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Research Article

A Highly Validated RP-HPLC Method for Pyrazoline Derivative Having Anti-inflammatory Activity

Sivagami. B[1]*, Chandrasekar. R[1], Selvamani.P [2], Ramesh.R[3], Sudakar.P [4]

[1,]* Department of Pharmaceutical Analysis, M.N.R. College of Pharmacy, Sangareddy, Medak Dist, 502294, Hyderabad, A.P. India.
[2]Anna University, Tiruchirapalli, Tamilnadu, India
[3]Syngene International Ltd., Biocon, Banglore, India
[4]Jaipur National University, Jaipur, Rajasthan, India

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ABSTRACT

A highly validated sensitive and specific reverse phase high performance liquid chromatography (RP-HPLC) method have been developed for the determination of synthesized product of pyrazoline derivative. The chromatographic separation was achieved using HPLC column Eclipse XDB C18 (150mm X 4.6mm X 5µm) column at isocratic mode. The mobile phase consists of 0.1% trifluoroacetic acid and methanol in the ratio of 20: 80. The flow rate and column temperature was maintained as 1.0 mL min^{-1} and 25 ± 2ºC respectively throughout the analysis. The injection volume was maintained as 5.0µL and the detection was carried out at 206 nm. The current method demonstrates good linearity over the range of 50-80 µg mL^{-1} and regression coefficient (r^2) was found to be 0.998. The limit of detection (LOD) and limit of quantification (LOQ) of the compound was found to be 4 µg mL^{-1} and 15µg mL^{-1} respectively. The method was validated in accordance with ICH guidelines which include accuracy, precision, specificity, linearity, ruggedness, robustness, stability and system suitability. In addition, the current method has been utilized for quantification and routine analysis of other pyrazoline derivatives.

Keywords: Pyrazoline derivative, Antimicrobial activity, RP-HPLC, Validation

1. INTRODUCTION

Pyrazoline is a new, upcoming molecule in the market, which posess anti-inflammatory, analgesic and antibacterial activity. Drugs which contain all three activities are not common and only very few RP-HPLC methods were developed for the pyrazoline compounds.

The purpose of the present study was to examine whether molecular modification might result in detection of new potential antirheumatic drugs having antimicrobial activities (1) Microbial infections often produce pain and inflammation. Chemotherapeutic, analgesic and anti-inflammatory drugs are prescribed simultaneously in normal practice. The compound possessing all three activities is not common. Pyrazoline is a dihydro form of pyrazole which is 5-membered ring containing adjacent nitrogen atoms. Several pyrazoline derivatives reported to possesses anti-inflammatory (2), anti-fertility (3), anti-implantation (4) and insecticidal (5) activity. Pyrazolines were also found as effective chemical bleaching agents, luminescent and fluorescent agents (6, 7).

Literature survey revealed that many pyrazoline derivatives have found their clinical application as NSAIDS. Antipyrine was the first pyrazolone derivative used in the management of pain and inflammation. Phenylbutazone and its potent metabolite oxyphenbutazone, a prototype of pyrazolinedione NSAIDs, are potent anti-inflammatory agents. However their use became restricted due to their GI side effects. Feprazone, the 4-

*Corresponding Author.
R. Chandrasekar, M. Pharm, Assistant Professor
Department of Pharmacognosy, MNR College of Pharmacy,
MNR Educational Trust, MNR Nagar, Fasalwadi, Sangareddy-502294
Medak Dist, Hyderabad, A.P.India
Tel.: +91-9705867855;
Email: rchand.siva@gmail.com

(methylbutenyl)-analogue is comparable to phenylbutazone in efficacy, but with less side effects on GI tract. Several related pyrazolidine-3, 5-diones, pyrazolin-3-ones and pyrazolin-5-ones are also available as NSAIDs; besides these many pyrazoline derivatives are also reported in literature as having potent anti-inflammatory activity. In view of these observations and in continuation of our research programme on the five membered hetero cyclic compounds, we report here some of the new pyrazoline derivatives, which have been found to possess an interesting profile of anti-inflammatory, analgesic, anti bacterial activity, with significant reduction in their ulcerogenic potential. Te chemical structure of the compound is shown in Fig.1.

But to the best of our knowledge, no HPLC method is available to determine the three critical components in a HPLC method which includes sample preparation, analysis and standardization of the compound. The solubility of the compound was determined with various solvents and was found to be polar, RP-HPLC method was used to develop and evaluate the method of performance in each component and adequate separation of all analytes.

The scope of the present work was to expand the optimization of the chromatographic conditions. The chromatographic separation was achieved using trouble-free isocratic mode using tri-fluoroacetic acid and methanol. A simple and single step procedure was followed for the sample preparation. The elution was made using C18 column with normal flow rate and temperature. With low amount of sample volume, the detection was done at simple UV/Visible detector. Finally, the target compound was eluted successfully with low runtime of 10 min.

Moreover, the current method has been validated for the compound in terms of accuracy, precision, specificity, linearity, ruggedness, robustness, solution stability and system suitability as per the recommendations of ICH guidelines. In addition, the proposed method was effectively applied for the routine analysis, quality control (QC) and in near future it will be applied in bioavailability and pharmacokinetic studies also.

The main objective of the present work is to develop a simple HPLC method for new compound. The scope of the present work is to expand the optimization of the chromatographic conditions, to develop RP-HPLC method for final compound and its intermediates. The developed method was also validated for maintaining specific and repeatability.

Different mobile phases were tried for selecting the ideal mobile phase. Among the various mobile phases (0.1% TFA in water: Methanol) with ratio 20:80 was found to be ideal as mobile phase, since it gave good resolution and well defined peak shapes with perfect optimization. The flow rate was found to be optimized at 1.0 ml/min. Detection was carried out by UV detector at 206 nm.

The linearity and range for the samples were established and it was found to be in the range of 50 to 150 μg/mL for the final compound. The correlation coefficient was found to be 0.9995, which indicates a perfect correlation. The developed method was validated for precision and system suitability. Similarly the RSD value for precision was also found to be within the acceptable limit.

2. MATERIALS AND METHOD

2.1. Chemicals

1- (2, 5-dihydroxyphenyl) ethanone, purchased from Sigma-Aldrich (Banglore, India). Solvents (AR grade) required for synthesis and extraction were purchased from Rankem Ltd. (New Delhi, India). Solvents (HPLC grade) used for chromatographic separation were obtained from Merck Ltd. (Mumbai, India).

2.2. Instrumentation

Agilent 1200 series HPLC system (Agilent Technologies, California, US) comprising of a dual piston reciprocating pump, DAD detector, an auto injector, and in-line degasser was employed. This separation was achieved using Eclipse XBD-C18 (Agilent Technologies, California, US). The data was acquired using ChemStation software (Agilent Technologies, California, US).

2.3. Liquid chromatography

The chromatographic separation was achieved using Eclipse XBD-C18 (250 mm × 4.6 mm, 5 μm) column at isocratic mode. The mobile phase consists of 0.1% trifluoroacetic acid and

methanol (20:80). The flow rate and column temperature was maintained as 1.0 mL min^{-1} and 25 ± 2ºC respectively throughout the analysis. The injection volume was maintained as 5.0 and the detection was carried out at 206 nm with DAD detector.

2.4. Sample preparation

This stage focuses on the selection of solvent and the sample preparation procedures. The use of mobile phase in the sample preparation is preferred which will ensure that there are no compatibility issues between the sample solution and the HPLC conditions. Sample solution was prepared by dissolving 60.1 mg of analyte in 100 mL of methanol to obtain solution (stock) having a known concentration. The stock solution was further diluted to get the calibration samples of 50, 80, 100, 120 1nd 150 µg $mL^{-1.}$ From this serial of calibration samples, the low, medium and high QC samples have been selected as 50, 100 and 150 µg $mL^{-1.}$ respectively for the method validation.

3. RESULTS AND DISCUSSION

3.1. Method development

The chromatographic separation was achieved with Eclipse XBD-C18 (250 mm × 4.6 mm, 5 µm) column using the mixture of water and methanol as a mobile phase. Based on the sample nature, different mobile phase composition was tried which gave broad peaks with poor resolution. Furthermore the sample elution time was more and peak symmetry was not good. To specify the LC conditions, different volume fractions were tested and the optimum conditions were obtained using 0.1% trifluoroacetic acid in Milli-Q water and methanol in the ratio of 20:80 v/v at the flow rate of 1.0 mL min^{-1} at the isocratic mode. Throughout the process, 5.0 µL was maintained as volume of injection and detection was performed at 206 nm with simple UV/Visible detector. The optimized LC condition resulted in less time consumption for sample preparation with no tedious extraction procedure and the retention time for the synthesized compound was achieved at 5.6 min. This condition was suitable for analysis of various intermediates giving sharp peak, peak symmetry, good resolution and run time shorter for the synthesized compound. A typical chromatogram of the synthesized drug product has been shown in Fig. 2.

3.2. Method validation

This purpose of method validation was to demonstrate the suitability for routine application of the developed methodology in accordance with the ICH guidelines. Method validation was treated as a final verification of the method performance. An important part is system suitability test which covers in the detail number of theoretical plates, peak asymmetry, resolution of individual components and repeatability of injection that was evaluated by retention time and peak area.

3.2.1. Accuracy and Precision

Accuracy was determined by calculating the percentage deviation observed from the analysis of QC samples and expressed as relative error (R.E). Injection repeatability was assessed using various levels of QC samples and the intra and inter- run precision was expressed as % RSD. The results were found to be in the range of 0.26.

3.2.2. Specificity

The specificity of the method was determined by comparing the chromatogram of diluents and known concentration of the synthesized compound with the diluents. As per the acceptance criteria, there is no interference at the retention time of synthesized compound due to the intermediate products or any other substance.

3.2.3. Linearity and Range

To evaluate the linearity of this proposed method, the standard stock solution was prepared and further diluted to get serial concentrations of 50, 80, 100, 120 and 150 µg mL^{-1} .The linearity of the method was determined by plotting the peak area versus the nominal concentration of the synthesized product. Results have shown that this method is linear over the range of 50-150 µg $mL^{-1}$with regression coefficient (r^2)0.995. Typical regression equation for the calibration plots was y = 64.011× + 174.27 and the calibration graph was shown in Fig. 3.

3.2.4. Limit of detection and quantification

The LOD and LOQ for the current method were performed on samples containing very low concentrations of analytes in accordance with the ICH guidelines. By applying the visual evaluation method, LOD was found to be 4 µg mL^{-1}, by establishing the minimum level at which the analyte can be reliably detected. LOQ was found to be 15µg mL^{-1} and considered as the lowest concentration of analyte in standards that can be reproducibly measured with acceptable accuracy and precision.

3.2.5. Solution stability

For the assessment of solution stability, the low and high QC samples were used. The obtained results of refrigerated and fresh samples were compared and it has been expressed in terms of % RSD. The % RSD was found to be less than 2.0%. There by indicating that the solution was sufficiently stable with varying conditions.

3.2.6. Robustness and Ruggedness

To ensure the insensitivity of the developed HPLC method, deliberate changes were made in method parameters like mobile phase composition, flow rate, column temperature and different columns. No significant changes were found in the obtained results (Table 2) and it shows the reliability of the compound by the peak area responses with % RSD not more than 2.0. Same like, the percentage recovery was calculated with sample solution with different days, different instruments and different analysts. As shown in the data in Table 2 excellent recoveries were made on various above mentioned conditions. The % RSD for the peak area responses were less than 2.0 which showed the ruggedness of the current method.

3.2.7. System suitability

Every day before the commencement of the sample analysis, freshly prepared system suitability samples were injected in the HPLC system and the response recorded. From the obtained results, the column parameters like retention time, capacity factor, theoretical plates, and asymmetry factor were calculated and compared with the previous day results. No significant changes were found with the everyday system suitability results. The obtained results were shown in the table 3.

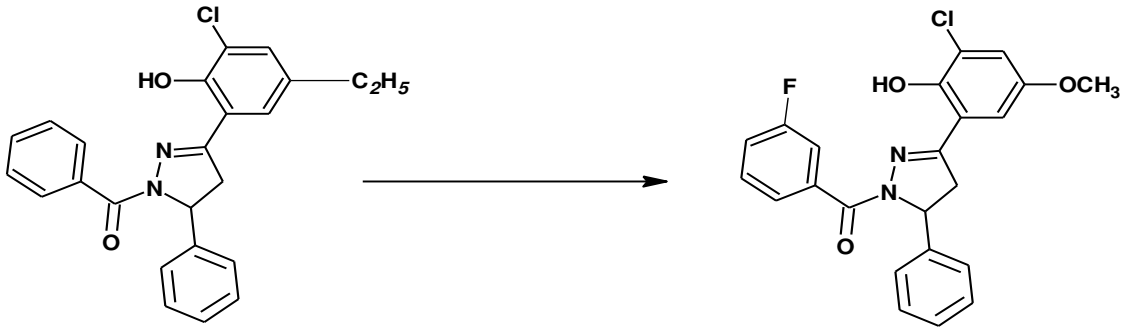

Fig. 1: Chemical structure of 2-(1-benzoyl-5-phenyl-4,5-dihydro-1h-pyrazol-3-yl)-6-chloro-4-methoxyphenol derivatives

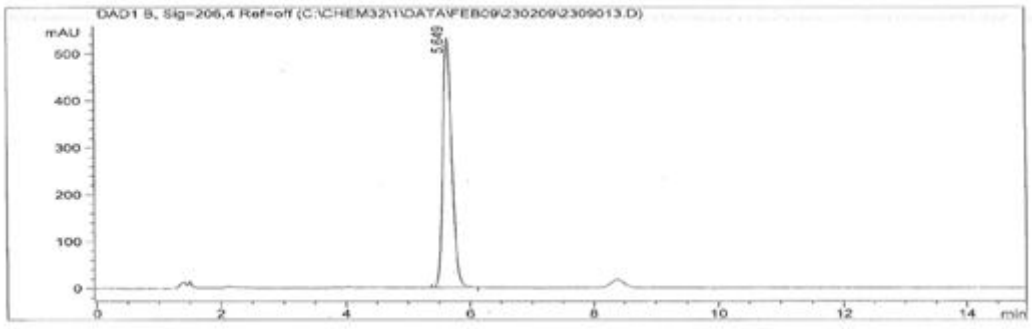

Fig. 2: A representative chromatogram of synthesized compound

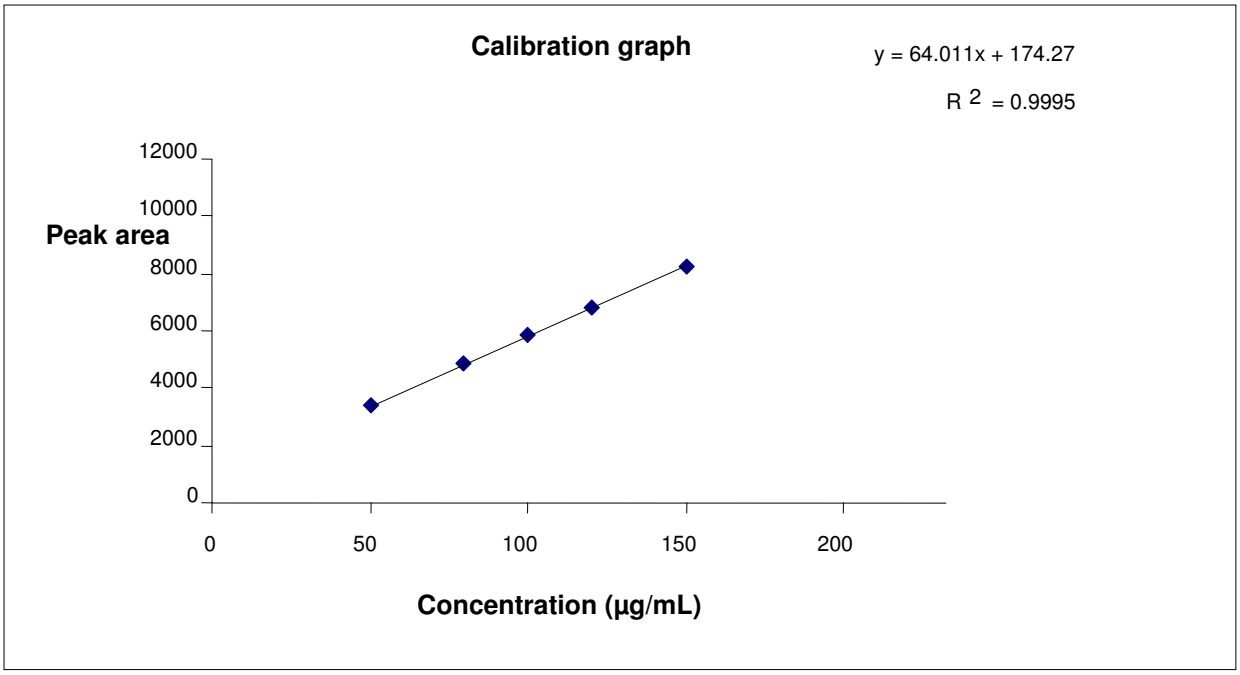

Fig. 3: A typical linearity graph for the synthesized compound

Table 1: Robustness study of synthesized compound with variations in chromatographic parameters

Chromatrographic Paremeter	Mobile Composition			Flow rate (ml/min)		
	80:20	75:25	85:15	0.9	1.0	1.1
No. of injections	5	5	5	5	5	5
Average (Area)	5352.208	7228.416	5881.146	7172.2	5358.6	5892.3
S.D	85.28	86.29	13.56	8.96	15.29	3.66
% R.S.D	1.59	1.19	0.23	0.12	0.29	0.06

Table 2: Ruggedness study on synthesized compound with various conditions

Various Conditions	Day		Instrument		Analyst	
	1	2	1	2	1	2
No. of injections	5	5	5	5	5	5
Average (Area)	6311.28	6310.88	6311.28	6178.84	6311.28	8183.90
S.D	3.43	4.37	3.43	4.63	3.43	15.17
% R.S.D	0.054	0.069	0.054	0.76	0.054	0.19

Table 3: Chromatographic parameters for system suitability of synthesized compound

System suitability parameters for synthesized compound	Value
Retention Time	5.6
Peak area (% RSD)	0.26
Retention / Capacity factor (K)	3.946
Pear purity	999.795
Theoretical plate counts	7438
Tailing / symmetry factor	0.97

Significance of the work

Based on the literature, no compound is synthesized based on the nucleus of Pyrazoline derivative. The synthesis of the compound 2-(1-benzoyl-5-phenyl-4,5-dihydro-1h-pyrazol-3-yl)-6-chloro-4-methoxyphenol derivatives paves a way for the other pyrazoline derivatives. Further, this would lead to the synthesis of other cost effective novel lead molecules.

The proposed RP-HPLC method can be efficiently utilized for the quantification and routine analysis, quality control and in near future can be applied in bioavailability and pharmacokinetic studies also.

4. CONCLUSION

From the above experimental data results and parameters it was concluded that the developed RP-HPLC method has the following advantages: The sample preparation requires less time, no tedious extraction procedure was involved in the analytical process, suitable for the analysis of intermediates and run time required for recording chromatograms were less than 20 minutes. Hence, the chromatographic method developed of final compound was found to be simple, precise and cost effective and it can be effectively applied for routine analysis in research institutions, quality control department in industries, approved testing laboratories, bio-pharmaceutics and bio-equivalence studies and in clinical pharmacokinetic studies in near future. From the current work it was finally concluded that the developed RP-HPLC method is found to very simple, reliable and selective providing satisfactory precision. The methods are suitable for routine quantitative analysis.

5. ACKNOWLEDGEMENTS

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