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BIOANALYTICAL RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF CURCUMIN IN PLASMA SAMPLES

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ABSTRACT

The present study was aimed at developing a reversed phase high performance liquid chromatography (RP-HPLC) method for determination of curcumin (CRM) in plasma and hydrochlorothiazide was used as an internal standard. The separation was achieved by using C-18 column (Qualisil BDS C18, 250 mm x 4.6 mm I.D.) coupled with a guard column of silica, mobile phase was consisting of acetonitrile: water with 0.1% formic acid (40:60 v/v). The flow rate was 0.3 ml/min and the drug was detected using PDA detector at the wavelength of 423 nm. The experimental conditions, including the diluting solvent, mobile phase composition, column saturation and flow rate, were optimised to provide high-resolution and reproducible peaks. The developed method was validated in terms of linearity, recovery, precision, ruggedness, sensitivity (LOD and LOQ) and stability study (short and long-term stabilities, Freeze/thaw stability and post-preparative).

KEYWORDS

Isocratic, Curcumin, RP-HPLC, Validation and Plasma samples.

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INTRODUCTION

Curcumin ((1E, 6E)-1, 7-Bis (4-hydroxy-3-methoxyphenyl)-1, 6 heptadiene-3, 5-dione), a polyphenol known as diferuloylmethane. Molecular formula of CRM C_{21} H_{20} O_6 and Molecular weight of curcumin (CRM) is 368.39 g/mol. CRM is highly lipophilic drug having a log P value is 1.82 respectively. Dissociation constant of CRM was 8.3±0.04. CRM is a bright yellow-orange powder material. CRM has maximum solubility in methanol and acetonitrile. Melting point of CRM was 185ºc respectively. Reported λmax of CRM is 423 nm.

This inhibits autophosphorylation of EGFR and blocks downstream signalling. There is no bio-analytical method for estimation of CRM in Plasma. Hence this study was aimed at developing a simple, rapid and sensitive method for estimation of analyte (CRM) in tissue samples (plasma) by using RP-HPLC[1-3].

MATERIAL AND METHODS

Material

Curcumin (CRM) supplied as a gift sample from Sunpure Extracts Pvt. Ltd (Delhi, India). All solvents used were of HPLC grade. Formic acid, Methanol and acetonitrile were obtained from MERCK. Chem. Ltd (Mumbai, India).

Plasma Samples

Plasma samples were obtained from Central Animal House Facility, R.C. Patel Institute of Pharmaceutical education and Research Shirpur. Registration number 651/PO/ReBi/S/02/CPCSEA. The rats were euthanasiazed by using CO_2 chamber (carcass disposal: Deep Burying under Soil). The rats were decapitated immediately after blood collection (Figure No.1). Blood samples were anticoagulated with heparin and centrifuged at 6000 rpm for 10 min to obtain plasma. All plasma samples were stored in a deep freezer at −70˚C until HPLC analysis.

Instrumentation

Analysis were carried out using an Agilent 1200 HPLC system (Agilent technologies, USA). The system was equipped with quaternary pump and photo diode-array detector (PDA). All data were acquired and processed using EZ chrome elite software version 3.3.2.

Chromatographic conditions

Chromatographic separation was performed by using C-18 column (Qualisil BDS C18, 250 mm x 4.6 mm I.D.) coupled with a guard column. Isocratic elution was performed with acetonitrile: water with 0.1% formic acid (40:60 v/v) at a flow rate of 0.3 mL/min. The mobile phase was selected to give proper resolution of peaks[4].

Plasma Samples processing and quality control (QC) samples

Certified reference standards of CRM was weighed accurately and transferred 100 mg of CRM as working standard into 100 ml of volumetric flask, add about 100 ml of methanol and sonicated (1000 µg/mL solution). The working standard solutions was 100-400 ng/mL solution[5].

Preparation of standard solutions of internal standard (IS)

Internal standard such as hydrochlorothiazide, add 100 mg of IS in 100 ml of methanolic working solution (1000 µg/ml). The working standard solutions was 30 µg/mL solution

Preparation of Plasma samples

The whole procedure was carried out at room temperature. To 100 µl of CRM standard solution 100 µl of blank plasma sample, 100 µl of IS hydrochlorothiazide (30 µg/ml) were spiked and added extraction solvent 2 mL of acetonitrile was added and vortexed mixture for 20 min. This sample was ultracentrifuged at 10,000 rpm for 10 min. The supernatant layer was collected and 20 µl was analyzed by HPLC system[6-8].

Method development

Methods development was important to judge the quality, reliability and consistency of analytical results. It is the process for proving that analytical method is acceptable for use to measure the concentration of drugs[7].

Method validation

Validation of an analytical method is the process which is established by laboratory studies to evaluate the performance uniqueness of the procedure meet the requirements for its intended use. The validation process for analytical procedures begins with planned and systematic collection by the applicant of the validation data to support analytical procedures. The following are typical analytical performance characteristics which may be tested during methods validation: linearity, recovery, precision, sensitivity (LOD and LOQ) and stability study (short and long-term stabilities, Freeze/thaw stability and post-preparative). The linearity of a bioanalytical 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 sample within a given range (100-400 ng/mL). Percent recovery of the proposed method was determined on the basis of standard addition method. The percent recovery as well as average percent recovery was calculated. Recovery should be assessed using minimum 9 determinations over minimum 3 concentrations level covering specified range. Recovery study was performed three different level 80 %, 100 % and 120 %. The precision is the measure of either the degree of reproducibility or repeatability of analytical method. It provides an indication of random error. Intra-day precision was determined by analysing, the three different concentrations 200 ng/ml, 300 ng/ml and 400 ng/ml for plasma samples analysis, for three times in the same day and Inter-day variability was assessed using above mentioned three concentrations of plasma samples were analysed by three different days, over a period of one week. Sensitivity refers to the smallest quantity that can be accurately measured. It also indicates the capacity of the method to measure small variations in concentration. Sensitivity of the proposed method were estimated in terms of Limit of Detection (LOD) and Limit of Quantitation (LOQ). For plasma sample analysis 100-400 ng/ml. The linear regression equation of the calibration curve was used to determine the LOD and LOQ. The stability of CRM in plasma samples was assessed under different storage conditions. Stability was expressed as the concentration ratio of analytes in sample under each storage condition against those in the freshly prepared sample. All stability assessments were assayed at three concentrations. Three samples were determined for short-term stability by putting them on the bench top at room temperature for 12 h and 24 h, respectively, prior to extraction. To evaluate freeze/thaw stability, three samples were subject to three freeze-thaw cycles with each cycle stepping from defrosting at room temperature to freezing at -20°C for 12 h. To determine the post-preparative stability, the extracted samples were stored in the sampler for 24 h. The long-term stability was performed by processing and analysing samples of plasma and brain kept at -20°C for 40 days[8-12].

RESULTS AND DISCUSSION

Method development

Mobile phase consisting of acetonitrile: water with 0.1% formic acid (40:60 v/v) was tried and drug was resolved properly. This method showed the best peak shape and ideal detection response. Furthermore, strong organic solvent in the reversed-phase chromatography can reduce static retention and shorten analysis time. Acetonitrile: water with 0.1% formic acid (40:60 v/v) mobile phase was optimized to give proper resolution of peaks (Figure No.2).

METHODS VALIDATION

Linearity

For plasma sample analysis linearity concentration in the range was100-400 ng/mL. The correlation coefficients (R^2) of CRM in plasma was found to be 0.9996.

Recovery, Precision and Sensitivity study

Recovery studies of plasma samples for the proposed method were carried out, respective data is obtained and mentioned in (Table No.1) Recovery study was determined at three levels 80 %, 100 %, 120 % at each level three determinations were performed. Intra-day and Inter-day precision of plasma sample analysis was reported in (Table No.1). The % RSD for CRM was less than 2.0 %. The results are showing that the proposed method was precise. Sensitivity of the proposed method were estimated in terms of Limit of Detection (LOD) and Limit of Quantitation (LOQ). The linear regression equation of the calibration curve was used to determine the LOD and LOQ. Limit of detection, limit of quantitation of plasma sample analysis were reported in (Table No.1) respectively[13-14].

Stability study

The results demonstrated that CRM were stable in plasma sample at room temperature for 12 h, in the sampling for 24 h and after three freeze-thaw cycles. All analytes were stable after stored at room temperature for 24 h. Even when stored in a long-

term freezer set at -20°C for 40 days, all analytes remained stable. Stability data for CRM shown in (Table No.2) and the results suggested that the tissue sample containing CRM can be stored under common laboratory conditions without any significant degradation of all analytes. Stability of CRM was investigated using different concentrations of QC plasma samples. Excellent recoveries of CRM were observed at different storage conditions and no significant loss of CRM in either plasma was observed.

Table No.1: Recovery, Precision and Sensitivity study

Recovery						
S.No	**Analysis**	**Drug**	**Initial amount (ng/ml)**	**Added Amount (ng/ml)**	**% Recovery**	**% RSD (n = 3)**
1	**Plasma**	CRM	200	188	99.57	0.66
2			200	200	96.19	0.25
3			200	202	100.37	0.16
Precision						
Analysis		**Drug**	**Con. (ng/ml)**	**Mean ± SD**	**% RSD (n = 3)**	**Mean ± SD**
4	**Plasma**	CRM	200	200.25 ± 0.27	0.11	200.22 ± 0.22
5			300	299.29 ± 0.74	0.16	292.74 ± 0.50
6			400	400.11 ± .056	0.35	391.11 ± 0.43
Sensitivity						
Analysis		**Drug**	**LOD**			**LOQ**
7	**Plasma**	CRM	52.66 ± 0.11			195.22 ± 0.28

Table No.2: Stability study (Plasma analysis)

S.No	**Nominal (ng/ml)**	**3 freeze-thaw cycles**	**short-term room temperature**		**post-preparative stability (24 h)**	**long-term room temperature (40 d)**
			(12 h)	**(24 h)**		
1	200	99.56 ± 1.41	98.16 ± 3.38	100.13 ± 5.19	102.13 ± 2.99	98.33 ± 6.63
2	300	100.08 ± 0.29	100.09 ± 1.36	100.52 ± 3.16	103.32 ± 0.35	103.86 ± 14.26
3	400	100.65 ± 1.63	99.56 ± 2.87	99.24 ± 6.62	100.54 ± 0.49	99.89 ± 8.58

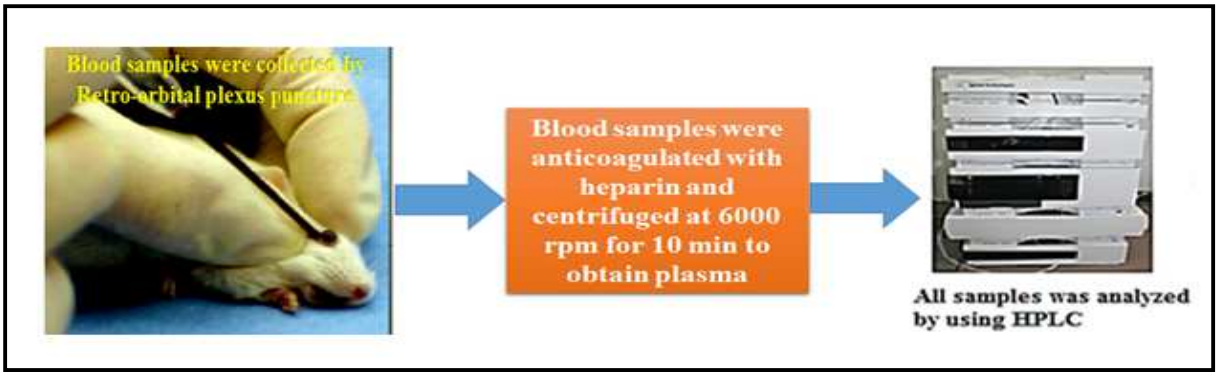

Figure No.1: Plasma sample collection and analysis steps

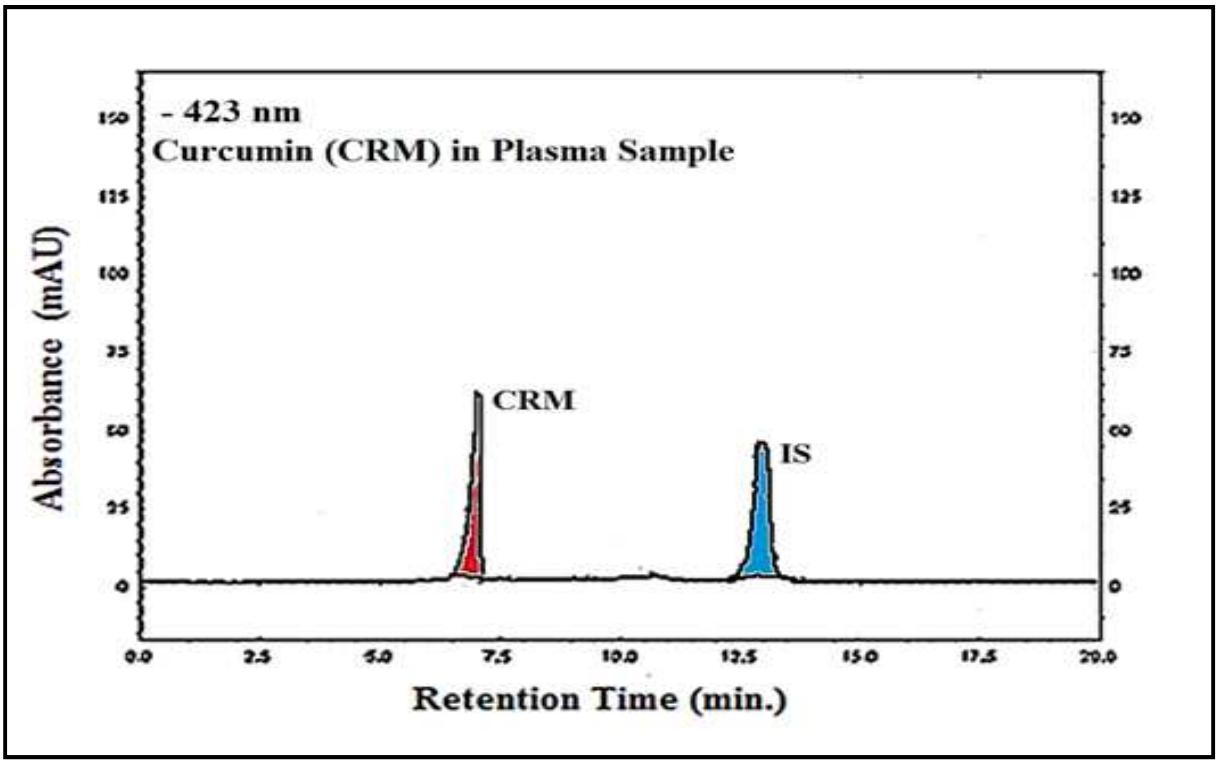

Figure No.2: Typical chromatogram of CRM in Plasma Samples

CONCLUSION

In this study, we developed and validated a highly sensitive and specific RP-HPLC method for the quantitative analysis of CRM in plasma samples. Validation of analytical method for estimation for CRM was determined by evaluating linearity, precision, recovery, sensitivity (LOD-LOQ) and stability (short and long-term stabilities, Freeze/thaw stability and post-preparative) in order to establish the suitability of analytical method. The method was validated in compliance with ICH guidelines is suitable for estimation of analytes with excellent recovery, precision, linearity and stability.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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