Development and validation of simultaneous estimation method for curcumin and piperine by RP-UFLC

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Abstract: Curcumin and piperine are proven for their potent medicinal benefits to treat various diseases and they are most commonly used combination in various Indian systems of medicine such as Ayurveda, Siddha and Unani. The objective of the present work is to develop a simultaneous estimation of curcumin and piperine by reverse phase Ultra-fast liquid chromatographic (RP-UFLC) method. The chromatographic separation was performed on a C8 column (250 x 4.6 mm, 5µ i.d.) stationary phase using a mobile phase of 25mM potassium dihydrogen ortho phosphate buffer (pH 3.5) and acetonitrile (30: 70 v/v) at a flow rate of lml/min at detection wave length of 280nm. The calibration curve was plotted in the concentration range of 0-2200ng/ml and found to be linear for both curcumin (r^2=0.996) and piperine (r^2=0.999). The method was validated for parameters such as accuracy, sensitivity, precision, linearity, specificity, ruggedness and robustness as per ICH guidelines. The developed simple, precise and specific method can be used as a quality control tool for qualitative and quantitative estimation of curcumin and piperine in various food products, herbal medicines and nutraceuticals.

Keywords: Curcumin, piperine, ICH guidelines, RP-UFLC, herbal medicinal products.

INTRODUCTION

Curcuma longa (Zingiberaceae) contains an active principle curcumin, chemically known as 1, 7 bis (4-hydroxy-3-methoxy phenyl)-1,6-heptadiene-3,5 dione (fig. 1) (Ireson *et al.,* 2002). Turmeric is commonly used colouring agent in foods due to presence of curcumin. It has been used for treatment of several ailments since centuries due to its therapeutic benefits on autoimmune, cancer, cardiovascular, neurodegenerative and pulmonary diseases, where inflammation is involved as major mechanistic pathway (Aggarwal and Hari kumar, 2008). Even though curcumin possess a wide range of physiological and pharmacological properties, several studies revealed its limitation of therapeutic applicability in terms of low bioavailability in the intestine (Sharma *et al.,* 2001; Ireson *et al.,* 2002; Garcea *et al.,* 2004). Piperine chemically known as 1-(5-(1, 3-benzodioxol-5-yl)-1-oxo-2, 4-pentadienyl (fig. 1), is a potent alkaloid available from *Piper longum* and *Piper nigrum* (Piperaceae). Piperine has been reported to possess CNS depressant, antipyretic, anti-inflammatory activities. It also improves digestion and appetite, active against cold, cough dyspnoea, colic, dysentery, worms and piles (Lee *et al.,* 1984; Khajuria *et al.,* 1997; Koul and Kapil, 1993). Preclinical and clinical studies reveal that the poor bioavailability of various other drugs has been improved by addition of piperine (Shoba *et al.,* 1998; At al *et al.,* 1985). Several spectroscopic and chromatographic methods were reported for the estimation of curcumin and piperine individually in UV, HPLC, GC, fluorescence spectroscopy, electrophoresis, LC-MS and HPTLC (Chauhan *et al.,* 1998; Singh *et al.,* 2007; Jayaprakash *et al.,* 2005; Koichi *et al.,* 2001; Kailong *et al.,* 2005; Liu *et al.,* 2006). Simultaneous estimation of curcumin and piperine in biological samples, herbal formulations were reported by HPLC (Krishna veni *et al.,* 2009; Niraj *et al.,* 2011; Shanmugam *et al*., 2013) and HPTLC methods (Verma and Joshi, 2006).

In most cases phytoconstituents were estimated by HPTLC technique but still limitations such as saturation of mobile phase, humidity in the working atmosphere, intensity of the application band and detection limit hurdle analysis. These can be overcome by HPLC and currently only few methods are available for simultaneous estimation of both drugs out of which most of the studies lack proper validation. Phytoconstituents are mostly methanol soluble and this solvent creates a back pressure exceeding 4000 psi in a conventional HPLC pump hence UFLC have been selected for the study which can withstand pressure up to 6800 psi (Gannu *et al.,* 2009). Hence a simple, rapid and cost-effective simultaneous RP-UFLC method was developed for the estimation of curcumin and piperine useful as a quality control tool for analyzing food products, herbal medicines and nutraceuticals.

MATERIALS AND METHODS

Materials

Standard drugs curcumin and piperine were purchased from Sigma Aldrich, Mumbai. HPLC grade Acetonitrile (ACN), ortho phosphoric acid, ammonium acetate and

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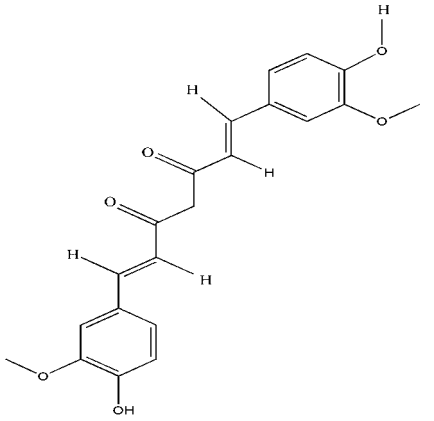
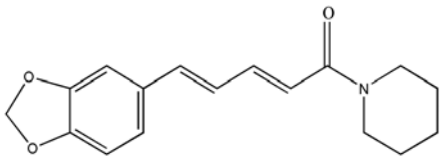
triethylamine were purchased from Merck (Germany). Milli-Q water was obtained from in-house milli Q-unit by employing triple distillation technique.



Curcumin



Piperine

Fig. 1: Chemical structures of curcumin and piperine.

Instrument and Chromatographic conditions

Shimadzu (Kyoto, Japan) prominence Ultra-Fast Liquid Chromatography (UFLC) instrument equipped with LC-20AD solvent delivery gradient pump with an injection loop volume of 20µL, 7725i rheodyne injector attached to a data station of LC Solution software using a Photodiode array detector (PDA) was utilized for method development and validation. Separation was achieved on a Phenomenex C8 column (250 x 4.6 mm, 5µ i.d.) using a mobile phase of 25 mM potassium dihydrogen ortho phosphate buffer (pH 3.5) and acetonitrile (30: 70 v/v) at 1 ml/min flow rate. The drugs were detected at wave length of 280 nm with a sample volume of 20µl. Systronics (Mumbai) pH meter was used for adjusting pH of the mobile phase.

Preparation of standard solutions of curcumin and piperine

Both Curcumin and piperine were accurately weighed about 10mg each and transferred into 10ml volumetric flask. It was solubilized and made up the volume with ACN to prepare a solution of 1mg/ml concentration. The prepared stock solution was stored at -20^{o}C±2^{o}C until analysed.

Detection of wavelength

Curcumin and piperine (each 100µg/ml) solutions were scanned in the UV region (200-400nm) by using PDA detector. Better response was achieved at 280 nm.

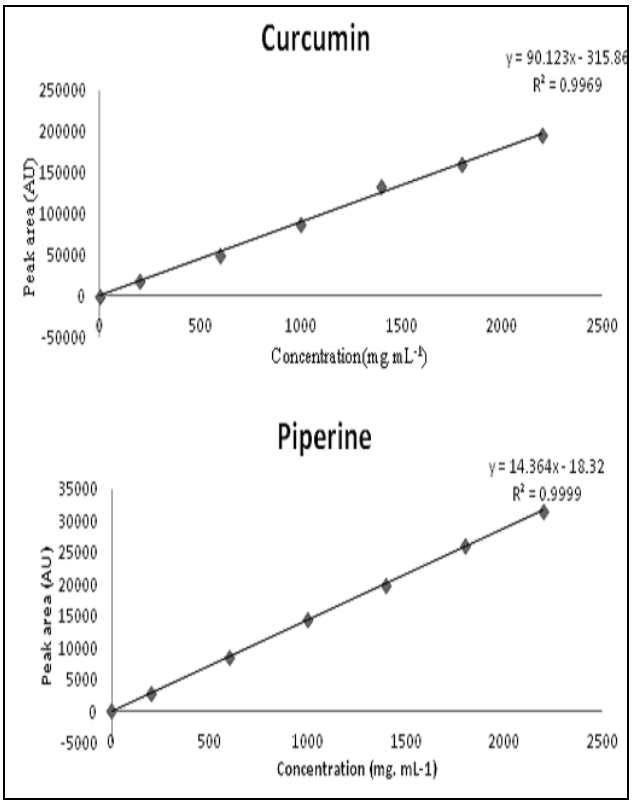


Fig. 2: Calibration curve for standard curcumin and piperine.

Mobile phase preparation

Potassium dihydrogen ortho phosphate of about 1.7011gm was weighed accurately and solubilized in mill Q water (500ml) and pH was adjusted with ortho phosphoric acid to 3.5 (solution A). Mixture of solution A and ACN (30:70 v/v) were mixed, degassed and used as mobile phase.

Development of calibration curve for curcumin and piperine Standards

Working solutions for calibration curve were prepared from the stock solution by an adequate dilution using ACN in the range of 0-2200 ng/ml for both curcumin and piperine respectively.

Method validation

The optimized method was validated as per International Conference on Harmonization (ICH) guidelines (ICH, 1995) for linearity and range, accuracy, selectivity, precision, sensitivity, specificity, ruggedness, robustness and system suitability.

Specificity

Specificity of the method is tested for any changes in the response of the drug in presence of any impurities and degradants. The standard retention time of the drugs is compared with that of the sample response as shown in fig 3.

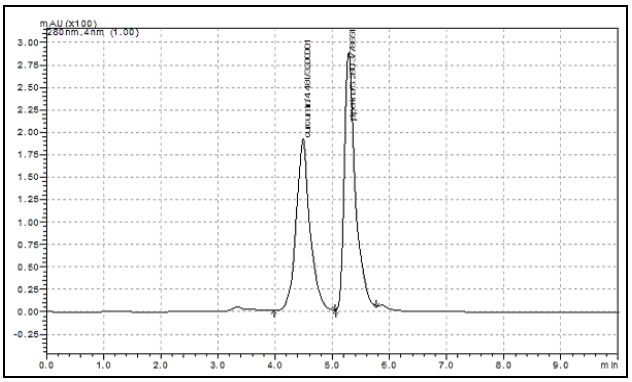


Fig. 3: Typical chromatogram for standard curcumin and piperine.

Precision

Precision of the method is determined to check repeatability. Inter-day and intra-day precision studies were carried out at three different concentrations (200, 1000 and 2200 ng/ml) by injecting each sample six times and coefficient of variation (% CV) was determined as per the formula

%CV = (Standard Deviation /Mean) x 100

Accuracy

The percentage difference between the expected and measured drug concentrations is calculated to determine accuracy of the method. The study was carried out by addition of known concentration of the standard solution to the sample solution and analyzed to determine the recovery and coefficient of variation (% CV).

Sensitivity

The sensitivity is measured in terms of limits of reliable quantitation in which the minimum level of the analyte can be detected. Lower level of quantification of the curcumin and piperine were evaluated based on signal-to-noise ratio.

Linearity

The calibration curve was plotted in the concentration range of 0-2200 ng/ml for both curcumin and piperine. The calibration curve was plotted for corresponding peak areas against each standard concentration where the slope value and correlation coefficient (r^2) values were calculated separately for each drug.

Robustness and ruggedness

These parameters were studied by carrying out experiments on diverse instrument models, operators and slight changes in the chromatographic conditions such as pH, flow rate and column temperature.

Detection Limit (LOD) and Quantitation Limit (LOQ)

Limit of detection (LOD) is the smallest concentration of analyte that gives response accurately but can be quantified necessarily. Limit of quantification (LOQ) is the least concentration of the analyte that gives accurate response. LOD and LOQ were measured based on the signal-to-noise ratio.

RESULTS

Detection of wavelength

Detection of the wavelength was carried out for Curcumin and piperine (each 100µg/ml) solutions in the UV region (200-400nm) by using PDA detector. Better response of isobathic point was achieved at 280 nm.

Accuracy

Accuracy results indicate that the recovery of the percentage difference between the expected and measured drug concentrations of curcumin and piperine are consistent at all levels and within the range. The percentage coefficient of variation (% CV) is found to be less than 3%.

Precision

The method was examined for both curcumin and piperine from six repeated injections at three different concentrations of 200, 1000 and 2200 ng/ml. The low relative standard deviation (RSD) values indicate the reproducibility of the method and results are shown as in tables No.2, 3 and 4.

Specificity

The developed method showed good separation between the standard curcumin and piperine. The peak of sample was identified by comparing with the retention time of the standard drug solution. On comparison of peaks, retention times and spectra of the standards revealed that they are not affected by other interferences hence the developed method was specific.

Sensitivity

The method developed is sensitive and the limits of reliable quantitation have been set at a concentration of 60 ng/ml.

Linearity

The calibration curves are found to be linear in the concentration range of 0-2200 ng/ml and correlation coefficient for curcumin and piperine were 0.996 and 0.999 respectively (fig. 2) and all the calibration curve results are well within the specified limits (table 6).

Ruggedness and robustness

The method is found to be rugged and robust since there are no changes in the chromatogram by changing the instrument, operator and chromatographic conditions and the parameters are given in table 5.

LOD and LOQ

LOD and LOQ for curcumin and piperine are found to be 6 ng/ml and 60 ng/ml respectively.

Table 1: Accuracy studies

S. No.	Curcumin (ng/ml)			Piperine (ng/ml)		
	Measured concentration	Actual Concentration	% Nominal	Measured concentration	Actual Concentration	% Nominal
1	18809	18876	99.6451	2872	2840	101.127
2	18976	18876	100.53	2986	2840	105.141
3	18896	18876	100.106	2864	2840	100.845
4	18123	18876	96.0108	2753	2840	96.9366
5	18765	18876	99.412	2845	2840	100.176
6	17976	18876	95.232	2811	2840	98.9789
Mean	98.48926			100.534		
SD	2.267898			2.726127		
%CV	2.302686			2.711646		

Accuracy studies were performed for determination of concentration of each sample (n=6) and % CV was determined.

Table 2: Precision studies: Intra-day precision

S. No.	Curcumin (ng/ml)			Piperine (ng/ml)		
	200 (ng/ml)	1000 (ng/ml)	2200 (ng/ml)	200 (ng/ml)	1000 (ng/ml)	2200 (ng/ml)
1	201.09	1001.87	2201.85	201.22	1000.72	2200.11
2	199.63	999.53	2199.36	209.16	988.05	2200.45
3	198.74	989.54	2205.34	200.66	998.21	2199.69
4	201.26	1000.73	2205.26	192.94	1002.25	2207.83
5	199.51	1000.93	2150.15	199.34	993.75	2199.34
6	202.96	998.72	2170.84	203.93	999.05	2200.25
Mean	200.5317	998.554	2188.799	201.2083	997.005	2201.278
SD	1.536612	4.552693	22.98969	5.34403	5.248473	3.236406
%CV	0.766269	0.455929	22.10909	5.344027	4.932158	3.186535

Intra-day precision studies for curcumin and piperine was performed in three different concentrations (n=3) and % CV was determine.

Table 3: Precision studies: Inter-day precision-1

S .No.	Curcumin			Piperine		
	200 (ng/ml)	1000 (ng/ml)	2200 (ng/ml)	200 (ng/ml)	1000 (ng/ml)	2200 (ng/ml)
1	200.5244	1001.87	2201.84	201.219	1000.72	2200.11
2	202.28	999.53	2199.58	200.8	1001.97	2201.64
3	201.87	999.42	2200.68	200.66	999.67	2199.68
4	199.78	1000.73	2205.26	202.68	999.46	2199.2
5	202.49	1001.26	2194.53	199.34	999.32	2200.87
6	201.27	1000.94	2194.03	199.75	999.74	2200.25
Mean	201.369067	1000.63	2199.32	200.742	1000.15	2200.29
SD	1.05694221	0.97104	4.34628	1.1784	1.01965	0.86567
% CV	0.52487814	0.09704	0.19762	0.58702	0.10195	0.03934

Intra-day precision studies for curcumin and piperine was performed in three different concentrations (n=3) and % CV was determine.

Table 4: Precision studies: Inter-day precision-2

S. No.	Curcumin (ng/ml)			Piperine (ng/ml)		
	200	1000	2200	200	1000	2200
1	200	1000.76	2207.98	195.51	998.28	2207.49
2	195.2	999.532	2195.43	201.49	967.23	2199.55
3	196.53	955.14	2201.55	203.17	1020.77	2193.42
4	203.49	1016.27	2205.26	196.14	1010.64	2213.4
5	200.62	1007.59	2216.72	201.01	975.86	2196
6	205.1847	987.63	2170.84	201.7	1015.54	2214.17
Mean	200.170783	994.487	2199.63	199.837	998.053	2204.01
SD	3.85620312	21.4765	15.7714	3.19621	22.0133	8.941
%C/V	1.92645653	2.15955	0.717	1.59941	2.20562	0.40567

Inter-day precision 2 study for Curcumin and piperine was performed in three different concentrations (n=3) and % CV was determined.

Table 5: System suitability studies

S. No	Parameters	Curcumin	Piperine
1	Theoretical plate	1751.399	4022.327
2	Tailing Factor	1.0	1.1
3	Asymmetric Factor	1.4	1.39
4	LOD (ng/ml)	6	6
5	LOQ (ng/ml)	60	60
6	Linearity range	0-2200 (ng/ml)	0-2200 (ng/ml)
7	Regression equation	Y=90.123 x-315.86	Y=14.364 x-18.32
8	Correlation coefficient	0.9969	0.9999
9	Retention time	4.4	5.2

Table 6: Calibration and linearity

S. No	Concentration (ng/ml)	Perk area of Curcumin	Perk area of piperine
1	0	0	0
2	200	18809	2872
3	600	49427	8614
4	1000	87876	14356
5	1400	134256	19876
6	1800	160987	25987
7	2200	195321	31584

Calibration curve was plotted for both curcumin and piperine (n=6) and linearity was determined.

DISCUSSION

Method optimization

Simultaneous method development is focused on simple, sensitive and rapid determination of the compounds. Initially solubility of the drugs is determined later selection of the column is done based on the polarity of the drugs such as C8 and C18 etc. Suitable wavelength for the detection of the standard mixture of curcumin and piperine is selected based on the response of the solution to the concentration of the analyte. The optimized chromatographic conditions are tuned by changing the organic phase (methanol and acetonitrile), aqueous phase (buffer), pH, flow rate and mobile phase concentration. The optimized chromatographic conditions revealed good separation of curcumin and piperine using a mobile phase of 25 mM potassium dihydrogen ortho phosphate buffer (pH 3.5) and acetonitrile (30:70 v/v). Curcumin and piperine retention times are found to be 4.4 min and 5.2 min respectively (fig. 3). The calibration curves for curcumin and piperine are found to be linear within the range of 0-2200 ng/ml.

Analytical method development was carried out as per ICH, Q_2R_1 guidelines. The developed method was evaluated for validation parameters such as accuracy, precision, specificity, precision, linearity, ruggedness and robustness. Accuracy was calculated in terms of percentage nominal where n=6 and the mean, standard deviation and percentage coefficient of variation (% CV) were calculated and % CV values were less than 3%. Precision of the method was carried out in two different days with three different concentrations (LQC, MQC and HQC) and mean, standard deviation and % CV were calculated and the low RSD values reflect the method was precise. Specificity of the method shows good separation and no interferences between the selected phytoconstituents and the matrices. Sensitivity of the developed method was selected based on lower limit of quantification. Good linearity and range were achieved with a concentration range of 0-2200 ng/ml with regression values of 0.996 and 0.999. Ruggedness and robustness was checked by differing chromatographic conditions, different operators and instruments, but there was no change in the developed method.

CONCLUSION

The developed simultaneous RP-UFLC method is simple, sensitive, specific and rapid for qualitative and quantitative estimation of curcumin and piperine either individually or in combination in food products, herbal medicines and nutraceuticals as per the regulatory needs.

REFERENCES

Aggarwal BB and Hari kumar KB (2008). Potential the rapeutic effects of curcumin, The anti-inflammatory agent, against neurodegenerative, cardiovascular, pulmonary, metabolic, autoimmune and neoplastic diseases. *Int. J. Biochem. Cell Biol.,* **41**: 40-59.

Atal CK, Dubey RK and Singh J (1985). Biochemical basis of enhanced drug bioavailability by piperine: Evidence that piperine is a potent inhibitor of drug metabolism. *J. Pharmacol. Exp. Ther.,* **232**: 258-262.

Chauhan SK, Kimothi GP, Singh BP, Agrawal S (1998). Development of HPLC method to determine piperine in different piper species. *Ind. Drugs,* **35**: 408-411.

Gannu R., Yamsani VV, Yamsani SK Bedi KL, Johri RK, Sharma SC. (2005). Development of Ultra fast liquid chromatographic method for simultaneous determination of nitrendipine and carvone in skin diffusate samples. *J. Pharm. and Biomed. Anal.,* **50**: 1080-1084.

Garcea G, Jones DJL, Singh R, Dennison AR, Farmer PB, Sharma RA, Steward WP, Gescher AJ, Berry DP. (2004). Detection of curcumin and its metabolites in hepatic tissue and portal blood of patients following oral administration. *Br. J. Cancer,* **90**: 1011-1015.

ICH Q2B (1995). Validation of analytical procedures: Methodology. International conference on Harmonisation, London, pp 1-12.

Ireson C, Orr S, Jones DJ, Verschoyle R, Lim CK, Luo JL, Howells L, Plummer S, Jukes R, Williams M, Steward WP, Gescher A.. (2001). Characterization of metabolites of the chemopreventive agent curcumin inhuman and rat hepatocytes and in the rat *in vivo* and evaluation of their ability to inhibit phorbol ester induced prostaglandin E2 production. *Cancer Res.,* **61**: 1058-1064.

Ireson CR, Jones DJ, Orr S, Coughtrie MW, Boocock DJ, Williams ML, Farmer PB, Steward WP, Gescher AJ. (2002). Metabolism of the cancer chemopreventive agent curcumin in human and rat intestine. Cancer. *Epidemiol. Biomarkers. Prev.,* **11**: 105-111.

Jayaprakash GK, Rao LJM, Sakariah KK (2005). A review on chemistry and biological activities of Curcuma longa. *Trends. Food. Sci. Tech.,* **1**: 533-548.

Khajuria A, Thush N, Zutshi U, Bedi, KL (1997). Antioxidant potential of piperine on oxidant induced alterations in rat intestinal lumen. *Ind. Drugs,* **34**: 557-563.

Koichi I, Yoshihiro Y and Hiroyuki N (2001). Evaluation of the turmeric (*Curcuma longa* L) based on flow injection analysis with ultraviolet and fluorometric detections. *Analy. Let.,* **34**: 1711-1718.

Koul IB and Kapil A (1993). Evaluation of the liver protective potential of piperine and active principle of black and long peppers. *Planta Med.,* **59**: 413-417.

Krishna Veni N, Meyyanathan SN and Rajinikanth BR, Elango. K (2009). A liquid chromatography method for the simultaneous determination of curcumin and piperine in food products using diode array detection. *Asian. J. Research. Chem.,* **2**: 115-118.

Lee EB, Shin KH and Woo WS (1984). Pharmacological study on piperine. *Arch. Pharma. Col. Res.,* **7**: 127-132.

Liu A, Lou H, Zhao L, Fan P. (2006). Validated LC-MS/MS assay for curcumin and tetrahydrocurcumin in rat plasma and application to pharmacokinetic study of phospholipid complex of curcumin. *J. Pharm. Biomed. Anal.,* **40**: 720-727.

Niraj V, Kanan G, Mohammad YK, Siddharth P, Pundarikakshudu K (2011). Simultaneous estimation of curcumin and piperine in their crude powder mixture and ayurvedic formulation using High performance thin layer chromatography. *Int. J. Res. Pharma and Biomed. Sci.,* **2**: 231-236.

Shanmugam. R, Gowthamarajan.K, Priyanka D.L, Madhuri.K, Hemnath. E, Dhanabal SP. (2013). Development and Validation of a RP- UFLC Method for Simultaneous Estimation of Quercetin and Rutin. *Hygeia.J.D.Med.vol.*,**5**: *113-120*

Sharma RA, McLelland HR, Hill KA, Ireson CR, Euden SA, Manson MM, Pirmohamed M, Marnett LJ, Gescher AJ, Steward WP. (2001). Pharmacodynamic and pharmacokinetic study of oral curcuma extract in patients with colorectal cancer. *Clin. Cancer. Res.,* **7**: 1894-1900.

Shoba G, Joy D, Joseph T, Majeed M, Rajendran R, Srinivas PS. (1998). Influence of piperine on the pharmacokinetics of curcumin in animals and human volunteers. *Planta. Med.,* **64**: 353-356.

Singh P, Bajpai M and Om P Mathur SC, Singh RM, Tiwari R, Singh GN (2007). Quantitative determination of piperine in *Piper nigrum* powder through HPTLC method. *J. Pharm. Res.,* **6**: 170-172.

Sun X, Gao C, Cao W, Yang X, Wang E. (2002). Capillary electrophoresis with amperometric detection of curcumin in chinese herbal medicine pretreated by solid phase extraction. *J. Chromatogr. A.*, **962**: 117-125.

Verma J K and Joshi AV (2006). Rapid HPTLC method for identification and quantification of curcumin, piperine and thymol in an ayurvedic formulation. *J. Planar. Chromatogr.*, **19**: 398-400.

Yuan K, Weng Q, Zhang H, Xiong J, Xu G. (2005). Application of capillary zone electrophoresis in the separation and determination of the curcuminoids in urine. *J. Pharm. And Biomed. Anal.,* **38**: 133-138.