Istanbul J Pharm 53 (1): 22-29
DOI: 10.26650/IstanbulJPharm.2023.1142916
Original Article

Development of quantification technique for multiconstituent phytoformulation with recap of effects of combination therapy

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Cite this article as: Raut, R., Shaji, J. (2023). Development of quantification technique for multiconstituent phytoformulation with recap of effects of combination therapy. *Istanbul Journal of Pharmacy, 53(1), 22-29*. DOI: 10.26650/IstanbulJPharm.2023.1142916

ABSTRACT

Background and Aims: The objective of this study was to extensively review therapeutic effects of co-administration of flavonoid curcuminoids and alkaloid piperine. Furthermore, the aim of this study was to develop a simple isocratic reversed-phase HPLC (RP-HPLC) method to quantify curcuminoids and piperine in the combined dosage form developed in-house.
Methods: The RP-HPLC quantification was performed on Inersil ODS-3V, 150 mm × 4.6 mm, 5 µm column using Acetonitrile: Buffer (35: 65 % v/v) as a mobile phase, at a flow rate of 1.5 mL/min. The curcuminoids and piperine were detected at wavelengths of 420 nm and 342nm, respectively. The method was validated according to the International Council for Harmonization (ICH) guideline Q2(R1).
Results: The individual curcuminoids and piperine peaks had theoretical plate number (N) > 4000 and a tailing factor (T) < 1.5 confirming well separation of the compounds. The calibration curve was linear from 0.6-18 µg/mL and 0.2-6 µg/mL for curcuminoids and piperine, respectively, with the correlation coefficient of >0.9990. The recovery and precision study values were in close agreement. The method was robust with relative standard deviation (RSD) less than 2%.
Conclusion: The literature survey indicated that the co-administration of piperine had influenced pharmacodynamic and pharmacokinetic activities of curcuminoids. The analytical method developed was found to be specific, sensitive, precise, and accurate for the estimation of curcuminoids and piperine in a single run.

Keywords: Co-administration, curcuminoids, piperine, RP-HPLC, validation

INTRODUCTION

For thousands of years, traditional medicines have been used in all parts of the world. Even today, they are used as a sole, complementary or alternative medicine. Most of them are polyherbal formulations containing number of herbs where herbs act as therapeutic agents and /or excipients. Variation in phytochemical composition of herbs and medicines is observed due to variation in genetic, seasonal, and geographical as well as herb processing methods. This sometimes results in poor quality of medicines with inferior or no therapeutic effect. Due to the complexity of chemical constituents, standardisation of herbal medicine is difficult. Thus, modern tendency is to isolate the therapeutic constituents and incorporate them in a dosage form. Isolation of morphine from opium is considered as the beginning of the new era. The number of isolated phytoconstituents is increasing day by day. (Krishnamurti, & Rao, 2016) Here, the preference is for the herbs which are used as a food which gives indication of safety. Also, these herbs are commercially cultivated, which ensures continuous availability of them. Turmeric is one such herb that is used as a spice in food and grown in many parts of the world with established medicinal values. It is often combined with black pepper in traditional medicines.

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Submitted: 10.07.2022
Revision Requested: 25.01.2023
Last Revision Received: 11.02.2023
Accepted: 28.03.2023
Published Online: 25.04.2023

Curcuma longa L. (turmeric) is a perennial plant of Zingiberaceae family. It grows around 1 meter. The rhizomes are cylindrical, yellow to orange, and highly branched. Rhizomes are washed, boiled, dried, and stored. As a spice, it is frequently ground to fine powder, and then used. As a home remedy, it is used for cuts, bruises, burns, swollen joints, skin diseases etc. It is taken internally as well as applied externally. The medicinal properties of the turmeric are attributed to the group of three yellow compounds (Figure 1) viz. curcumin, demethoxycurcumin and bisdemethoxycurcumin, collectively termed curcuminoids (Li et al.,2011). As most of the researchers find it difficult to isolate curcumin from the rest, three compounds are used as a mixture. Among curcuminoids, curcumin is in the highest quantity in the mixture, and in general, the mixture is simply referred as curcumin. Black pepper is the berry of trailing vine *Piper nigrum* L., family Piperaceae. Almost- ripened peppercorns are left to dry until they turn black. These are then ready to be consumed. Pungency and heat of the pepper is attributed to the piperine (Figure 1) found in it. People take black pepper orally for upset stomach, bronchitis, colic etc., and apply it on skin for discoloured skin (vitiligo) and nerve pain (Tasleem, Azhar, Ali, Perveen, & Mahmood, 2014).

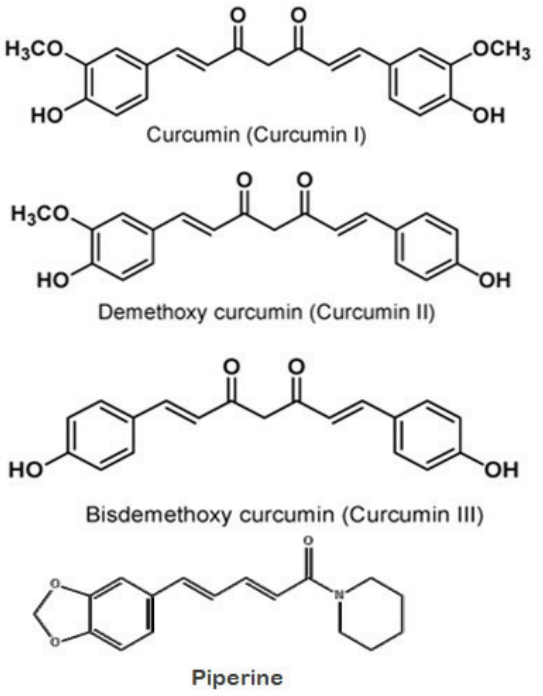

Figure 1. Chemical structures of curcuminoids and piperine.

Effects of co-administration of curcuminoids and piperine

Solubility and permeability of curcuminoids

Curcumin is a symmetric molecule. It has an o-methoxy phenolic group attached to an aromatic ring. Two such aromatic rings are joined by a chain of seven carbon atoms carrying α, β-unsaturated β-diketone moiety. With such structure, hydrogen bonding happens between the two curcumin molecules. The process repeats, and curcumin molecules get stacked. Probability of hydrogen bonding between curcumin and water reduces significantly. Hence, curcumin is a highly hydrophobic and poorly soluble compound. Piperine gets incorporated in stacked layers of curcumin as it has competing hydrogen bonding forces. This reduces hydrophobicity of curcumin and increases quantity of curcumin in solution. This solubilized curcumin become available for absorption (Patil, Das, & Balasubramanian, 2016).

Piperine was found to increase skin permeability to curcumin in proportion to its concentration. A transdermal double layered patch of curcumin and piperine was developed. Piperine containing layer was kept in contact with skin. Thus, skin received pretreatment with piperine before receiving curcumin. An increase in permeation of 1.89 times was observed with piperine at a concentration of 7.41% of composite membrane (Jantarat, Sirathanarun, Boonmee, Meechoosin & Wangpittaya, 2018).

Pharmacokinetics of curcuminoids

Following its oral administration, curcumin is poorly absorbed through GI tract. The oral bioavailability in rats was about 1%. A fiftyfold higher oral dose of curcumin failed to simulate serum concentrations as that of intravenous administration (Yang, Lin, Tseng, Wang, & Tsai, 2007). Curcumin metabolism was studied by using isolated hepatocytes. Curcumin, after metabolism in the liver, is mainly excreted through bile. Curcumin shows two phases in metabolism. Curcuminoids undergoes several reactions of reduction by a reductase in phase I of metabolism. In phase II of metabolism, both curcuminoids and its phase I metabolites become water soluble by conjugation with glucuronic acid and sulphate (Pan , Huang, & Lin ,1999; Holder, Plummer, & Ryan 1978) These conjugates are excreted through urine. Thus, only a small fraction of oral curcuminoids become available for pharmacological actions, which making curcuminoids poor therapeutic agents.

Piperine acts as a bioenhancer *via* multiple pathways. It enhances bile acid secretion. Bile acid forms micelles of lipid and lipid soluble compounds that promote its absorption. Upon oral administration, it increases intestinal brush border membrane fluidity and microvilli length, which results in increased intestinal permeability of compounds (Khajuria, Thusu, & Zutshi, 2002). It influences P-glycoprotein (p-gp) efflux pump (Han, Tan, & Lim, 2008). In most of the situations, P-gp efflux pump of intestinal epithelium decreases intracellular concentration of compounds by transporting it back to the lumen of the intestine. Inhibition of P-gp efflux pump by piperine helps to retain absorbed curcumin. Oral administration of piperine in rats strongly inhibits the metabolic enzymes such as hepatic arylhydrocarbon hydroxylase and UDP-glucuronyltransferase. This in turn maintains concentration of original unmetabolised drugs in the body (Atal, Dubey, & Singh J, 1985). In rats, oral dose of curcumin and piperine at 2 g/kg and 20 mg/kg, respectively increased bioavailability of curcumin by 154%-fold compared to curcumin alone. In humans, co-administration of 2g of curcumin and 20 mg of piperine enhanced curcumin bioavailability by 2000% compared to curcumin alone (Shoba et al., 1998).

Pharmacodynamics of curcuminoids

Curcumin is cardioprotective in nature. In an experimental set up, cardiotoxicity was induced in rats by administration of cyclophosphamide. Curcumin and piperine at 50 mg/kg and 20 mg/kg respectively, showed extremely significant ($P < 0.001$) to moderately significant ($P < 0.01$) influence on parameters of cardiac health compared to curcumin (200 mg/kg) alone-treated group. The studied parameters included lipid profile, antioxidant levels, electrocardiogram, and histopathological score (Chakraborty, Bhattacharjee & Kamath, 2017). The hypocholesterolemic effects of curcumin were potentiated by co- administration of piperine. With the help of a high-fat diet, cholesterol levels were increased in Sprague-Dawley rats. The study indicated the enhanced activity and gene expression of apolipoprotein AI, lecithin cholesterol acyltransferase, cholesterol 7 alpha-hydroxylase, and low-density lipoprotein receptor (Tu et al., 2014). The radioprotective effects of curcumin and piperine alone and in combination were assessed on ionizing radiation exposed human normal lymphocytes. An additive radioprotective effect was observed in combination treatment at low concentration, though at higher concentration observations were not the same (Ghelishli, Ghasemi, & Hosseinimehr, 2019). Pretreatment with curcumin (100 mg/kg) and piperine (20 mg/kg) separately as well as in combination were given to male Swiss albino mice, which received benzo(a) pyrene, a known genotoxic. Combination treatment exerted potent antigenotoxic effect compared to curcumin alone (Sehgal, Kumar, Jain, & Dhawan, 2011). The combined treatment of curcumin (100 mg/kg; p.o.) with piperine (20 mg/kg; p.o.) showed better suppression of diethylnitrosamine induced hepatocellular carcinoma in comparison to curcumin alone. The findings were based on the morphological, histopathological, biochemical observations of the liver, and serum of rats (Patial et al.,2015). The combination chemotherapy of curcumin and piperine for colorectal carcinoma in HCT116 cells was studied. 1.5 times enhancement in apoptosis was observed when HCT116 cells were treated with a combination of CurcuEmulsome and PiperineEulsomes compared to CurcuEmulsome alone (Bolat et al.,2020). Glioblastoma multiforme is aggressive brain cancer with low life expectancy in patients. In cellular studies, IC_{50} of only curcumin and only piperine loaded gold nanogel were recorded at 30 µM and 35 µM, whereas gold nanogel containing the combination of both agents showed IC_{50} at 21 µM (Javed, Zhao, Cui, Curtin, & Tian, 2021).). Prophylactic antimalarial study was carried out in *Plasmodium berghei* ANKA-infected mice, and curcumin at dose of 300 mg/kg in combination with piperine 20 mg/kg showed delayed onset of clinical symptoms, and survival rate was prolonged compared to the treatment of individual drugs (Khairani et al., 2022).

In a clinical trial carried out on 44 osteoarthritic patients in Mumbai, a combination of curcumin 500 mg and piperine 5 mg was administered to the patients orally twice daily for 12 weeks. At the end of the study, statistically significant reduction in pain and stiffness in patients who received a combination of curcumin and piperine was observed. The need for additional NSAIDs for pain management also reduced (Reddy, & Faruqui 2016).

Based on these literature inputs, we aimed for the development of a gel formulation containing curcuminoids and piperine in the laboratory. The gel formulation contained excipients like polysorbate 80, Kolliphor RH 40, Kollisolv PEG 400, isopropyl alcohol, Carbopol 974P, sesamol, eucalyptus oil, triethanolamine, and purified water. For any pharmaceutical product development, assays form the integral part of the process. An analytical method was developed to estimate concentration of curcuminoids and piperine by using HPLC. HPLC is the most popular technique in pharmaceutical industry as it offers great flexibility in the procedure. This technique needs to be validated before its routine use. The present article further discusses the validation of the HPLC method.

MATERIALS AND METHODS

The curcumin (total curcuminoids 97%) was purchased from Sigma-Aldrich Chemie GmbH. Further curcuminoids were extracted in– house and were found to contain total curcuminoids 98.30%. This was used in the formulation and analysis. The standard piperine (97.1%) was purchased from Sigma-Aldrich Chemie GmbH. HPLC grade water was purchased from J.K. Labs, Thane, Maharashtra. Analytical grade orthophosphoric acid was obtained from Loba-Chemie, India. Methanol and Acetonitrile of HPLC grade were purchased from E. Merck, India.

Chromatographic system

The HPLC analysis was carried out on Agilent 1260 Infinity II (Agilent Technologies Deutschland GmbH, Wald Bronn Germany) equipped with UV/Vis Detector. The mobile phase was Acetonitrile: Buffer (35: 65 % v/v), where buffer was prepared by adding 0.1 % orthophosphoric acid (OPA) in water. The analysis was performed in isocratic mode with flow rate of 1.5 mL / min. 100 µL test solution was injected in the system. Separation was carried out on Inersil ODS-3V(150 mm X 4.6 mm, 5 µm) column by maintaining column oven temperature 40°C. The detection was carried out using UV-Vis detector at wavelength of 420 nm for curcuminoids and 342 nm for piperine. Total run time was 50 mins. Schematic diagram of the whole procedures is depicted in Figure 2.

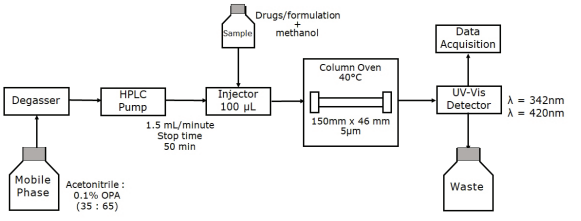

Figure 2. Schematic diagram of the HPLC system with parameters.

Stock and working solutions

Curcuminoids (5 mg) were weighed and transferred to volumetric flask. With sonication, it was completely dissolved in methanol. The volume was made up to get to the stock solution of 500 µg/mL curcuminoids . Similar procedure was followed to get the stock solution of 250 µg/mL piperine . 0.6 mL of curcuminoids stock solution and 0.4 mL of piperine stock solution were

transferred in 25 mL volumetric flask. The volume was made up to the mark with mobile phase to get the solution containing 12 µg/mL of curcuminoids and 4 µg/mL of piperine.

The required quantity of the test sample was weighed in a 50 mL beaker, and about 30-35 mL methanol was added. It was sonicated for 15 minutes with intermittent stirring every 5 minutes. The solution was transferred to a volumetric flask, and the volume was made up with methanol. The solution was filtered through 0.45µ Nylon syringe filter discarding 3-5 mL of sample. 1.6 mL of filtrate was diluted to 10 mL with mobile phase and mixed well to get the solution containing 12 µg/mL of curcuminoids and 4 µg/mL of piperine.

System suitability

Specificity of the analysis was established by injecting the mobile phase as well as the placebo solution that was obtained after giving the same treatment as the product into the HPLC system. Six replicate injections of the sample containing a mixture of curcuminoids and piperine at a concentration of 12 µg/mL and 4 µg/mL were analysed. Retention time (RT), asymmetry, theoretical plates, and resolutions were determined.

Linearity

Solutions of 6 different concentrations were prepared by taking 5% to 150 % of working concentration. Each level injected in triplicate. Linearity graph was plotted as concentration against mean peak area. Intercept, slope, and regression coefficient were calculated. The limit of detection and limit of quantification were calculated using the formula LOD = 3.3 Q/S and LOQ = 10 Q/S where Q is the standard deviation of the intercepts, and S is the slope of the calibration curve.

Accuracy

Accuracy of the proposed method was confirmed by spiking the placebo at 50%, 100%, and 150% concentrations, and then analysing it to determine the amount of the drug recovered.

Precision

Precision study was carried out in terms of repeatability (intraday precision) and inter-day precision. The gel containing curcuminoids and piperine was repeatedly analysed. Intraday precision study was carried out by estimating corresponding responses three times on the same day. For inter-day precision study, analysis was carried out by another analyst on another day in the same laboratory. The precision of proposed method was obtained by calculating the relative standard deviation (RSD) values observed for intra-day and inter-day analysis with acceptance criteria of RSD less than 2%.

Robustness

Robustness indicates the capacity of an analytical method to remain unaffected by minor changes in the method parameters. Effects of variation in experimental conditions like column temperature (± 2°C) and flow rate (± 10%) were evaluated.

RESULTS AND DISCUSSION

In reverse phase HPLC analysis, stationary phase is hydrophobic in nature, and accordingly, it retains hydrophobic molecule for longer time. Ionised species are comparatively hydrophilic in nature and are eluted fast. Elution of compounds is also affected by polarity of mobile phase. An increase in the polarity of the mobile phase results in longer retention times. Thus, careful selection of mobile phase is essential for good separation of compounds. Curcumin, demethoxycurcumin, and bisdemethoxycurcumin differ in the number of methoxy groups. They have molecular weights 368.38, 338.35, and 308.33, respectively. They have 3 labile protons and correspondingly have 3 pKa values. The first pKa is reported to be in the pH range of 7.5 to 8.5 (Priyadarsini, 2014). Piperine has a molecular weight of 285.34, and pKa of 12.22 (Lide, 2007). At a given pH, these compounds ionise to different extents because of the inherent structural difference. We optimised composition of mobile phase so that 3 curcuminoids and piperine were retained on the stationary phase for different time and eluted at different time without overlapping. We fixed it to acetonitrile: 0.1 % OPA in water at the ratio of 35: 65 % v/v.

The developed method was specific with no interference with placebo or mobile phase. The analysis was carried out in isocratic mode at flow rate of 1.5 mL / min, and the method was found to be reproducible. The phytoconstituents were analysed at two different wavelengths 420 nm for curcuminoids and 342 nm for piperine, and the detection specificity enhanced (Figure 3). The peaks of piperine, bisdemethoxycurcumin, demethoxycurcumin, and curcumin had good symmetry and were well-separated (Table 1). The method was found to be linear for curcuminoids and piperine in the range of 0.6 to 18 µg/mL and 0.2 to 6 µg/mL, respectively (Figures 4 and Figure 5). The LOD and LOQ for curcuminoids were 0.03523 and 0.1067 µg/mL, and for piperine 0.01277 and 0.0387 µg/mL, respectively.

Accuracy was studied in terms of recovery by spiking placebo at levels of 50%, 100% and 150% of the phytoconstituents. For recovery studies, % RSD was less than 1 (Tables 2 and Table 3). Similarly, intraday and inter day variation was found to be optimum with % RSD less than 1 (Table 4). The change in flow

Table 1. System suitability.

Name	Retention time	Asymmetry	Theoretical Plates	Resolution
Piperine	23.78 ± 0.01	1.02± 0.006	5746± 16.82	0
Bisdemethoxycurcumin	27.25 ± 0.031	1.29 ± 0.012	5054.67 ± 83.63	2.47± 0.01
Desmethoxycurcumin	31.50 ± 0.006	1.06 ± 0.012	5037.67 ± 42.59	2.58 ± 0.01
Curcumin	36.34 ±0.01	1.04 ± 0.015	4989.33 ± 27.47	2.52 ± 0.006

(Data given in this table is presented as mean ± SD, n=3, SD = Standard Deviation)

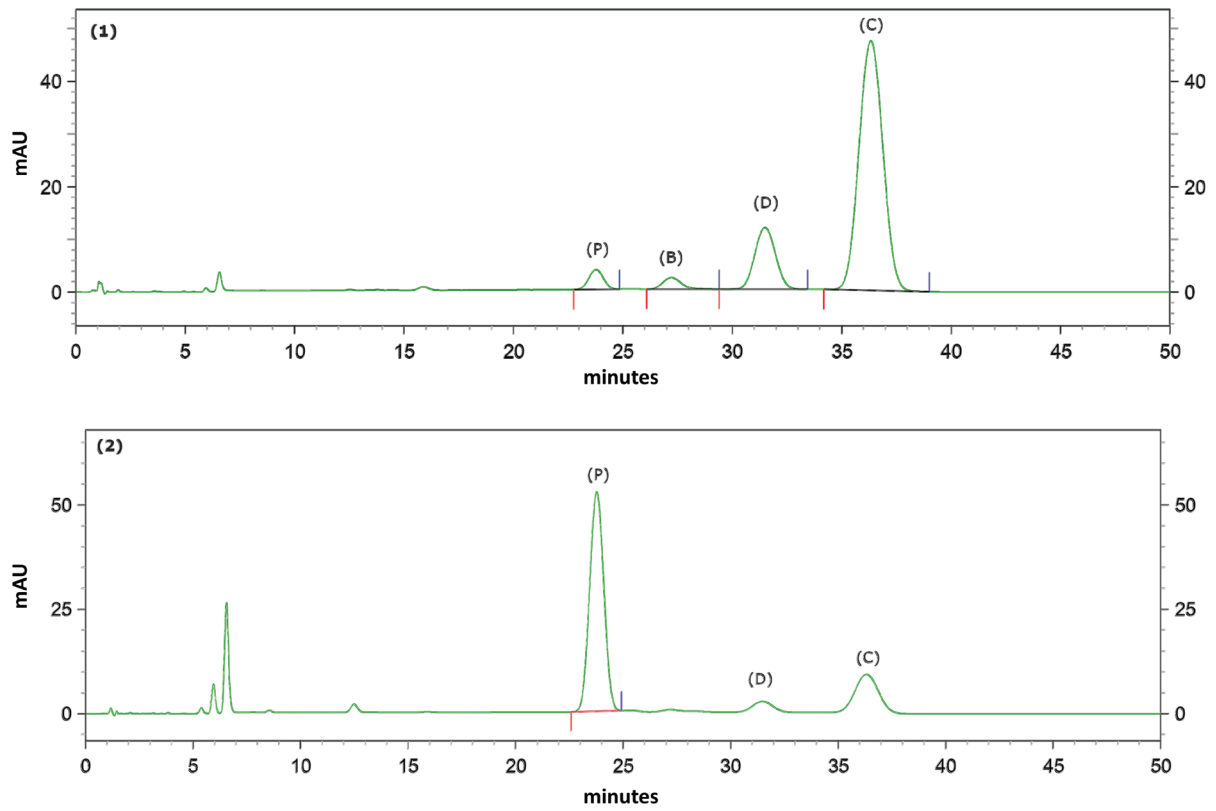

Figure 3. Chromatographic separation of curcuminoids and piperine with an isocratic mobile phase composed of an acetonitrile: buffer (35: 65 % v/v) at flow rate of 1.5 ml/min at (1) 420 nm and (2) 342 nm. Peaks representation (P)=piperine, (B)=bisdemethoxycurcumin, (D)=demethoxycurcumin, (C)= curcumin.

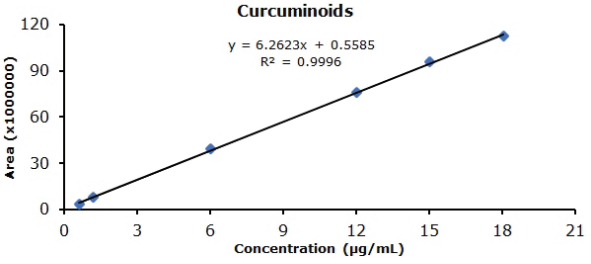

Figure 4. Linearity plot of curcuminoids. (Data given in mean ± SD) (n=3).

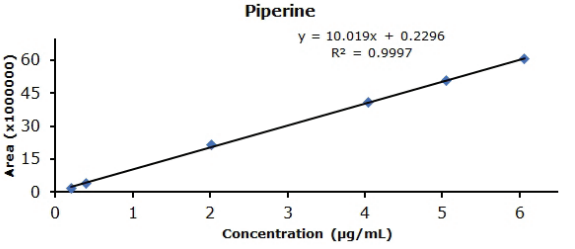

Figure 5. Linearity plot of piperine. (Data given is mean ± SD) (n=3).

Table 2. Recovery results for curcuminoids (acceptance limit recovery %= 98-102%).

Level in (%)	Amount spiked (µg/ml)	Amount recovered (µg/ml)	% Recovery	%RSD
50%	5.90	5.92	100.31	Mean= 100.37 % RSD=0.153
50%	5.90	5.91	100.25	
50%	5.90	5.93	100.54	
100%	11.80	11.81	100.12	Mean= 100.43 % RSD= 0.997
100%	11.80	11.75	99.62	
100%	11.80	11.98	101.55	
150%	17.69	17.70	100.02	Mean= 100.05 % RSD=0.211
150%	17.69	17.67	99.85	
150%	17.69	17.74	100.27	

rate by 10% and change in temperature by 2°C did not affect the analysis (Table 5). Good resolution, peak symmetry, and reproducibility outweighed its long run time.

As per USP 36-NF 31 curcuminoids are defined as "a partially purified natural complex of diarylheptanoid derivatives isolated from Turmeric, *Curcuma longa* L. It contains NLT 95.0% of curcuminoids, calculated on the dried basis, as the sum of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin." Also, as the dosage forms like capsule, USP directs that it should contain NLT 90.0% and NMT 110.0% of the labelled amount of curcuminoids, calculated as the sum of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin. Hence, it is important that an analytical method should detect and quantify individual three curcuminoids.

Scientists have used a mixture of curcuminoids for studies. Nowadays, they work on the effects of curcumin, demethoxycurcumin, and bisdemethoxycurcumin individually (Sato et al., 2014; Naksuriya, van Steenbergen, Torano, Okonogi, & Hennink, 2016; Liu et al 2021; Kumar, Lal, Nemaysh, & Luthra, 2018). There is a scope to use individual curcuminoids in combination with piperine. This underlines also the need to have an analytical method which can detect individual curcuminoids with piperine.

Analytical methods and validations are reported for quantification of curcumin and piperine in plasma. (Sethi et al., 2009; Rodriguez et al., 2021). Content of curcumin & piperine, and rutin, quercetin, curcumin, and piperine was estimated by RP-UFLC (Ramaswamy et al., 2014; Ramaswamy, Gowthamarajan, Dwarampudi, Bhaskaran, & Kadiyala, 2021) and RP- HPLC (Kuber,

Table 3. Recovery results for piperine (acceptance limit recovery%= 98-102%).

Level in (%)	Amount spiked (µg/ml)	Amount recovered (µg/ml)	% Recovery	%RSD
50%	2.01	2.00	99.22	Mean =99.75 %RSD= 0.526
50%	2.01	2.02	100.27	
50%	2.01	2.01	99.75	
100%	4.03	4.04	100.42	Mean =100.35 %RSD= 0.394
100%	4.03	4.02	99.92	
100%	4.03	4.06	100.70	
150%	6.04	6.03	99.83	Mean =99.60 %RSD= 0.296
150%	6.04	6.02	99.71	
150%	6.04	6.00	99.27	

Table 4. Precision studies for the developed method.

	Intra-day		Inter-day	
	% Curcuminoids	% Piperine	% Curcuminoids	% Piperine
Sample 1	99.29	98.23	99.56	100.19
Sample 2	98.87	98.33	99.73	99.92
Sample 3	99.00	99.43	101.04	98.79
Mean	99.06	98.66	100.11	99.64
SD	0.214885	0.668233	0.810486	0.743026
%RSD	0.217	0.677	0.810	0.746

Table 5. Robustness studies for the developed method.

Robustness Parameter	Level	Assay		% RSD	
		% Curcuminoids	% Piperine	Curcuminoids	Piperine
Flow rate	+10%	100.48	98.54	0.31	0.64
	-10%	100.30	99.81	0.30	0.79
Temperature	+2°C	100.07	100.14	0.78	0.70
	-2°C	100.24	99.95	0.04	0.44

(Data given in this table is presented as mean where n=3, RSD = Relative Standard Deviation)

2018). HPLC analytical method was developed for estimation of curcumin and piperine in nanoparticulate dosage form (Khismatrao, Bhairy, & Hirlekar, 2018). Curcumin, piperine, and camphor in an ayurvedic formulation were quantified (Shaikh and Jain 2018). All the analytical method development and validation studies mentioned above were not separated individual curcuminoids. An analytical method was developed for simultaneous quantification of curcumin and piperine in a microparticulate formulation with linearity in the range of 1.25 to 15 µg/mL for piperine and 2.50 to 30 µg /mL for curcumin. Though they could detect three separate peaks for individual curcuminoids, the resolution was less than 2, and only content of curcumin was quantified for validation (Setyaningsih et al., 2021). Reviewer guidance document for validation of chromatographic methods released by Center for Drug Evaluation and Research (CDER) recommends chromatographic resolution to be greater than 2. In this context, the analytical method developed by us has many advantages in terms of specificity and sensitivity.

CONCLUSION

From the literature, it can be concluded that curcuminoids- piperine combination therapy has many advantages. More studies need to be carried out with respect dose and dosage form for various ailments. A validated HPLC method for the simultaneous quantification of curcuminoids (curcumin, demethoxycurcumin, and bisdemethoxycurcumin) and piperine has been established in this study. Accurate, sensitive, and reproducible quantification was possible with the developed method.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- R.R. J.S.; Data Acquisition- R.R.; Data Analysis/Interpretation- R.R. J.S.; Drafting Manuscript- R.R.; Critical Revision of Manuscript- R.R. J.S.; Final Approval and Accountability- R.R. J.S.

Conflict of Interest: The authors have no conflict of interest to declare

Financial Disclosure: Authors declared no financial support.

Acknowledgements: Authors are thankful to Prin. K.M. Kundnani College of Pharmacy, Mumbai, India for their support in this work.

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