BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION BY QUALITY BY DESIGN APPROACH FOR CURCUMIN BENZATHIAZOLE ANALOG

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Abstract:

Objective: The goal of the ongoing effort is to create and validate a quick, sensitive, and easy-to-use RP-HPLC method for the quantification of the anticancer drug curcumin benzothiazole analogue in human plasma using the Quality by Design methodology. **Materials and method:** The investigation employed a Waters Alliance HPLC system with a PDA detector and a Phenomenax C18 column. By using 33 factorial designs, Box-Behnken Design rationally optimised the process. Retention time, theoretical plate area, and wavelength were chosen as dependent variables; the mobile phase, pH, and wavelength were selected as independent variables. **Results:** It was discovered that a detection wavelength of 235 nm had the highest response when the mobile phase was tuned using an isocratic mode in the ratio of 40:60 (acetonitrile and acetic acid in 0.2 filtered water; pH - 2.5). The protein precipitation technique was used to remove the substance from plasma. With a runtime of 10 minutes, the retention duration of curcumin analogues was discovered to be 2.7 min. The approach was validated using ICH Guidelines. The recovery was greater than 95% in all of the quality

control samples, the LOD and LOQ values were found to be 22 ng and 68 ng, respectively. To further prove the specificity of the developed method, common medications like paracetamol, cetricin, aceclofenac, pantaprazole, and metformin were spiked into blank plasma before being processed by the method. The established technique can be used to assess the compound's oral bioavailability in biological fluids. **Conclusion:** An accurate, precise and rapid method was developed for curcumin benzothiazole analogue. This can be further applied for formulation and in vivo studies.

Keywords: Bioanalytical method, Curcumin analogs, RP-HPLC, protein precipitation, QbD, Box Behnken design

Introduction

Curcumin is a miracle compound with a wide range of pharmacological activities. Chemically it appears in the form of keto-enol, which makes it unstable and quickly metabolized in the biological system with a half-life of less than 1 hour (1,2). Curcumin's potential as a health-improving agent has recently been the subject of extensive research. Dasatinib, resveratrol, and 5-fluorouracil were combined with curcumin to show increased cytotoxic action (3). Molecular fusion was one of the strategies to construct conjugated compounds (4). Hence, the modification of the keto-enol portion of curcumin with a heterocyclic ring formation increases its potency. Many researchers have worked on curcumin to overcome its disadvantages by modifying its structure (5,6). The benzothiazole curcumin (Fig. 1) derivative was designed and synthesized by Selvam et al., who then reported on its anticancer effect against head and neck cancer (7). When combined with hydrazinobenzothiazole, curcumin creates a potential anticancer agent. We previously explored these curcumin analogues' docking studies in the active sites of COX enzyme, COX inhibitory activity, antioxidant, and anti-inflammatory characteristics (8), and the benzothiazole analogue showed strong cancer cell suppression via STAT inhibition (9).

One of the curcumin analogs (hydrazine curcumin) was formulated by Satyavert et al., and *in-vivo* bioavailability studies were carried out in rat. A bioanalytical technique has been created to estimate a chemical in biological samples (10,11). Numerous curcumin analogues still need to be investigated in the biological matrix. To confirm the activity and mechanism of action, in vitro cell line investigations and molecular level studies were carried out. In vivo investigations should be the emphasis of the following stage of study.

The bioanalysis of the compound is an important step in the drug development. To evaluate the pharmacokinetic and pharmacodynamics of a drug candidate, developing a bioanalytical method is important (12–14). Selvam et al. successfully synthesized curcumin benzathiazole analogue and reported its anti-cancer properties (7,8). According to the literature, no HPLC method for benzathiazole curcumin analogue (Figure 1) in biological samples has been developed.

The quality by design (QbD) approach uses the Box Behnken design (BBD) using the principles of Design of Experiment (DOE) which is a tactical design to optimize the chromatographic conditions, so we can avoid trial and error process to save time (15,16). DoE, A QbD commences upon prioritization of high-risk and powerful input variables among the so many possibilities and defining an "optimal" analytical solution in the form of a design space. When compared to other designs, the BBD is the one that is most frequently employed to optimise a chromatographic process. For a more straightforward, speedier, and reliable way to estimate the formulation of omeprazole, Vayeda et al. have devised an HPLC method using DOE (17). By using the Quality by Design methodology, Sumant et al. developed and validated an RP-HPLC technique for ferulic acid quantification (18). Vishakha et al. used the QbD method to measure curcumin using the RP-HPLC method (19,20).

The goal of the current study is to design and validate an RP-HPLC bioanalytical method for the determination of benzathiazole curcumin analogue using the DOE approach that is straightforward, sensitive, affordable, robust, accurate, precise, and rugged. This approach will be useful to estimate the pharmacokinetic parameters of benzathiazole curcumin analogue, such as half-life and the longest time required to attain maximum concentration when the drug is administered orally.

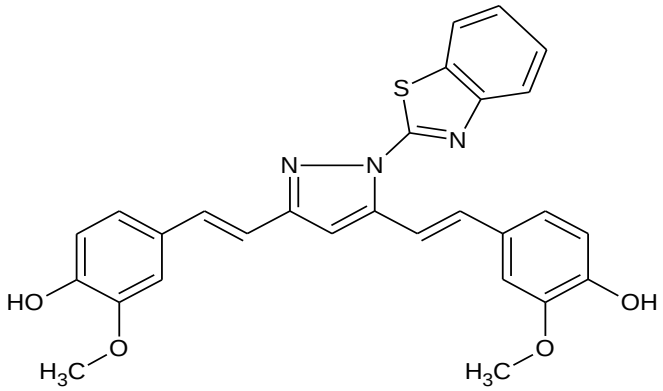

Figure 1: Curcumin benzathiazole analog

Materials and Methods

Chemicals and reagents Curcumin and 2-hydrazino benzothiazole were purchased from Himedia Laboratories and TCI chemicals, India. Acetic acid and acetonitrile were purchased from Merck in Mumbai, India, and were of HPLC-grade. Throughout the experiment, a Millipore device was used to collect Milli Q water. The usage of additional solvents, chemicals, and reagents was HPLC grade. The Karpagam Medical College Hospital Blood Centre in Coimbatore, Tamil Nadu, India donated human plasma.

Chromatographic instrumentation and conditions The Waters Alliance HPLC system (21 CFR Part 11 compliance software) with a PDA detector and a Phenomenax C18 column with a 250 mm x 4.6 mm particle size of 5 µm was used to develop the bioanalytical approach. Acetonitrile and 0.2% acetic acid are both present in the mobile phase, which has a flow rate of 1 ml/min and a detection wavelength of 235 nm.

Preparation of stock solution and working standards The compound was used to make the stock solution, which had a concentration of 1 mg/ml. As a working standard, different concentrations of the chemical (10 µg/ml to 200 µg/ml) were made from stock solution by serial dilution with acetonitrile.

Preparation of spiked solution Compound solutions at various concentrations were spiked into the plasma. To extract the chemical from plasma, 1000 µl of acetonitrile (a precipitating agent) was added to the drug samples that had been spiked before they were placed in Eppendorf tubes and vortexed for 15 minutes. For 10 minutes, the combined samples were centrifuged at 4000 rpm. A 0.2µ filter was used to filter the separated supernatant layer before it was fed into the HPLC system.

Quality by design-based method development The HPLC method was optimized by employing Design of Experiments (DoE) for studying the effect of selected process parameters and their responses by Box Behnken design using Design-Expert software version 13.0, (Stat-Ease Inc., USA). The best chromatographic procedure was determined using a straightforward 33 complete factorial design with 3 variables and 3 answers (Tables 1A & 1B), resulting in 17 experimental runs. The crucial values

required to get the desired response from the independent variables were identified and assessed using the ANOVA test (19). To develop a reasonable method, the various elements were employed to create a matrix of 17 separate trial runs (table 2).

Table 1A: List of independent variables in Box Behnken design for method optimization.

Factor	**Name**	**Units**	**Minimum**	**Maximum**
A	pH	-	2	3
B	Mobile Phase	%	40	80
C	Wavelength	nm	230	240

Table 1B: List of dependent variables in Box Behnken design for method optimization

Response	**Name**	**Unit**
R1	Peak area	-
R2	Theoretical plate	-
R3	Retention time	Min

Table 2: Design matrix by box Behnken design for HPLC method development

Run	**Factor A: pH**	**Factor B: Mobile Phase**	**Factor C: Wavelength**
1	3	80	235
2	2.5	80	230
3	3	60	240
4	2.5	40	240
5	2.5	60	235
6	2	60	230
7	2.5	40	230
8	2	60	240
9	2.5	80	240
10	2	40	235
11	2.5	60	235
12	2.5	60	235
13	3	40	235

14	2.5	60	235
15	2	80	235
16	2.5	60	235
17	3	60	230

Linearity & Range Utilizing eight different concentrations of spiked solution (1 to 20 μg/ml), the method's linearity was established. To reduce errors, the procedure was carried out three times. A graph was used to evaluate the linearity (area response Vs concentration).

Precision & Accuracy Precision and accuracy were assessed by determining the Low Quality Control (LQC), Middle Quality Control (MQC) and High Quality Control (HQC) in the concentration range of 20, 50 and 180 μg/ml respectively each in triplicates. To assess precision and accuracy, intraday (within a day) and interday (day to day) sampling were used.

Selectivity Six lots of compound-free blank plasma were gathered, processed using the protein precipitation method, and then injected into the HPLC apparatus to check for any plasma interference. Rejected lots included those that exceeded the Lower Limits of Quantification (LLOQ) (9.96 μg/ml) concentration by more than 20%.

Specificity Five over-the-counter medications were spiked in blank plasma and processed using the protein precipitation method, including paracetamol, cetricin, aceclofenac, pantaprazole, and metformin. We shall reject any attempts to tamper with our RT of interest..

Recovery Absolute recovery was performed by injecting an analytical standard solution and extracted standard from biological matrices by protein precipitation technique in all levels of quality control samples. The global recovery must be precise in all three levels.

Result and Discussion

Bioanalytical Method development and validation The development of bioanalytical methods was impacted by a number of variables, including stationary phase selection,

mobile phase optimization, optimum detection approaches, and detector type. As a result, the above parameters were optimised to start the bioanalytical process. Based on the physiochemical nature (pH, pka, and polarity) of the curcumin benzathiazole analog, it was found that the C_{18} column would be the most suitable for separation (Phenomenax C_{18} 250 mm x 4.6 mm i.d 5µm particle size).

Acetonitrile and 0.2% acetic acid in water served as the ideal mobile phase condition (60:40). The photodiode array was the best detector to employ for the detection since it covers the entire wavelength range of 190 to 800 nm with a single injection and has the highest absorption at 235 nm.

Mobile phase concentration and other conditions were optimised to achieve symmetrical peaks and good theoretical plates. The plasma interference was taken into consideration, and the RT obtained was about 2 min. The developed method was ideal for its application to pharmacokinetic studies.

Optimization of extraction procedure. The three most successful methods for extracting compounds from plasma were protein precipitation, solid phase extraction, and liquid-liquid extraction. Considering the polarity and affinity towards the protein, the plasma protein binding was assumed to be high. Thus, the protein precipitating method was chosen over the other two methods. As protein precipitating agents, acetonitrile, methanol, and zinc sulphide were tested; among which acetonitrile was preferred. The validation of bioanalytical methods in line with the Guidance for Industry bioanalytical method.

Optimization of mobile phase condition. Acetonitrile: Water in the ratios of 80:20, 60:40 and 40:60 were among the mobile phases which were optimized and shown in the figure 2. pH 2 by acetic acid in water, pH 2.5 by acetic acid in water, pH 3 by acetic acid in water were among the variables in pH range. Additionally, wavelengths 230 nm, 235 nm and 240 nm were also employed. As a result, chromatographic conditions in trials were used for validation.

. The mobile phase was optimized in isocratic mode at a flow rate of 1 ml/min, with the detection wavelength of 235 nm being determined to have the highest response (40:60 Acetonitrile and Acetic acid in 0.2µ filtered water; pH - 2.5). A perfect chromatogram was chosen, as seen in figure 3.

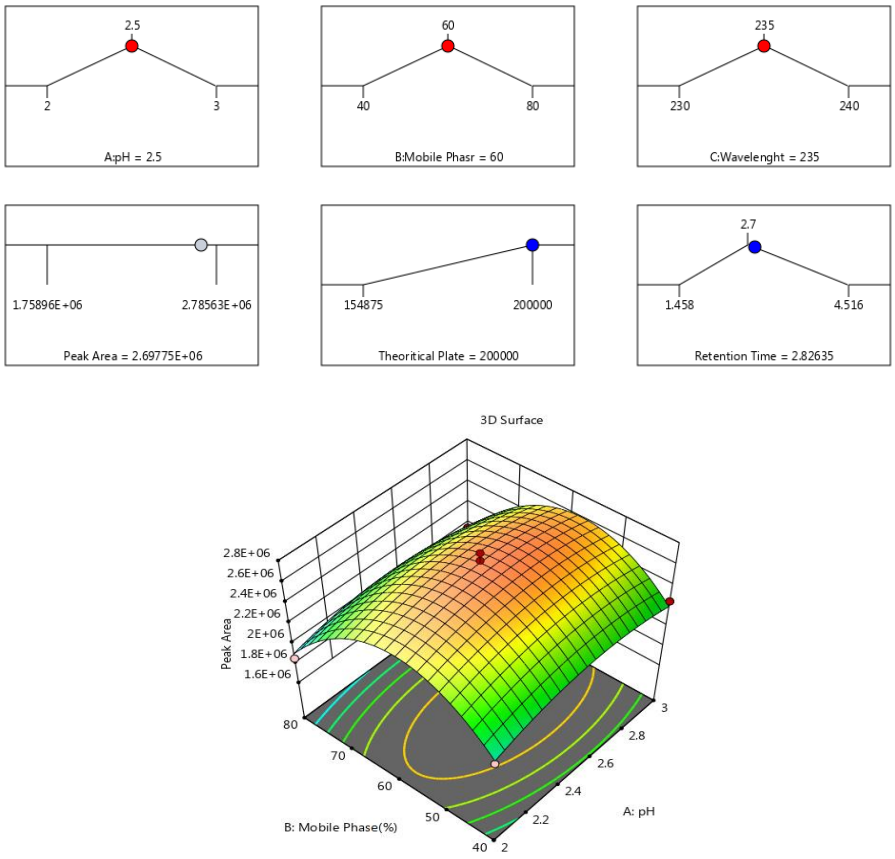

Figure 2: Optimization of conditions

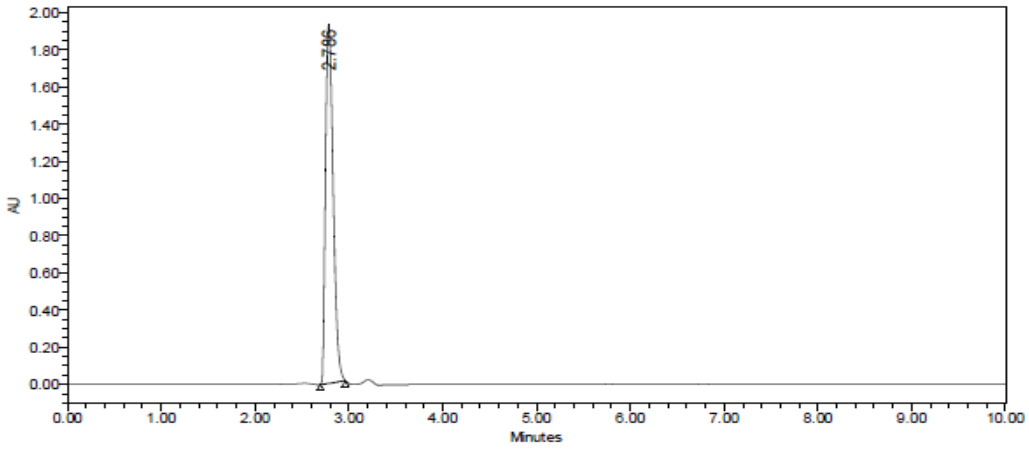

Figure 3: Ideal Chromatogram

Box Behnken Design for optimization of various parameters. The design summary and optimized method are presented in tables 3 and 4, respectively.

Table 3: Design summary for optimization

Study type	**Design type**	**Design model**	**Total runs**
Response surface	Box Behnken design	Quadratic	17

Table 4: Solution obtained for optimized method

Run	Factor A: pH	Factor B: Mobile Phase	Factor C: Wavelength	Response 1: Peak area	Response 2: Theoretical plate	Response 3: Retention time
16	2.5	60	235	2717546	20000	2.715

System suitability. Parameters for system suitability were set to evaluate the instrument's performance. Retention time average was 2.715 ± 0.001, percentage CV was 0.036 % and peak area average was 2670526 ± 44502 with a 1.666 % CV. The data showed that the technique complies with the system appropriateness requirements in terms of percent CV and standard deviation. Figures 4 and 5 depict the typical chromatogram of system appropriateness for the curcumin benzothiazole analogue.

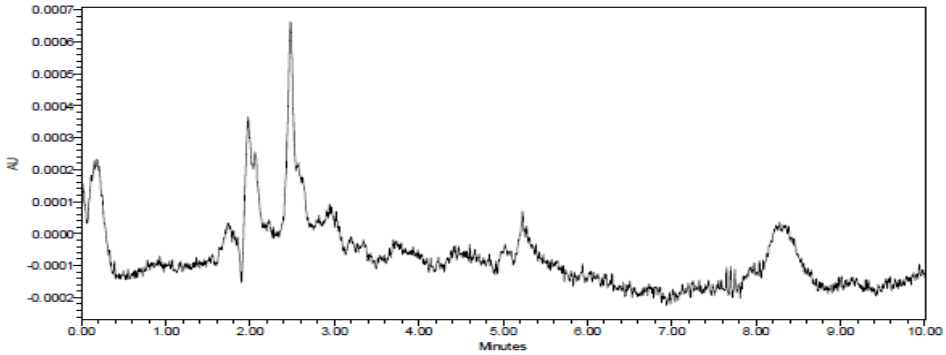

Figure 4: Blank Chromatogram

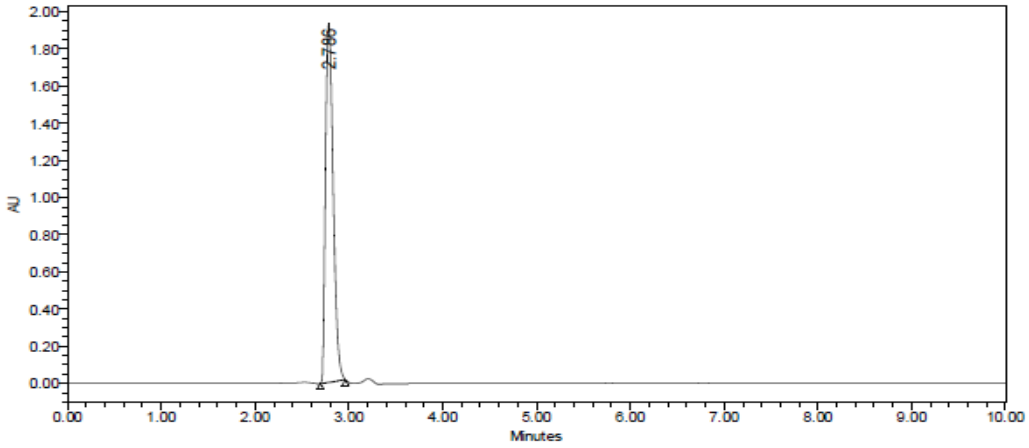

Figure 5: System Suitability chromatogram

Specificity and Selectivity. The protein precipitation method was used to analyse six blank plasmas. All lots displayed interference that was less than 20% of LLOQ. It implies that lots used for bioanalysis didn't have any interference and it was selective for curcumin benzothiazole analog. It suggests that the samples used for bioanalysis had no interference and were focused on the curcumin benzothiazole derivative. In order to further demonstrate the method's precision, popular medications like paracetamol, cetricin, aceclofenac, pantaprazole, and metformin were spiked into blank plasma before being processed by the protein precipitation method. The procedure was particular for the curcumin analogue, as indicated in table 6.

Table 5: Selectivity and Specificity

Sample	**RT**	**Area**	**% Interference**
Blank 1	2.714	622	0.23 %
Blank 2	2.718	480	0.184 %
Blank 3	2.712	221	0.0850 %
Blank 4	2.714	384	0.147 %
Blank 5	2.718	403	0.155 %
Blank 6	2.710	697	0.2682
LLOQ (9.9 µg/ml)	2.715	259848	--

Linearity & Range. To plot the calibration curve, varying concentration of working standard (9 to 200 µg/ ml) and the peak area data were used. Our analysis of the calibration curve revealed that the response was linear, with a regression value of 0.995. Table 6 contains the findings and regression analysis results related to the curcumin benzothiazole analogue, and Figure 6 displays the linearity graph.

Table 6: Linear regression analysis results for curcumin benzothiazole analog

Parameters	**Values**
Range (µg/ml)	9 - 200
Correlation coefficient (r^2)	0.9994
Slope	24928
Intercept	15712

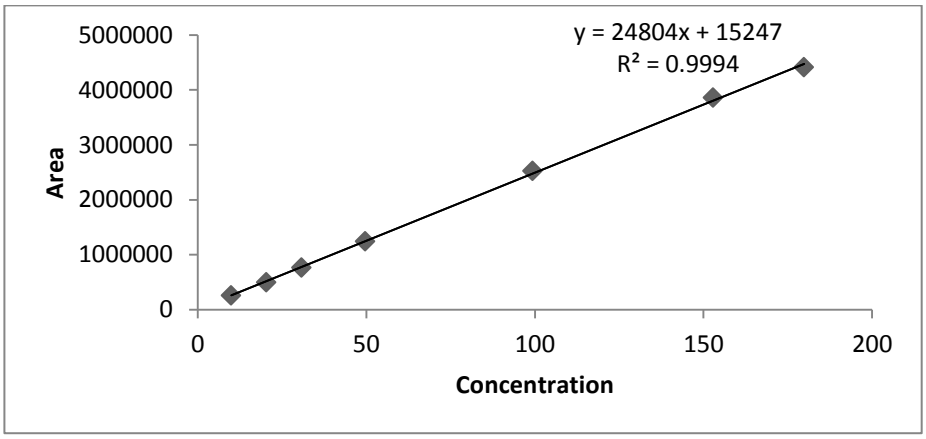

Figure 6: Graph representing peak-area versus concentration (µg/ ml)

Sensitivity. To forecast the method's sensitivity, the limit of detection (LOD) and limit of quantification (LOQ) were both assessed. The lower limit of detection was determined by 3.3 x (standard deviation of intercept/mean slope of linear curve) and lowest limit of quantification was determined by 10 x (standard deviation of intercept/mean slope of linear curve). The values for the LOD and LOQ were established to be 22 ng and 68 ng, respectively.

Precision & Accuracy. The accuracy of the developed method was assessed for LQC, MQC and HQC in the concentration range of 20, 50 and 180 µg/ml, respectively, for intra-day and inter-day changes. LQC, MQC, and HQC concentrations were each examined in triplicate over the course of two days. The computed percentage coefficient of variation (% CV) and standard deviation values demonstrate that the method's precision and accuracy were within acceptable bounds (table 7).

Table 7: Results of Accuracy & Precision

	Concentration	**Area**	**Accuracy (%)**	**Precision (CV %)**
(Intra day)				
LQC	479765	20.70	101.82	1.39
MQC	1256523	50.13	100.90	0.84
HQC	4700445	180.59	100.42	0.42
(Inter day)				
LQC	468456	20.27	99.71	1.35

MQC	1256983	50.14	100.93	0.64
HQC	4690309	180.21	100.21	0.59

Robustness & Ruggedness. Small variations in the chromatographic settings, such as flow rate, pH, and mobile phase composition, are not expected to have a significant impact on the method's robustness and ruggedness. After statistical analysis, it was determined that the results were significant.

Recovery. Three samples with varying concentrations (20, 60, and 140 g/ml) were made and spiked into plasma to study the compound recovery. A protein precipitating agent was applied, each sample was vortex-mixed, and then it was centrifuged. The supernatant was taken and injected to the HPLC. Areas of peak were identified after the method was repeated three times. The peak area of the pre- and post-extraction processes was compared, and the compound recovery was computed. Samples 20, 60, and 140 µ g/ml had mean extraction recoveries of 92.7 ± 5.6, 97.7 ± 5.8 and 95.6 ± 5.0%, respectively.

Conclusion

For the curcumin benzathiazole analogue, a straightforward, accurate, precise, quick, sensitive, and economical bioanalytical approach was designed and validated. Through the use of 3 x 3 variables and a quality by design methodology, the process was optimised. With a regression value of 0.995, our analysis of the calibration curve showed that the response was linear. For the easy recovery of the chemical from plasma, a protein precipitation approach was used. To further verify the specificity of the developed method, common medications like paracetamol, cetricin, aceclofenac, pantaprazole, and metformin were spiked into blank plasma. For the purpose of estimating pharmacokinetic data, the described approach can be utilised to quantitatively measure curcumin benzathiazole analogue in animals. Future formulations of the benzathiazole curcumin analogue could be made and analysed using this technique, and those formulations could then be employed in clinical trials.

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