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Research Article

Analytical Method Development and Validation of Warfarin by Quantitative Estimation Method in Bulk and Dosage Form

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ABSTRACT

The present study was undertaken to develop and validate a simple, reliable, accurate, precise, rapid, and cost-effective Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) method for the quantitative determination of Warfarin in bulk drug and tablet dosage forms. Warfarin is a widely prescribed oral anticoagulant used for the prevention and management of thromboembolic disorders. Owing to its narrow therapeutic index, the development of a sensitive and reliable analytical method is essential to ensure the quality, safety, and therapeutic effectiveness of the drug. Chromatographic separation was achieved using a Cosmosil C18 column (250 × 4.6 mm, 5 µm particle size) with a mobile phase comprising methanol and potassium phosphate buffer (80:20, v/v). Detection was performed using a UV detector at 275 nm, producing well-resolved and symmetrical chromatographic peaks with an acceptable retention time. The developed analytical method was validated in accordance with the International Council for Harmonisation (ICH) guidelines by evaluating parameters such as linearity, accuracy, precision, specificity, robustness, limit of detection (LOD), and limit of quantification (LOQ). The method demonstrated excellent linearity over the concentration range of 5–25 µg/mL, with a high correlation coefficient indicating a strong linear relationship between concentration and peak area. Precision studies showed low %RSD values, confirming the repeatability and reproducibility of the method. Accuracy was established through recovery studies, which yielded satisfactory recoveries within the prescribed acceptance limits. Furthermore, the method proved to be specific and robust, showing no interference from formulation excipients or minor variations in chromatographic conditions. The validated RP-HPLC method was successfully applied to the quantitative estimation of Warfarin in commercially available tablet formulations. The assay results complied with the labeled claim, demonstrating the suitability of the developed method for routine quality control and assay determination. Therefore, the proposed RP-HPLC method can be recommended for regular pharmaceutical analysis of Warfarin in quality control laboratories and

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research settings due to its simplicity, reliability, accuracy, and reproducibility.

INTRODUCTION

Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) is one of the most reliable and widely employed analytical techniques for the qualitative and quantitative determination of pharmaceutical compounds. In RP-HPLC, separation is achieved using a non-polar stationary phase, typically a C18 column, and a relatively polar mobile phase consisting of water or buffer mixed with organic solvents such as methanol or acetonitrile. The technique offers excellent sensitivity, specificity, precision, accuracy, and reproducibility, making it the preferred method for pharmaceutical analysis. RP-HPLC is extensively used for assay determination, impurity profiling, dissolution testing, stability studies, and method validation in accordance with ICH guidelines, ensuring the quality and consistency of drug productsWarfarin is a widely prescribed oral anticoagulant belonging to the coumarin class of drugs. It acts by inhibiting the vitamin K epoxide reductase complex (VKORC1), thereby reducing the synthesis of vitamin K-dependent clotting factors II, VII, IX, and X. Warfarin is extensively used for the prevention and treatment of deep vein thrombosis, pulmonary embolism, atrial fibrillation-associated stroke, and thromboembolic complications in patients with prosthetic heart valves. Due to its narrow therapeutic index and significant inter-patient variability, accurate quantification of warfarin in bulk drug substances and pharmaceutical dosage forms is essential to ensure product quality, efficacy, and patient safety.

MATERIALS AND INSTRUMENTS

Materials

Warfarin was obtained as a gift sample from Swapnroop Agency, Aurangabad, and was used as the reference standard for the development and validation of the analytical method. All chemicals and reagents employed during the study were of analytical reagent (AR) or HPLC grade to ensure high purity and reproducibility of the analytical results. HPLC-grade methanol, acetonitrile, ethanol, and acetone were procured from Merck Pvt. Ltd., Mumbai. HPLC-grade water was obtained from J.K. Labs, Thane, while potassium phosphate and orthophosphoric acid were purchased from Ozone International, Mumbai and MolyChem Pvt. Ltd., Mumbai, respectively. All solvents were filtered through a 0.45 µm membrane filter and degassed by sonication before use.

Instruments

The chromatographic analysis was performed using an HPLC 3000 Series Binary Gradient System (Analytical Technologies Ltd.) equipped with a UV-3000-M UV detector and a P-3000-M reciprocating pump capable of operating up to 40 MPa. Separation was achieved using a Cosmosil C18 column (250 × 4.6 mm i.d., 5 µm particle size), and data acquisition and processing were carried out using the HPLC Workstation software. Sample weighing was performed using a Wenser High Precision Balance (Model: PGB100) with a maximum capacity of 100 g and a readability of 0.001 g. Degassing of the mobile phase and sample solutions was carried out using a Wenser Ultra Sonicator (Model: WUC-4L) with a 4 L capacity to ensure the removal of dissolved gases and to obtain consistent chromatographic performance.

Evaluation of Analytical Method Validation as per ICH Guidelines

The developed RP-HPLC method for the quantitative estimation of Warfarin was validated

according to ICH guidelines with respect to linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), range, selectivity, robustness, and ruggedness.

Linearity:

Linearity was established by preparing standard solutions of Warfarin in the concentration range of 5–25 µg/mL**.** The peak area was plotted against concentration, and a calibration curve was constructed to demonstrate a direct proportional relationship.

Precision:

Precision was evaluated by analyzing replicate samples under identical conditions. Repeatability, intra-day precision, and inter-day precision were determined, and the results were expressed as %RSD, indicating good reproducibility of the method.

Accuracy:

Accuracy was determined by the standard addition (recovery) method at 80%, 100%, and 120% concentration levels. The percentage recovery confirmed the accuracy and reliability of the developed method.

Limit of Detection (LOD):

LOD represents the lowest concentration of Warfarin that can be detected but not necessarily quantified. It was calculated using the formula LOD = 3.3σ/**S**, where σ is the standard deviation of the response and S is the slope of the calibration curve. The LOD for Warfarin was found to be **0**.02534 µg/mL.

Limit of Quantification (LOQ):

LOQ is the lowest concentration of Warfarin that can be quantified with acceptable precision and accuracy. It was calculated using the formula LOQ = 10σ/S**.** The LOQ for Warfarin was found to be 0.06680 µg/mL.

Range:

The validated analytical range of the developed RP-HPLC method was 5–25 µg/mL**,** over which acceptable linearity, precision, and accuracy were achieved.

Selectivity:

Selectivity was assessed by analyzing Warfarin in the presence of excipients. No interference from excipients or impurities was observed at the retention time of Warfarin, confirming the specificity of the method.

Robustness:

Robustness was evaluated by introducing small deliberate variations in chromatographic conditions, such as flow rate (±0.1 mL/min) and detection wavelength (±5 nm)**.** These variations did not significantly affect the chromatographic performance, demonstrating the robustness of the method.

Ruggedness:

Ruggedness was evaluated by performing the analysis under different conditions, including different analysts and different days. The method produced consistent and reproducible results, confirming its suitability for routine quality control analysis.

METHODOLOGY

Solubility Study

The solubility of Warfarin was evaluated in various polar solvents, including water, methanol, acetonitrile, ethanol, and acetone. Warfarin was found to be highly soluble in methanol, freely soluble in water, acetonitrile, and ethanol, and insoluble in acetone. Based on the solubility profile, methanol was selected as the primary solvent for further studies.

Selection and Optimization of Mobile Phase

Different mobile phase combinations were investigated to obtain satisfactory chromatographic performance. Among the various solvent systems tested, Methanol: Potassium Phosphate Buffer (80:20, v/v) produced a sharp, symmetrical peak with good resolution and acceptable retention time. Therefore, it was selected as the optimized mobile phase for the RP-HPLC method.

Selection of Detection Wavelength

A standard solution of Warfarin (1000 µg/mL) was prepared and scanned in the UV range of 200–400 nm. The maximum absorbance (λmax) was observed at 258 nm, which was selected as the detection wavelength for all chromatographic analyses.

Selection of Retention Time

The HPLC system was equilibrated with the optimized mobile phase until a stable baseline was achieved. Standard Warfarin solution was injected repeatedly, and the retention time along with system suitability parameters such as theoretical plates and tailing factor were evaluated to ensure consistent chromatographic performance.

Optimization of Chromatographic Conditions

Chromatographic conditions were optimized by varying the mobile phase composition. The optimized method employed a Cosmosil C18 column with Methanol: Potassium Phosphate Buffer (80:20, v/v) as the mobile phase, providing well-resolved peaks with satisfactory peak symmetry and reproducibility.

Preparation of Buffer Solution

Potassium phosphate buffer was prepared by dissolving the required quantity of potassium phosphate in HPLC-grade water, and the pH was adjusted to **6.8** using orthophosphoric acid.

Preparation of Mobile Phase

The optimized mobile phase consisting of Methanol: Potassium Phosphate Buffer (80:20, v/v) was prepared, filtered through a 0.45 µm membrane filter, and degassed by ultrasonication before use.

Preparation of Standard Stock Solution

A standard stock solution of Warfarin was prepared by accurately weighing **100 mg** of the drug, dissolving it in the mobile phase, and making up the volume to **100 mL**. Appropriate dilutions were prepared from the stock solution for analysis.

System Equilibration and Sample Injection

The filtered mobile phase was loaded into the HPLC system, and the instrument was equilibrated until a stable baseline was obtained. Standard and sample solutions were filtered and injected into the HPLC system for chromatographic analysis.

Optimization of RP-HPLC Method

The developed chromatographic conditions were optimized based on peak symmetry, resolution, sensitivity, and reproducibility. The optimized method demonstrated satisfactory

chromatographic performance and was selected for method validation.

Assay of Tablet Formulation

Commercial Coumadin tablets (Label claim: 5 mg Warfarin) were analyzed using the developed RP-HPLC method. Twenty tablets were accurately weighed, powdered, and an amount equivalent to 100 mg of Warfarin was transferred to a volumetric flask. The powder was dissolved in the mobile phase, sonicated, filtered, and suitably diluted. The prepared sample solution was injected into the HPLC system, and the drug content was calculated from the calibration curve. The assay results were found to be within the acceptable limits of the labeled claim.

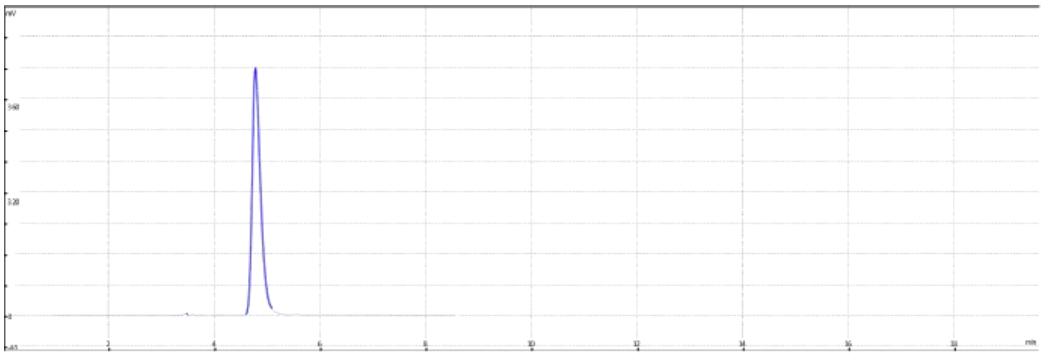
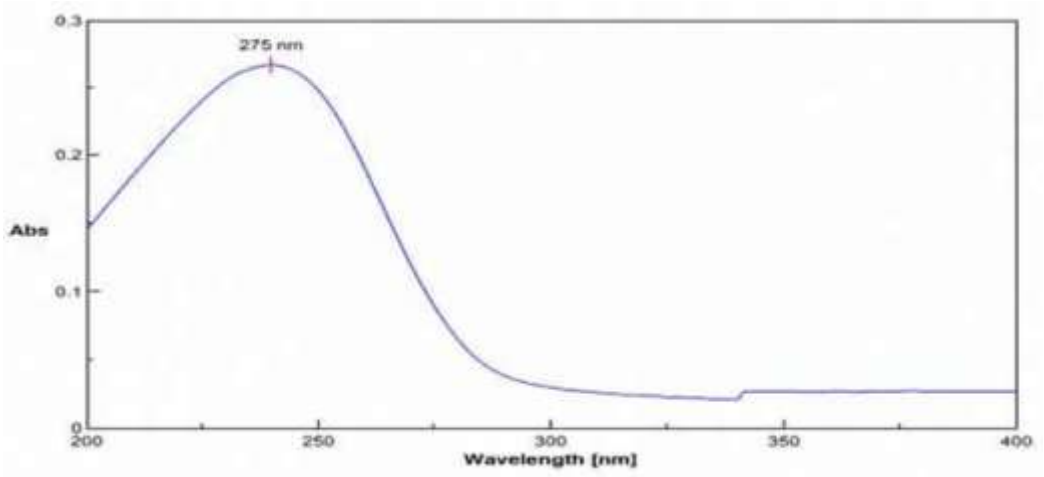


Fig: Trial 1: Methanol+potassium phosphate buffer (70:20)

Satisfactory and better results M.P. selected for further analysis so method is accepted

Determination of wavelength of Warfarin:-

The standard solution of Warfarin was scanned at different concentrations in the range of 200-400nm and the wavelength was found to be 275 against reagent blank.[41]



Analysis of Tablet Formulation

Table 1. Analysis of tablet formulation.

Sr. no	Wt. of stand. (mg)	Peak area of (µv.sec)	Mean	Label claim (%)	SD	%RSD
	WARF	WARF	WARF	WARF	0.01456	0.01455
1		269734	100.02			
2	160 mg	269745	100			
3		269756	99.97	99.77		

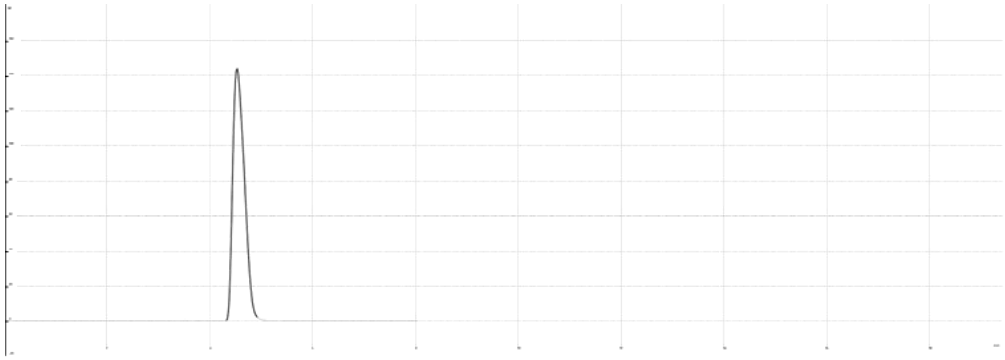


Figure 2. Chromatogram of tablet solutions of Warfarin

Sr. No	Name	Retention time (Min)	Peak area (µv.sec)	NTP	Asymmetry
1.	Warfarin	4.477	2697346	7079	1.17

Evaluation of Analytical Method Validation as Per ICH Guidelines:-

Linearity:

Table 2 . Linearity data for Warfarin

Standard conc.	5ug/ml	10ug/ml	15ug/ml	20ug/ml	25ug/ml
Replicates	Peak Area (µV.sec)				
1.	169639	259579	365290	469754	574536
2	169745	225697	355345	469756	575446
3	169797	225706	355377	469767	575777
4	169701	225776	355392	469923	575679
5	169745	225791	355491	469977	575972
Mean	169765	225732	355371	469797	575506
SD	70.9577	71.126	47.644	91.164	675.041
%RSD	0.04769	0.03151	0.01367	0.03940	0.1190

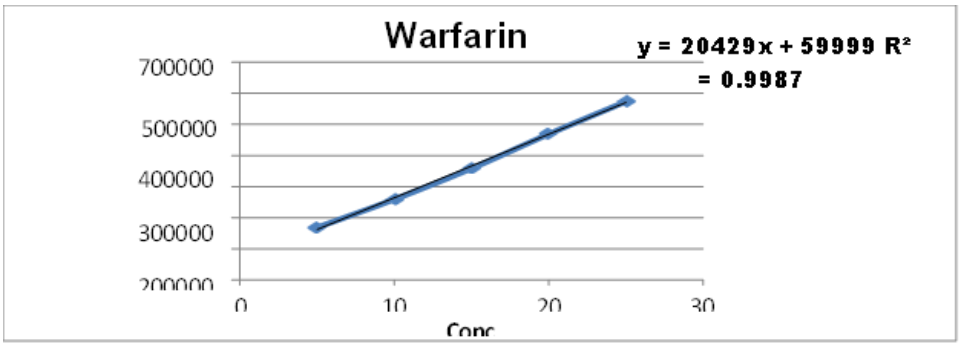


Figure 3.calibration curve for Warfarin

Precision:

Table 3: Inter-day variability of Warfarin

Conc. (µg/ml)	Peak area (µV.sec)			Mean area (µV/sec)	± SD	%RSD
	Day 1	Day 2	Day 3			
5	151179	153392	153397	152656.33	1279.41	0.7370
15	152691	152757	152767	152705	9775.34	0.06469
25	150679	150972	150997	150776.33	17973.40	0.1191

Table 4: Intra-day variability of Warfarin

Conc. (µg/ml)	Peak area(µV.sec)			Mean area (µV/sec)	± SD	% RSD
	Day 1	Day 2	Day 3			
5	151177	151177	151209	151174.66	21.7191	0.0144
15	152567	152679	152701	152652.33	74.144	0.04757
25	150946	151094	150956	150997.66	72.7123	0.05477

Accuracy:

Table 5: Recovery study of Warfarin

Recovery Level	Warfarin					
	Area (µV.sec)	Amt. added (mg)	Amt. recovered (mg)	% Recovery	Average recovery %	% RSD
70%	49346	7	7.50	97.79	99.57	0.0724
	49349	7	7.77	99.77		
	49417	7	7.97	99.97		
100%	151179	10	10	100	99.97	0.6777
	152691	10	9.95	99.94		
	150679	10	10	100		
120%	259164	12	11.99	99.97	99.9	0.1166
	257636	12	11.97	99.96		
	257646	12	11.97	99.97		

Limit of detection(LOD):

Table no.6: Limit of detection

Parameters	Warfarin µg/ml
LOD	0.02534

Table no.7 : Limit of Quantification

Parameters	Warfarin µg/ml
LOQ	0.07670

Robustness:

Limit of Quantitation(LOQ):

Table 8: Robustness study of system suitability parameter change in flow rate (ml/min)

System suitability parameter	Drug	Change in flow rate (ml/min)		RSD	
		0.7ml/min	1 min/ml	0.7min/ml	1 min/ml
Peak area	Warfarin	102776	102117	0.17	0.15
Theoretical plates.	Warfarin	7323	7120	0.62	0.25
Tailing Factor	Warfarin	0.99	1.07	0.51	0.79
Retention Time (Min)	Warfarin	4.79	5.55	0.56	0.55

Table 9: Robustness study of system suitability parameter change in wavelength (nm)

System suitability parameter	Drug	Change in Wavelength		RSD	
		270	270	270	270
Peak area	Warfarin	102575	102671	0.17	0.15
Theoretical plates.	Warfarin	9021	7525	0.62	0.25
Tailing Factor	Warfarin	1.03	0.99	0.51	0.79
Retention Time (Min)	Warfarin	4.35	4.46	0.56	0.55

Ruggedness

Injection No.	Analyst 1 Peak Area	Analyst 2 Peak Area
1	245621	244970
2	245790	245310
3	246110	245745
4	245775	245420
5	245970	245690
6	245760	245510

Parameter	Analyst 1	Analyst 2
Mean Peak Area	245773	245459
SD	163.5	296.2
%RSD	0.066%	0.12%

SUMMARY AND CONCLUSION

The present research work was successfully carried out for the development and validation of a simple, accurate, precise, economical, and reproducible RP-HPLC method for the quantitative estimation of Warfarin in bulk drug and pharmaceutical dosage form. The developed analytical method proved to be suitable for routine quality control analysis of Warfarin according to ICH guideline requirements.

In the present study, different chromatographic conditions were evaluated in order to obtain an optimized and satisfactory chromatographic separation. Various mobile phase combinations such as Methanol:Water, Acetonitrile:Water, Methanol:Acetonitrile, and Methanol:Potassium phosphate buffer were investigated during method optimization. Among all the trials, the mobile phase consisting of Methanol:Potassium phosphate buffer (80:20 v/v) provided sharp, symmetrical, and well-resolved peaks with acceptable retention time and good chromatographic performance. Therefore, this mobile phase was selected as the optimized mobile phase for further analysis.

The developed RP-HPLC method employed Cosmosil C18 column (250 × 4.6 mm ID, 5 µm particle size) using Methanol and Potassium phosphate buffer as mobile phase with UV detection. The method showed satisfactory chromatographic behavior with excellent peak symmetry and reproducibility. The optimized chromatographic conditions resulted in a retention time of approximately 4–5 minutes for Warfarin, indicating rapid analysis and reduced solvent consumption.

The wavelength selected for analysis was found to be 275 nm after scanning the standard solution in the UV region between 200–400 nm. The selected wavelength showed good sensitivity and adequate response for quantitative estimation of Warfarin. The method demonstrated good specificity without interference from excipients, impurities, or solvent peaks, indicating the suitability of the developed method for routine pharmaceutical analysis.

The developed method was validated according to ICH guidelines for various analytical validation parameters such as linearity, precision, accuracy, specificity, robustness, limit of detection (LOD), and limit of quantification (LOQ). The linearity study showed a good correlation coefficient over the concentration range of 5–25 µg/ml, indicating a direct proportional relationship between concentration and peak area. The calibration curve demonstrated excellent linearity with correlation coefficient close to unity, confirming the reliability of the method for quantitative analysis

The precision study including intra-day and inter-day precision demonstrated low %RSD values, which indicated that the developed method possesses good repeatability and reproducibility. The low standard deviation values confirmed that the method is precise and reliable for routine analysis. Accuracy studies performed by recovery method at 80%, 100%, and 120% levels showed excellent recovery values within acceptable limits,

confirming the accuracy of the developed method. The percentage recovery values were found near 100%, indicating that the method is free from interference and capable of accurate estimation of Warfarin in dosage forms

The limit of detection (LOD) and limit of quantification (LOQ) values indicated that the developed method is highly sensitive for the estimation of Warfarin even at lower concentrations. The robustness study revealed that small deliberate variations in chromatographic conditions such as flow rate and wavelength did not significantly affect the analytical performance of the method. Hence, the method was found to be robust and reliable during normal laboratory conditions

The developed RP-HPLC method was successfully applied for the analysis of marketed tablet formulation of Warfarin. The assay results were found within acceptable pharmacopoeial limits and close to the labeled claim, demonstrating the applicability of the method for pharmaceutical dosage forms. The chromatograms obtained during tablet analysis showed well-defined and resolved peaks without any interference from formulation excipients.

Overall, the developed RP-HPLC method was found to be simple, rapid, accurate, precise, selective, economical, and reproducible for quantitative estimation of Warfarin in bulk and pharmaceutical dosage forms. The method can be effectively utilized for routine quality control analysis, assay determination, and stability studies in pharmaceutical industries and research

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