estradiol acetate as an internal standard was used in extraction to compensate variations in extraction recovery and personal errors since it is stable, non-reactive and commercially available in high purity.[8,14] This compound, β-17-estradiol acetate, is not structurally similar to curcumin and piperine but its behavioral characteristics and properties conform to the chemical requirement for internal standards in HPLC.[21] Ireson et al.[1] reported a HPLC method in gradient mode with a limit of detection between 1.84 and 3.68 ng mL^{-1} and limit of quantification 7.37 ng mL^{-1} but the run time is more than 30 min. The only chromatographic method reported for simultaneous determination of curcumin and piperine is by HPTLC developed by Verma and Joshi,[17] and applied for the estimation of curcumin and piperine in ayurvedic formulation. However, HPTLC has limited application in therapeutic monitoring due to low sensitivity as compared to the present HPLC method. The mean concentrations of curcumin in healthy volunteers at different time interval were in accordance with the values reported earlier.[7] The concentration of piperine in rat plasma has been determined:[16] however, piperine concentration in human plasma is not reported so far. The concentration of piperine was determined in human plasma at various time intervals in this communication for the first time. The developed method should find application in monitoring of curcumin and piperine concentrations in clinical studies due to their enormous potential for a variety of diseases.

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