CHAPTER-5

Analytical Method Development of Curcumin

5.1 EXPERIMENTAL METHODS

5.1.1 Pre-formulation studies

Pre-formulation studies focus on the physiochemical properties of the compounds that affect the drug performance and development of an efficacious dosage form. A thorough understanding of these properties, ultimately provide a rational for formulation design. Drug identification test and analytical method development were done in this phase to provide a useful support in development of dosage form. The following studies were performed:

- Organoleptic characteristics and melting point of curcumin
- Analytical method development and validation of curcumin by UV-visible spectrophotometer
- Analytical method development and validation of curcumin by high performance liquid chromatography (HPLC)
- Solubility studies of curcumin

5.1.1.1 Organoleptic characteristics and melting point of curcumin (CUR)

The Organoleptic characteristics test were performed to confirm the color, odor and taste of the CUR. The melting point of CUR was determined by capillary tube method (Aulton, 2007). Briefly, small amount of CUR was incorporated in capillary tube and placed into the melting point apparatus and melting temperature of CUR was recorded from 175-185°C.

5.1.1.2 Analytical Method Development and Validation of CUR by UV-Visible Spectrophotometer

5.1.1.2.1 Selection of media

The selection of media was done on the basis of CUR solubility. Phosphate buffer solution (PBS), pH 7.4 was selected for preparation of calibration curve.

5.1.1.2.2 Scanning for λ_{max}

10 mg of CUR was dissolved in little volume of PBS, pH 7.4 and finally diluted to 10 ml in volumetric flask to get a concentration of 100 µg/ml. This was treated as stock solution. Various aliquots of stock solution were diluted further to get different concentrations. Resultant solutions were scanned for λ_{max} in the visible range of 400-800 nm using UV-visible spectrophotometer.

5.1.1.2.3 Sample Preparation

Aliquots of the stock solution of CUR (100 µg/ml) were pipetted out into a series of 10 ml volumetric flasks and further diluted with the PBS of pH 7.4 to get a final concentration range of 1-6 µg/ml of sample solution. The absorbance of the resultant solutions was measured at 420 nm. Freshly prepared solutions were made for the calibration curve on three consecutive days.

5.1.1.2.4 Method Validation

The validation of an analytical procedure is the process of confirming that the analytical procedure employed for a test of drug samples is suitable for its intended use. Assay validation of calibration curve was carried out as per FDA guidelines and ICH Q2A and Q2B guidelines. In validation procedure, calibration curve prepared in PBS, pH 7.4 and run in triplicate for 3 days to determine intra and inter-day variations (Chaurasia *et al.,* 2011).

To be fit for intended purpose, the method must meet certain validation characteristics. Typical validation characteristics, which should be considered

are: specificity, linearity, range, accuracy, precision, limit of detection and quantitation.

5.1.1.2.4.1 Specificity

The specificity is the ability of an analytical procedure to measure accurately an analyte in the presence of components that may be expected to be present in sample matrix. The specificity is a measure of discriminating ability. Lack of specificity of an analytical procedure may be compensated by other supporting analytical procedures.

5.1.1.2.4.2 Linearity

The linearity is the ability of analytical procedure to produce test results which are proportional to the concentration (amount) of analyte in samples within a given concentration range, either directly or by means of a well-defined mathematical transformation. Linearity should be determined by using a minimum of six standards whose concentration span be 80-120% of the expected concentration range.

5.1.1.2.4.3 Range

The specified range is normally derived from the linearity studies. The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample for which it has been demonstrated that the analytical method has suitable levels of precision, accuracy and linearity.

The following minimum specified ranges should be considered:

- For the assay of the active constituent: normally from 80-120% of the test concentration/label concentration; and
- For the determination of an impurity: from the specification level of the impurity to 120% of the specification.

5.1.1.2.4.4 Accuracy and Precision

The accuracy of an analytical method is defined as the similarity of the results obtained by the analytical method to the true value and the precision as the degree of that similarity (Chow & Shao, 2002). Accuracy of the method was performed by recovery study of three drug-spiked placebo formulations of six concentrations (one near to the limits of quantitation) on a single assay day to determine intra-day precision and accuracy. In addition, analyses of six samples of six concentrations on three consecutive days were used to determine inter-day precision and accuracy.

Recovery is defined as the ratio of the observed result to the expected result expressed as a percentage.

The accuracy of a method may vary across the range of possible assay values and therefore must be determined at several different fortification levels. The accuracy should cover at least 3 concentrations (80, 100 and 120%) in the expected range.

Acceptance criteria: the expected recovery depends on the sample matrix, the sample processing procedure and on the analyte concentration. The mean % recovery should be within the following ranges are shown in Table 5.1:

Table 5.1 Acceptance criteria for percentage mean recovery

% active/impurity content	Acceptable mean recovery
≥10	98-102%
≥1	90-110%
0.1-1	80-120%
<0.1	75-125%

Accuracy can also be associated with the term bias. A biased estimate is systematically either higher or lower than the true value. The acceptable values of % bias should lies within ±15% at all concentrations (Bressolle *et al.,* 1996). The % bias was assessed by determining percent error, which was calculated as:

$$\% \, Bias = \frac{Measured \; Concentration - Expected \; Concentration}{Expected \; Concentration} \times 100$$

The precision of an analytical method or a test procedure is referred to as the degree of closeness of the result obtained by the analytical method or the test procedure to the true value. In practice, the total variability associated with the assay result is often considered as the primary measure for the assessment of assay precision. Precision was studied to find out intra and inter day variation in the calibration curves of drug prepared in PBS, pH 7.4. The precision was assessed by relative standard deviations (R.S.D.) with the acceptable values should be ≤15% at all concentrations (Shah *et al.,* 1992) which was calculated as:

$$\% \, R.S.D. = \frac{Standard \; Deviations}{Mean \; Measured \; Concentrations} \times 100$$

5.1.1.2.4.5 Limits of detection

The limit of detection (LOD) is the lowest amount or concentration of the analyte in a sample that is detectable, but not necessarily quantifiable.

LOD should be normally determined so that producer's and consumer's risks are less than 5%. The LOD may be calculated using the standard deviation of responses of blank samples or samples containing an analyte close to the detection limit and the slope of the calibration curve close to the detection limit. The following equation used to determine the LOD using the standard deviation of responses of blank samples and the slope of the calibration curve:

$$\mathrm{LOD} = \frac{3.3 \times \sigma}{\mathrm{Slope}}$$

Where, LOD: Limit of Detection

σ: the standard deviation of responses of blank samples

Slope: slope of the calibration curve

The noise level may be used as the standard deviation of responses of blank samples in chromatographic methods. It should be ensured that the LOD of the analytical procedure is lower than the specified limit for testing.

5.1.1.2.4.6 Limits of quantitation

The limit of quantitation (LOQ) is the lowest amount or concentration of the analyte in a sample that can be determined. The precision expressed as the relative standard deviation of samples containing an analyte at the LOQ is usually 10%.

The LOQ may be calculated using the standard deviation of responses of blank samples or samples containing an analyte close to the LOQ and the slope of the calibration curve close to the LOQ. The following equation used to determine the LOD using the standard deviation of responses of blank samples and the slope of the calibration curve:

$$\mathrm{LOQ}=\frac{10\times\sigma}{\mathrm{Slope}}$$

Where, LOQ: Limit of Quantitation

σ: the standard deviation of responses of blank samples

Slope: slope of the calibration curve

The noise level may be used as the standard deviation of responses of blank samples in chromatographic methods. It should be ensured that the LOD of the analytical procedure is lower than the specified limit for testing.

5.1.1.3 Analytical method development and validation of CUR by High performance liquid chromatography (HPLC) in biological samples

5.1.1.3.1 Chromatographic systems and conditions

The HPLC system (Shimadzu Corporation, koyoto, Japan), equipped with UV-Visible detector (SPD-20A), a pump (LC-20AD), a degasser (DGU-20A_3) and a rheodyne manual injector (SIL-20A) attached with 20 µl sample loop. A C_{18} reverse-phase column (Phonomenex, 250mm×4.6mm, Particle size 5 µ, Enable) and a C_{18} guard column were utilized for drug separation and quantification. The isocratic elution was performed at a flow rate of 1 ml/min with a mobile phase consisting of acetonitrile and 2% (v/v) acetic acid at the ratio of 80:20. The mobile phase was filtered through 0.45 µm nylon filter (Himedia, India) and degassed in ultrasonic bath for 10 min prior to use. The detection and quantification was performed by monitoring the elute at 425 nm wavelength.

5.1.1.3.2 Stock and working standard solutions

Stock standard solutions of CUR (1 mg/ml) and 4-hydroxybenzophenone (1 mg/ml; internal standard, IS) were prepared by dissolving 10 mg of drug in 10 ml of acetonitrile (ACN) (containing 0.02% BHT), respectively. Different working standard solutions of CUR (concentrations ranging from 5 to 1000 ng/ml) were obtained by diluting the stock solutions with ACN (0.02% BHT). These solutions were protected from light and stored at -20°C between experiments, and it was found to be stable for at least 3 weeks (Ma *et al.,* 2007). The 4-hydroxybenzophenone solution was subsequently diluted with ACN to make a working IS solution of 10 µg/ml.

Calibration standards for plasma were prepared daily for three days by spiking 90 µL of blank plasma with 10 µL of the appropriate working standard solutions resulting in concentrations of 5 to 1000 ng/ml (Ma *et al.,* 2007).

5.1.1.3.3 Plasma sample preparation procedures

A liquid-liquid extraction method was employed for extraction of drug from the plasma (Maiti *et al.,* 2007). Plasma containing CUR and internal standard (4-hydroxybenzophenone, 10 µg/ml) was thawed. 100 µl was transferred to a 1.5 ml eppendorf tube and 900 µl ACN was added to precipitate plasma proteins. This mixture was then vortexed for 5 min and centrifuged at 5000 rpm for 10 min. Supernatant was transferred to a glass tube and evaporated in a vacuum oven at 40°C. The residue was reconstituted in 100 µl mobile phase and 20 µl of reconstituted samples was injected onto the HPLC column for drug content after filtration through 0.2 µm syringe filter.

5.1.1.3.4 Recovery

The recoveries of CUR from plasma were determined by spiking an equal amount of the drug into the corresponding blank sample and ACN (BHT 0.02%) (solvent used in the final chromatographic step). Recoveries at three concentration levels 10, 200 and 1000 ng/ml for plasma were studied in six-times for each sample type. Percentage recoveries were calculated by comparing the absolute responses (peak areas) of CUR from sample extracts to the absolute responses (peak areas) of non-extracted standards [CUR in ACN (0.02% BHT) (Ma *et al.,* 2007).

5.1.1.3.5 Linearity, precision and accuracy

Calibration curves of CUR in plasma were established over the concentration range: 5-1000 ng/ml. For construction of the calibration curves, six different calibration standards were prepared and processed for the range 5-1000 ng/ml in plasma. Drug concentrations versus the corresponding peak areas of CUR were plotted. All calibration curves were done in triplicate. Precision (intra day and inter day variation) was evaluated by analyzing six replicate plasma samples at the following concentrations: 10, 200 and 1000 ng/ml. The variability was expressed as the R.S.D. (relative standard deviation).

To be acceptable, the values should be ≤15% at all concentrations (Shah *et al.,* 1992).

Accuracy of the assay method was calculated from the same samples as those used for intra day and inter day variation studies. The accuracy was expressed as % bias and for being acceptable, the values should be within ±15% at all concentrations (Ma *et al.,* 2007).

5.1.1.3.6 LOD and LOQ

LOD and LOQ were estimated from the signal-to-noise ratios (ICH Q2B, 1996). The detection limit was defined as the lowest concentration level resulting in a peak area of 3 times the baseline noise. The quantitation limit was defined as the lowest concentration level that provided a peak area with a signal-to-noise ratio higher than 10.

5.1.1.4 Solubility studies of CUR

Saturation solubility was determined in buffer solutions of different pH by shake flask method (Aulton, 2007). Distilled water, acidic buffer of pH 1.2, PBS, pH 6.8 and 7.4 were taken as different aqueous media. 50 ml of each solution was transferred in conical flask and an excess amount of CUR was added in each. All the conical flasks were shaken overnight on rotary shaker. In between, if drug was completely dissolved in any solution then, more amount of drug was added in the same. Finally, each solution was filtered through Whatman paper no.1 filter and diluted with respective media and drug concentration was determined UV-Visible spectrophotometrically at 420 nm.

5.2 RESULTS AND DISCUSSION

5.2.1 Organoleptic Characteristics

The pure CUR possesses orange-yellow color at neutral pH, odorless and pungent, sour as well as bitter taste. The physical appearance of pure CUR is solid. The melting point of pure CUR was found to be 180°C. It is noninflammable and light sensitive drug.

5.2.2 Analytical Method Development and Validation of CUR by UV-Visible Spectrophotometer

UV-visible spectrum of CUR was taken to determine the λ_{max} of CUR in PBS, pH 7.4. The 100 µg/ml solution of CUR in the test medium were scanned for absorption maxima in the visible range of 400-800 nm, and are illustrated in Table 5.2 and Figure 5.1, respectively and therefore chosen for the drug estimation for further studies.

Table 5.2 Scanned λ_{max} and absorbance value of CUR prepared in PBS, pH 7.4

Strength (µg/ml)	Scanned λ_{max}	Absorbance
100	420	0.977

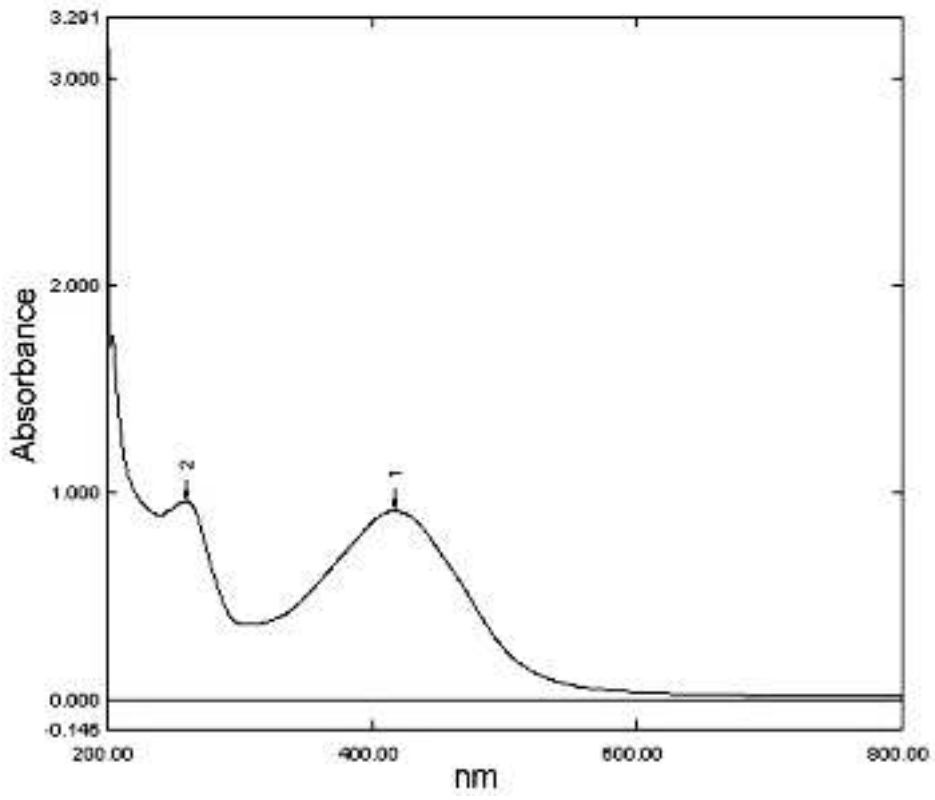

Figure 5.1 UV-Visible absorbance spectra of pure CUR in PBS, pH 7.4

Calibration curve of CUR were prepared in PBS of pH 7.4 on three consecutive days at λ_{max} 420 nm. The absorbance values (mean of three determinations) with their standard deviations at different concentration in the range of 1-6 µg/ml is shown in Table 5.3 and their linear plot is presented in Figure 5.2. Drug was found to obey Beer-Lambert's law in the concentration range of 1-6 µg/ml with regression coefficient (R^2) values 0.999. The regression equation was found to be y = 0.175X – 0.076 in PBS, pH 7.4.

Table 5.3 Absorbance for plot of calibration curve of CUR in PBS, pH 7.4

S. No.	Concentration (µg/ml)	Absorbance (mean±SD; n=3)
1	0	0.000 ± 0.0000
2	1	0.152 ± 0.0056
3	2	0.301 ± 0.0161
4	3	0.465 ± 0.0238
5	4	0.635 ± 0.0323
6	5	0.799 ± 0.0259
7	6	0.968 ± 0.0583

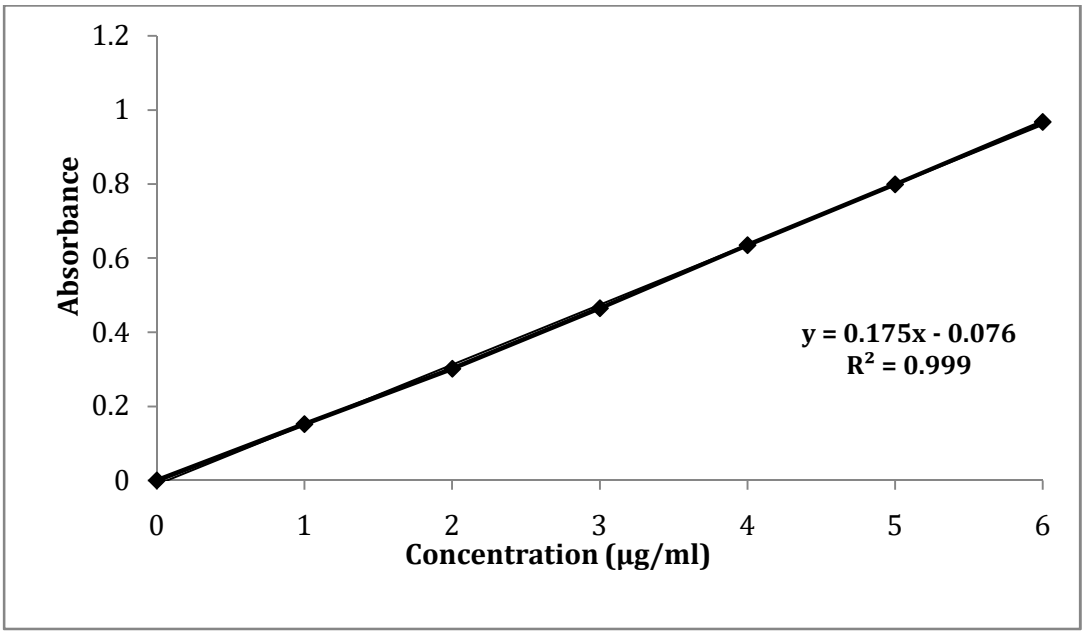

Figure 5.2 Calibration curve of CUR in PBS, pH 7.4.

Recovery of CUR from PBS, pH 7.4 was determined by spiking an equal amount of the drug into the corresponding known amounts of pre-analyzed standard solutions of CUR and subjected for UV-visible spectrophotometery at concentrations of 2, 4 and 6 µg/ml using three replicates at each concentration. The results of recovery studies are summarized in Table 5.4. The data proved the suitability of this recovery method for the analysis of PBS, pH 7.4 samples.

Table 5.4 Recovery studies of CUR from PBS, pH 7.4

S.No.	Concentration (µg/ml)	Concentration recovered (µg/ml) (mean±SD; n=3)	Recovery (%) (mean±SD; n=3)	R.S.D. (%)	Bias (%)
1	2	2.02±0.071	101.00±3.555	3.52	0.99
2	4	4.05±0.176	101.25±4.415	4.36	1.25
3	6	6.15±0.446	102.50±7.431	7.25	2.50

For intra-day and inter-day precision study, calibration curves prepared in PBS, pH 7.4 were run on triplicate in same day for three days as well as three consecutive days, respectively and Percentage Relative Standard Deviation (%R.S.D.) calculated for all the cases. The intra-day variability of the assay method (%R.S.D.) for CUR in PBS, pH 7.4 were found to be less than 8.11% and all data are shown in Table 5.5 whereas, corresponding inter-day variability were found to be less than 9.11%, and all data are summarized in Table 5.6. The data indicate that the assay method is reproducible within the same day and within different days; %R.S.D. are less than 15% for all sample types over the concentration ranges assayed (Shah *et al.,* 1992).

Table 5.5 The Intra-day precision of the method

Day 1	**Intra-Day**			
S. No.	**Expected Concentration (µg/ml)**	**Measured Concentration (µg/ml) (mean±SD; n=3)**	**% R.S.D.**	**% Bias**
1	0	0.000 ± 0.000	0.00	0.00
2	1	0.998 ± 0.023	2.32	-0.20
3	2	2.111 ± 0.077	3.65	5.55
4	3	3.211 ± 0.058	1.82	7.03
5	4	4.081 ± 0.198	4.87	2.03
6	5	5.024 ± 0.262	5.22	0.48
7	6	6.121 ± 0.496	8.11	2.01
Day 2				
1	0	0.000 ± 0.000	0.00	0.00
2	1	1.012 ± 0.021	2.06	1.20
3	2	1.988 ± 0.086	4.34	-0.60
4	3	3.021 ± 0.030	1.02	0.70
5	4	3.891 ± 0.128	3.28	-2.72
6	5	5.004 ± 0.054	1.09	0.08
7	6	6.031 ± 0.054	0.91	0.52
Day 3				
1	0	0.000 ± 0.000	0.00	0.00
2	1	1.109 ± 0.070	6.34	10.90
3	2	2.015 ± 0.027	5.44	0.75
4	3	3.088 ± 0.126	4.09	2.94
5	4	4.152 ± 0.178	4.29	3.80
6	5	5.001 ± 0.138	2.77	0.02
7	6	6.099 ± 0.101	1.66	1.65

Table 5.6 The Inter-day precision of the method

Day 1-3	Inter-Day			
S. No.	**Expected Concentration (µg/ml)**	**Measured Concentration (µg/ml) (mean±SD; n=3)**	**% R.S.D.**	**% Bias**
1	0	0.000 ± 0.000	0.00	0.00
2	1	0.918 ± 0.084	9.11	-8.20
3	2	1.911 ± 0.146	7.65	-4.45
4	3	3.371 ± 0.196	5.82	12.37
5	4	4.011 ± 0.163	4.07	0.27
6	5	5.091 ± 0.113	2.22	1.82
7	6	6.101 ± 0.079	1.31	1.68

Linearity of an analytical method is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range. Data from the regression line is helpful to provide mathematical estimates of the degree of linearity. Table 5.7 shows the linearity and range data for calibration curves prepared in PBS, pH 7.4.

LOD and LOQ of calibration curve were calculated which was based on the standard deviation of y-intercept of regression line (SD) and the slope (S) of the calibration curve. The LOD and LOQ values are shows in the Table 5.7.

Table 5.7 Other validation parameters for calibration curve prepared in PBS, pH 7.4

Parameters	Values
Linearity correlation coefficient	0.999
y- intercept (SD)	0.01277
Slope	0.175
Range	1-6 µg/ml
LOD	0.240 µg/ml
LOQ	0.729 µg/ml

The developed method of estimation of CUR using UV spectrophotometry technique is sufficiently rapid, simple, sensitive, precise as well as accurate that complies with FDA guidelines. The linearity, accuracy, precision, limit of detection and quantification and specificity-selectivity of the method were established. In addition to analysis of drug during assay and release studies, this rapid and reproducible analytical method was successfully used for analysis of drug-excipient compatibility in a newly developed formulation in our laboratory and also for their stability studies.

5.2.3 Analytical method development and validation of CUR by HPLC in biological samples

5.2.3.1 Chromatography spectrum

The selectivity of HPLC analysis was studied by analyzing plasma samples, which did not show any interfering components. A typical chromatogram of a drug-free plasma sample spiked with curcumin and IS is shown in Figure 5.3. Separation of curcumin and IS was achieved successfully. The retention times of curcumin and IS were approximately 7.7 and 7.0 min, respectively.

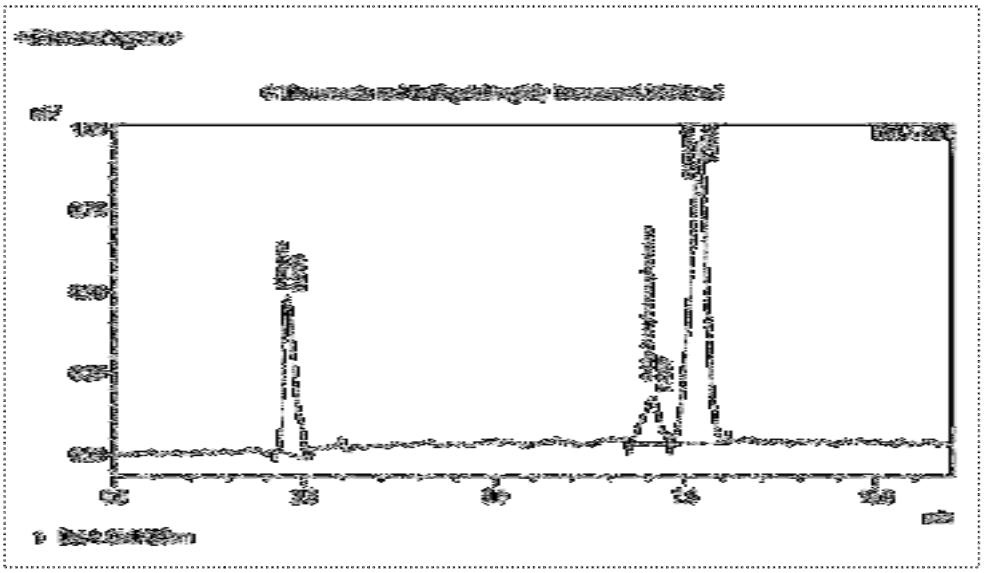

Figure 5.3 Typical HPLC chromatogram of blank plasma and blank plasma spiked with CUR and IS.

5.2.3.2 Extraction Efficiency

The absolute recovery of CUR from plasma was determined by comparing peak area obtained from extracts of spiked plasma samples with that obtained from the direct injection of known amounts of standard solutions of CUR. Assessed at concentration of 10, 200 and 1000 ng/ml using six replicates at each concentration, the recoveries were 93.87, 96.80 and 94.17%, respectively as shown in Table 5.8. The data proved the suitability of this extraction method for the analysis of plasma samples.

Table 5.8 Recovery of CUR from rat plasma

S. No.	Concentration (ng/ml)	Recovery (%) (mean±SEM; n=6)	R.S.D. (%)
1	10	92.074±1.881	2.042
2	200	94.778±2.271	2.396
3	1000	94.540±0.502	0.531

5.2.3.3 Precision and Accuracy

Table 5.9 shows a summary of intra- and inter-day precision and accuracy for CUR in rat plasma. The intra-day and inter-day of CUR ranged from 92.70 to 112.23% over a wide range of CUR concentrations with precision (%CV) less than 0.125 and 4.667%, respectively. Precision and accuracy studies indicated that the developed HPLC method is reproducible, accurate and therefore applicable to the pharmacokinetic studies of CUR in rats.

Table 5.9 The intra-day (n=6) and inter-day (n=6) precision of the method

	Intra-day			Inter-day		
Expected Concentration (ng/ml)	(mean±SEM; n=6) (ng/ml)	% CV	%Bias	(mean±SEM; n=6) (ng/ml)	%CV	%Bias
10	10.787±0.011	0.1044	7.876	8.910±0.415	4.667	-10.9
200	202.764±0.255	0.1259	1.382	200.526±0.540	0.269	0.264
1000	1008.378±0.539	0.0534	0.837	992.726±1.211	0.122	-0.727

5.2.3.4 Linearity

The linearity of an analytical procedure is its ability to obtain test results which are directly proportional to the concentrations of analyte in the sample. The linearity of the assay for the CUR was evaluated with a total of six calibration standards. The linear regression analysis of CUR was constructed by plotting the peak-area ratio of CUR to IS vs the concentration of CUR in ng/ml. The calibration curve was highly linear (R^2>0.999) within the range of 5-1000 ng/ml for CUR extracted from rat plasma samples as shown in Figure 5.4. Typical values for the regression parameters a (the slope) and b (the y-intercept) were calculated to be 0.002 and 0.0542, respectively.

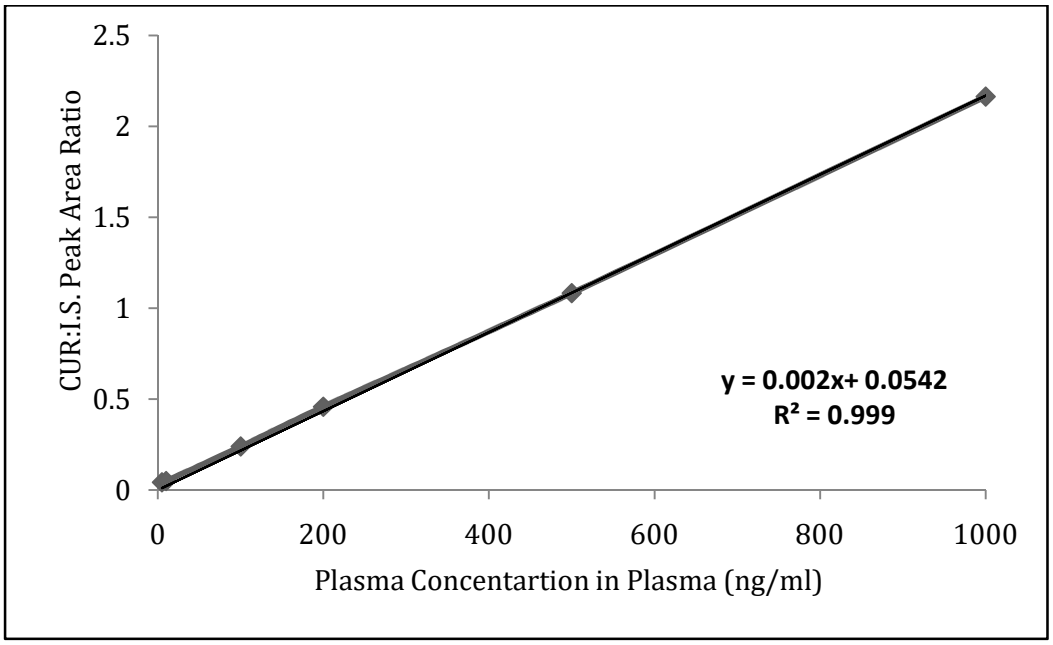

Figure 5.4 A representative standard curve for CUR in rat plasma extending from 5 to 1000 ng/ml.

5.2.3.5 LOD and LOQ

LOD and LOQ of calibration curve were calculated which was based on the standard deviation of y- intercept of regression line (SD) and the slope (S) of the calibration curve at levels approximating the LOD and LOQ, LOD = 3.3 (SD/S) and LOQ =10 (SD/S) (Chow & Shao, 2002). LOD and LOQ values of the assay method were found at 0.6604 ng/ml and 2.0014 ng/ml, respectively.

The described HPLC method is highly specific, sensitive and reproducible for the quantitative analysis of CUR in rat plasma samples. No interfering peak co-eluted with the CUR and IS were observed when rat plasma samples were analyzed. Good linearity, precision and accuracy for CUR proved the feasibility of this assay in the pharmacokinetic studies.

5.2.4 Solubility studies

The saturation solubility profile of pure CUR in different media at 25±2°C was determined. The solubility of pure CUR was found to be 0.0582±0.003, 0.9042±0.045, 0.9556±0.047 and 1.9978±0.099 µg/ml in water, acidic buffer (pH 1.2), phosphate buffer (pH 6.8) and PBS, pH 7.4, respectively as shown in Figure 5.5. The results proved that the pure CUR is able to provide a clear solution when dissolved in PBS, pH 7.4.

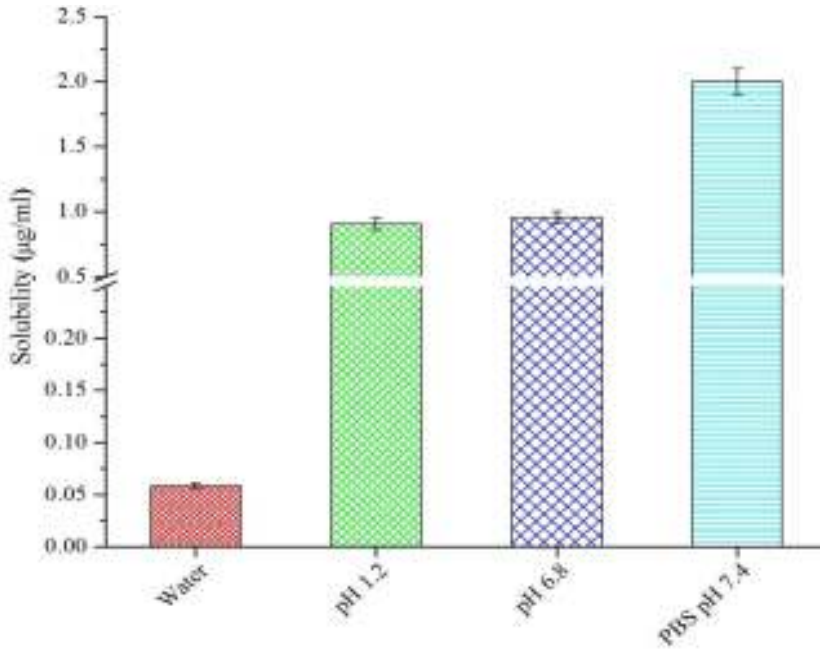

Figure 5.5 Solubility profile of pure CUR in different media at 25±2°C (Vertical bars represent ±SD; n=3).