RESEARCH ARTICLE

HPLC Analytical Research Method Development and Validation of Inosine Pranobex by using the QbD Approach.

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ABSTRACT

The study highlights the development of a high-performance liquid chromatographic (HPLC) method for estimating Inosine Pranobex using a Quality by Design (QbD) approach. This risk-based method development emphasizes risk assessment and management to achieve robustness. A 2-factorial experimental design, utilizing a central composite design, was used to optimize the mobile phase and flow rate, aided by Design Expert software. Optimal conditions included a C18 column (4.6 x 100 mm), a mobile phase of methanol to 0.1% OPA in a 60:40 ratio, and a flow rate of 1 ml/min, resulting in a retention time of 6.1 minutes. The method showed linearity (r2 = 0.999) in the 10–50 µg/ml range at a detection wavelength of 259 nm. Intraday and interday precision %RSD values were between 0.40–1.5 and 0.0–0.61, respectively. Robustness values were under 2%, and the assay result was 99.97 ± 0.03%. The chromatographic peak purity analysis confirmed the absence of co-eluting peaks with Inosine Pranobex. The method validation complied with ICH guidelines, demonstrating a successful HPLC method development with an improved understanding of performance-related variables.

Keywords: Inosine Pranobex, Inosine, HPLC, QbD

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INTRODUCTION

Inosine Pranobex, a compound consisting of Inosine, Acetamidobenzoic acid, and Dimethylaminoisopropanol, serves as both an antiviral agent and an immune system booster, functioning similarly to thymic hormones. It has demonstrated efficacy against viruses like HSV and HPV.[1,2] This medication has been studied for various viral-related conditions, including rheumatoid arthritis, multiple sclerosis, and alopecia. Themis Medicare launched it in 2019 under the brand name "Viralex" for treating COVID-19 after receiving governmental approval. While clinical trials have shown only moderate or limited therapeutic effects, the drug is generally well-tolerated, with no significant adverse effects or drug interactions reported.

Quality by design (QbD) concept

This methodology focuses on gaining a deep understanding of the product and process, along with effective risk management and control, to ensure superior product quality, regulatory flexibility, and continuous improvement.[5,6]

Literature review and current work

This study aims to develop and optimize the HPLC method for Inosine Pranobex in pharmaceutical dosage forms using the QbD approach.

MATERIALS

Inosine Pranobex was obtained from Yarrow Pharmaceutical Pvt. Ltd., Mumbai. All other reagents and chemicals used were of analytical grade, and the solvents were of HPLC grade. The marketed formulation VIRALEX-500 Tablets by Themis Medicare Ltd, Mumbai, was used for the assay.

Instruments and chromatographic conditions

The HPLC system included a G1310A ISOPUMP with a maximum pressure capacity of 400 bar, capable of using four mobile phases with a discharge rate of 0.001 ml – 5 ml. The C-18 column (100 mm × 4.6 mm, 2.5 µm particle size) was equilibrated with a mobile phase of methanol to OPA (60:40, v/v).[7] The flow rate was set at 1 ml/min, and the column was maintained at ambient temperature. Detection was carried out using a PDA detector at 259.0 nm, achieving satisfactory separation and peak symmetry for the drug under these chromatographic conditions.[7-9]

Preparation of reference standard solution

A 1000 µg/ml standard stock solution was prepared by dissolving 10 mg of Inosine Pranobex in 10 ml methanol. This stock solution was further diluted to a sub-stock of 100 µg/ml. A 10 µg/ml solution was prepared by diluting 0.1 ml of the sub-stock solution to 10 ml with methanol.

Selection of detection wavelength

A 10 µg/ml solution of Inosine Pranobex was scanned between 200–400 nm, and the wavelength maxima at 259 nm were selected as the detection wavelength.

Evaluation of experimental results and final method selection

The central composite design (CCD) approach was used to evaluate the method conditions. Initially, retention time and peak area conditions were assessed, leading to distinct chromatographic conditions for Inosine Pranobex. Acceptable ranges were identified from robust regions

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Table 1: Statistical Data Analysis (DOE) Two-Factorial Design [2^3]

Std	Run	Factor 1 A: Flow rate (ml/min)	Factor 2 B: Methanol (%)	Response 1 RT (min)	Response 2 Peak Area
8	1	1	65	6.1	3758.04
1	2	0.9	55	11.12	4272.7
3	3	1.1	55	9.2	3229.32
5	4	1	60	7.8	3742.55
6	5	1.1	60	7.1	3341.53
4	6	0.9	60	8.7	4278.26
7	7	0.9	65	6.9	4285.84
2	8	1	55	10.2	3742.55
9	9	1.1	65	5.6	3337.77

where deliberate variations in method parameters did not affect quality, ensuring method reliability during downstream validation testing. The best chromatographic conditions were optimized using Design Expert tools.[6,7]

Risk assessment

The optimized final method was selected based on its attributes, ensuring it remains effective throughout the product's lifecycle. A risk-based approach, in line with QbD principles outlined in the ICH Q8 and ICH Q9 guidelines, was used to evaluate the method's robustness and ruggedness.[6,7]

Control strategy implementation

After developing the method, a control strategy was implemented. An analytical target profile guided the development of the analytical control strategy, which is a planned set of controls derived from understanding various parameters, such as fitness for purpose, the analytical procedure, and risk management.[6,7]

Analytical method validation

Method validation provides documented evidence that a specific method is reliable and suitable for its intended use. The HPLC method developed for estimating Inosine Pranobex was validated according to ICH Q2 (R1) guidelines.

RESULTS

HPLC method development via QbD approach

Selection of Quality Target Product Profile (QTPP): Retention time and peak area were identified as QTPP for the proposed HPLC method.[6,7] Determination of Critical Quality Attributes (CQAs): The mobile phase composition of Methanol to OPA at a 60:40 ratio and a flow rate of 1 ml/min were determined as critical factors.[6,7]

Factorial Design: After defining the QTPP and CQAs, a central composite experimental design was used to optimize and select 2 key factors: mobile phase composition and flow rate.[6,7] A 2-factor design involving the mobile phase

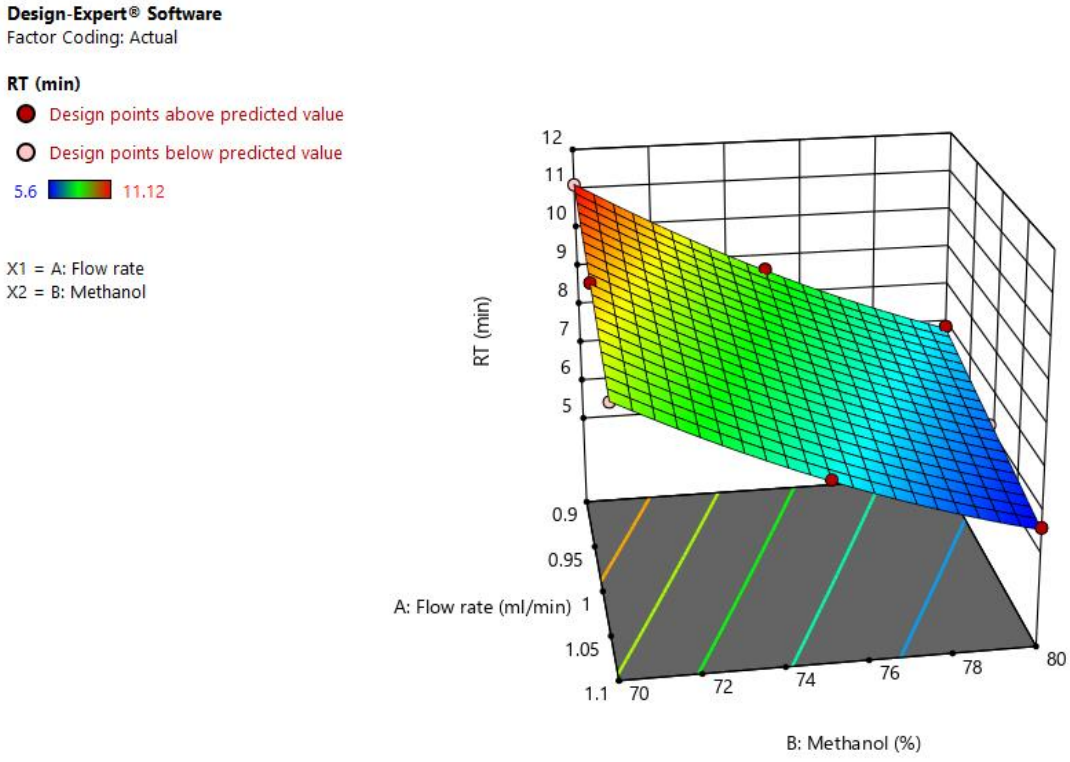

Figure 1: 3D graphical representation: DOE of Retention Time of Inosine Pranobex against mobile phase and flow rate

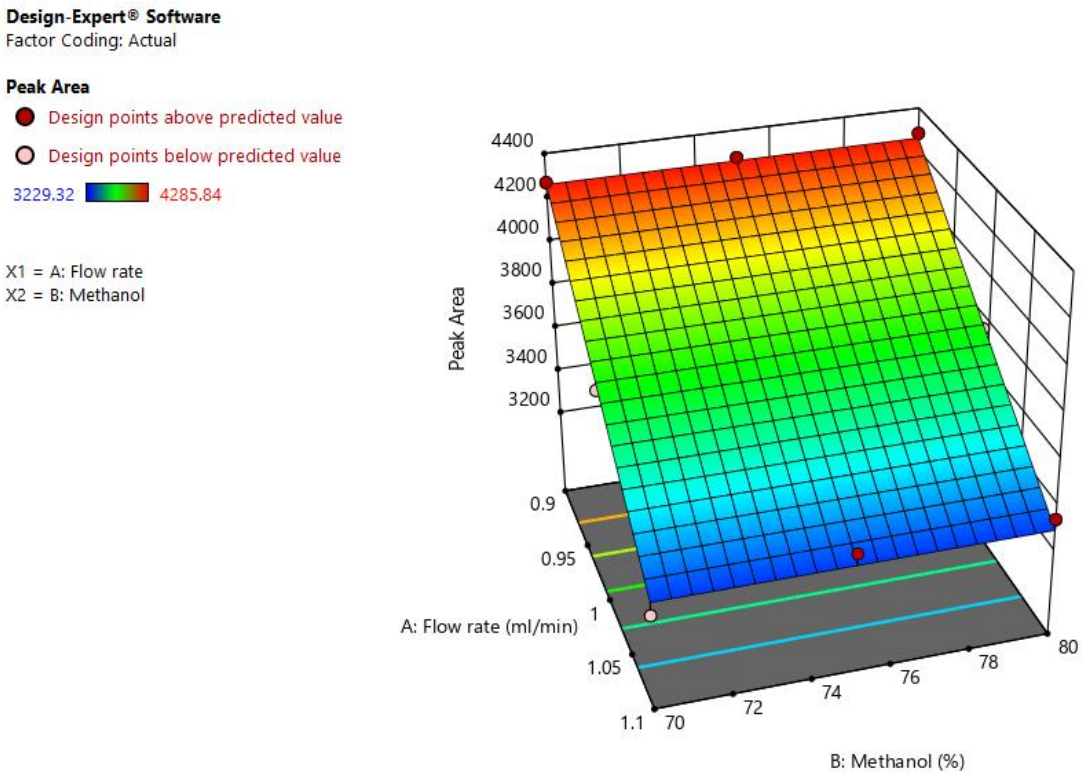

Figure 2: 3D graphical representation: DOE of Peak Area of Inosine Pranobex against mobile phase and flow rate

composition and flow rate at 3 different levels was implemented using Design Expert (Version 11.0).

The optimisation of various parameters is shown in Table 1 below:

A response surface methodology involving a central composite design and a quadratic design model with 9 runs was employed. The proposed central composite design (CCD) was utilized to evaluate the effects of flow rate and methanol composition on two responses: retention time and peak area. The results were summarized based on this experimental setup**.**

Retention time (for actual values) = +7.87-0.803A-1.99B+0.1550AB+0.3200B2

The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels are coded as -1. The coded equation

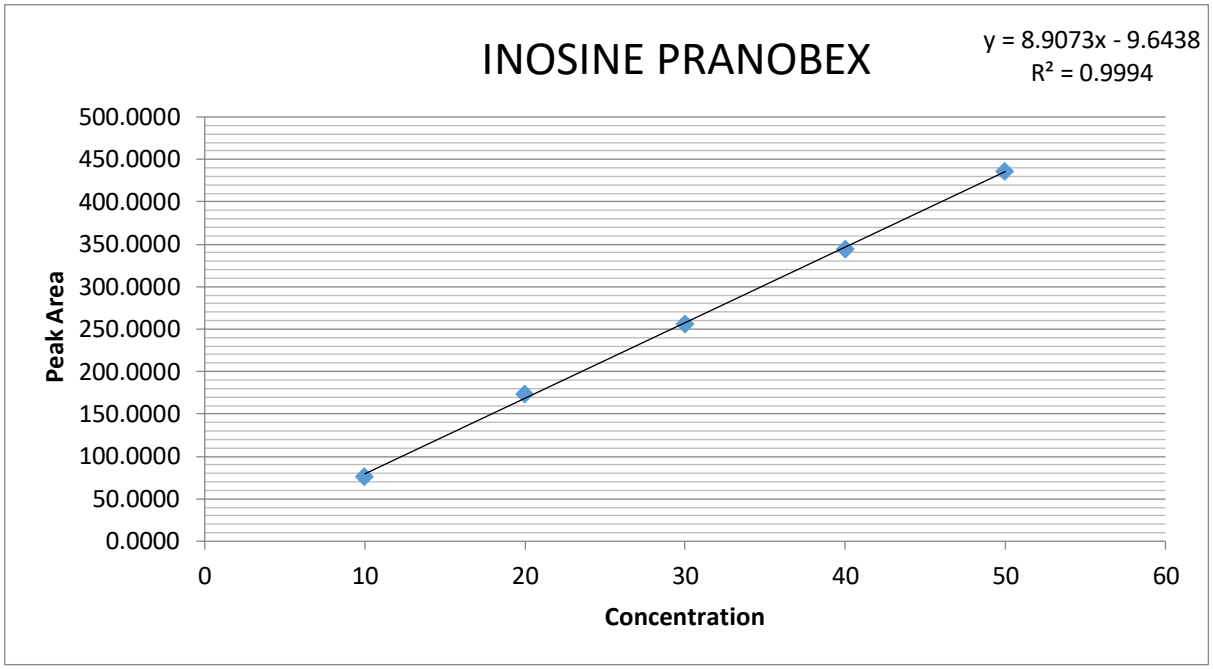

Figure 3: Linearity Curve

Table 2: Observation Table of Linearity (* - Avg. of 3 readings)

Conc. µg/ml	Average peak area (*)	(+/-) S.D. of Peak Area	% RSD of Peak Area
10	76.7401	0.72	0.94
20	173.9219	0.79	0.46
30	255.712	0.97	0.38
40	344.881	0.65	0.19
50	435.6277	0.08	0.02
Equation	y = 8.907x (-9.643)		
R^2	0.9994		

is useful for identifying the relative impact of the factors by comparing the factor coefficients.

PA= +3776.51-488.03A

The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels are coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.

Method validation

Linearity

The calibration curve constructed for Inosine Pranobex demonstrated linearity within the concentration range of 10–50 µg/ml. The regression equation for the calibration curve was determined to be y = 8.9073x – 9.6438, with a correlation coefficient of 0.994, indicating a strong linear relationship between peak area and concentration.[5,6]

Precision

The % RSD for repeatability of Inosine Pranobex, based on 6 measurements of a 100 µg/ml concentration, was less than 0.082. Interday and intraday precisions are detailed in Table 5. With % RSD values below 2, the developed method demonstrated a high degree of precision.

Accuracy

20 tablets were weighed and ground into a fine powder. An amount equivalent to 100 mg of Inosine Pranobex was transferred to a 100 ml volumetric flask, and 10 ml of methanol was added. The mixture was sonicated for 15 minutes to ensure complete dissolution, then diluted to the mark with the mobile phase. The solution was filtered through 0.42 µm Whatman filter paper. From the filtrate, 0.5 ml was taken and diluted to 10 ml to obtain a concentration of 100 µg/ml. This solution was analyzed by HPLC under the same chromatographic conditions used for assessing linearity. The mean value of 3 different assays was calculated.Accuracy was evaluated through a recovery study. Sample solutions were prepared by spiking at 3 levels: 80%, 100%, and 120%. The % recovery data obtained using the proposed HPLC method are presented in Table 6. Recoveries within the range of 98–102% demonstrate that the developed method is accurate, under ICH Q2 (R1) guidelines.

Robustness and ruggedness studies

Robustness and ruggedness were evaluated using a 100 µg/ml solution of Inosine Pranobex. Robustness was tested by making slight but deliberate changes to method parameters such as mobile phase composition, flow rate, and detection wavelength. The %RSD for peak area was found to be less than 2% under these varied conditions, indicating the method's robustness.

LOD and LOQ

The detection limit (LOD) and quantification limit (LOQ) were determined using the following equations, as per ICH guidelines:

$LOD=3.3\times\sigma/SD$

$LOQ=10\times\sigma/SD$

where σ denotes the standard deviation of the y-intercept of the regression line, and SD denotes the slope of the calibration curve.

The limits of detection (LOD) and quantification (LOQ) for Inosine Pranobex were found to be 0.23 µg/ml, and the LOQ was 0.71 µg/ml.

Assay

The optimized chromatographic method for Inosine Pranobex showed a resolved peak at a retention time of 4.83 minutes during the assay of tablet formulations. The percentage assay of the drug content was 99.97 ± 0.03 for the label claim of Inosine Pranobex. This result indicates that the method can accurately and specifically measure the drug in the presence of excipients in the tablet powder.

CONCLUSION

The principles of analytical Quality by Design (QbD) were applied to the development of the HPLC method for Inosine Pranobex. To identify the optimal system and establish the final design space, a multi-variable analysis of key process parameters, such as flow rate and mobile phase composition, was conducted. These parameters were evaluated and optimized at various levels using a central composite design.

This approach facilitated a deeper understanding of the factors affecting chromatographic separation and the method's capability to achieve its intended purpose. The insights gained contribute to the future development of chromatographic optimization.All validated parameters met the acceptance criteria. The method was confirmed to be linear, precise, accurate, specific, robust, and rugged for determining Inosine Pranobex. The QbD approach enhanced the understanding of method variables, minimizing the likelihood of failure during method validation and transfer.

Using the automated QbD method development via Design Expert software resulted in a more efficient and robust

Table 3: Observation Table of Inter-day and Intra-day precision

Con (µg/ml)	Intraday Precision		Inter-day Precision	
	Mean± SD	%Amount Found	Mean± SD	%Amount Found
20	169.25±2.17	100.42	170.20± 1.03	100.96
30	256.12±1.18	99.46	255.05±0.13	99.06
40	343.81±1.07	99.21	348.21±0.0	100.44

Table 4: Observation table of Accuracy study (% Recovery data)

Level (%)	Tab. Conc. taken (µg/ml)	Std. Conc. Taken (µg/ml)	Area Mean* (±) S.D.	Amt. recovered Mean *(±) S.D.	%Recovery Mean*(±) S.D.
80%	10	08	18.12(±) 0.06	8.12(±) 0.06	101.45(±) 0.82
100%	10	10	20.07(±) 0.50	10.07(±) 0.050	100.75(±) 0.50
120%	10	12	21.95(±) 0.013	11.95(±) 0.013	99.59(±)0.10

method in less time than manual development. Statistical analysis indicated that the method is reproducible, selective, accurate, and robust. This method will be implemented for routine quality control analysis in the pharmaceutical industry.

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