Development and Validation of a Stability Indicating HPLC Assay Method for Determination of Warfarin Sodium in Tablet Formulation

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**ABSTRACT**

The objective of the current study was to develop a simple, precise and accurate isocratic reversed-phase stability indicating high performance liquid chromatography (HPLC) assay method validated for determination of warfarin in solid pharmaceutical dosage forms. Isocratic reversed phase high performance liquid chromatography (RP-HPLC) separation was achieved on a SGE S silica (150 mm × 4.6 mm i.d., C8RS 5 μm particle size) column using mobile phase of acetonitrile and 50 mM sodium hydrogen phosphate dibasic dihydrate buffer pH 3.0 (50:50 v/v) at a flow rate of 1.0 mL min\(^{-1}\), the injection volume was 20.0 μL and the detection was carried out at 280 nm using photo-diode array detector. The drug was subjected to oxidation, hydrolysis, photolysis and heat in order to apply stress condition. The method was validated for specificity, linearity, precision, accuracy, robustness and solution stability. The method was linear in the drug concentration range of 40–160 μg mL\(^{-1}\) with a correlation coefficient 0.9995. The precision (relative standard deviation RSD) amongst six-sample preparation was 0.22% for repeatability and the intermediate precision (RSD) amongst six-sample preparation was 0.70%. The accuracy (recovery) was between 99.95 and 99.15%. Degradation products produced as a result of stress studies did not interfere with detection of warfarin and the assay can thus be considered stability indicating.
Introduction to warfarin sodium

Warfarin (also known under the brand names coumadin, jantoven, marevan, lawarin, and waran) is an anticoagulant. The name warfarin stems from the acronym WARF, for Wisconsin Alumni Research Foundation + the ending -arin indicating its link with coumarin.

Description

Chemically warfarin is (RS)-4-hydroxy-3-(3-oxo-1-phenylbutyl)-2H-chromen-2-one sodium salt [Figure 1]. Warfarin was first registered for being used as a rodenticide in the US in 1948, and immediately became popular; although it was developed by Link, the WARF financially supported the research and was assigned the patent [1].

![Chemical structure of warfarin](image)

**Figure 1.** (RS)-4-hydroxy-3-(3-oxo-1-phenylbutyl)-2H-chromen-2-one sodium salt
A few years after its introduction, warfarin was found to be effective and relatively safe for preventing thrombosis and embolism (abnormal formation and migration of blood clots) in many disorders. It was approved for use as a medication in the early 1950s and has remained popular ever since; warfarin is the most widely prescribed anticoagulant drug in North America [2].

Despite its effectiveness, treatment with warfarin has several shortcomings. Many commonly used medications interact with warfarin, as do some foods, and its activity has to be monitored by frequent blood testing for the international normalized ratio (INR) to ensure an adequate yet safe dose is taken.

Warfarin is a synthetic derivative of dicoumarol, a 4-hydroxycoumarin-derived mycotoxin anticoagulant found in spoiled clover-based animal feeds. Warfarin and related 4-hydroxycoumarin-containing molecules decrease blood coagulation by inhibiting vitamin K epoxide reductase, an enzyme that recycles oxidized vitamin K to its reduced form after it has participated in the carboxylation of several blood coagulation proteins, mainly prothrombin and factor VII. For this reason, drugs in this class are also referred to as vitamin K antagonists [3].

Literature review reveals that there are various analytical methods like LC-MS-MS [4], chiral HPLC [5], micellar electrokinetic mass spectrometry [6], UPLC-MS-MS [7] and dissolution methods [8] are available for the determination of warfarin in pharmaceutical formulations and in biological fluids are reported. These methods applied to the pharmacokinetic studies of warfarin sodium. So far, to our present knowledge, no validated stability indicating HPLC assay method for the determination of warfarin sodium in pharmaceutical formulation was available in literature. The present work deals with the forced degradation of warfarin sodium under stress condition like acid hydrolysis, base hydrolysis, oxidation, thermal and photolytic stress. This work also deals with the validation of the developed method for the assay of warfarin sodium from its formulations (tablets). Hence, the method is recommended for routine quality control analysis and also stability sample analysis.

**Experimental**

**Materials**

Warfarin sodium standard was provided by Luotian Hengxingyuan Chemical Co. Ltd, China. Tablets containing warfarin sodium 5 mg and the inactive ingredient used in drug matrix were obtained from market. Analytical grade sodium hydrogen phosphate dibasic dihydrate was purchased from Sisco Research Pvt. Ltd., Mumbai (India). HPLC grade acetonitrile was obtained from Spectrochem Pvt. Ltd., Mumbai (India). MiliiQ (Milipore India (Pvt) Limited) water purification system is used to obtain HPLC grade water. Analytical grade hydrochloric acid, ortho-phosphoric acid, sodium
hydroxide pellets and 30% v/v hydrogen peroxide solution were obtained from RANKEM, New Delhi (India).

**Instrumentation**

The chromatographic system used to perform the development and validation of this assay method was comprised of a LC-10ATvp binary pump, a SPD-M10Avp photodiode-array detector and a rheodyne manual injector model 7725i with 20 μL loop (Shimadzu, Kyoto, Japan) connected to a multi-instrument data acquisition and data processing system (Class-VP 6.13 SP2, Shimadzu).

**Mobile phase preparation**

The mobile phase consisted of acetonitrile 50 mM sodium hydrogen phosphate dibasic dihydrate buffer pH 3.0 (50:50, v/v). Buffer solution was prepared by dissolving 8.9 g sodium hydrogen phosphate dibasic dihydrate in 900 mL HPLC grade water and then adjusted to pH 3.0 with ortho-phosphoric acid and the final volume was, then, made up to 1000 mL with HPLC grade water. Mobile phase was filtered through a 0.45 μm nylon membrane (Millipore Pvt. Ltd. Bengaluru, India) and degassed for 15 minutes in an ultrasonic bath. HPLC grade water was used as diluent.

**Standard preparation**

A warfarin sodium standard solution containing 100 μg/mL was prepared in a 100 mL volumetric flask by dissolving 10.00 mg of warfarin sodium and then diluted to volume with diluent.

**Test preparation**

Twenty tablets were weighed and the average weight of tablet was determined. From that, five tablets were weighed and transferred into a 250 mL volumetric flask. About 50 mL diluent was added and sonicated for a minimum 30 minutes with intermittent shaking. Then, the content was brought back to ambient temperature and diluted to volume with diluent. The sample was filtered through 0.45 μm nylon syringe filter. The obtained concentration was 100 μg/mL of warfarin sodium.

**Chromatographic conditions**

Chromatographic analysis was performed on a SGE make SS wakosil II 5C8RS column (150 mm, 4.6 mm i.d., 5 μm particle size) column. The flow rate of the mobile phase was adjusted to 1.0 mL/min and the injection volume was 20 μL. Detection was performed at 280 nm on a Photo diode array detector.

Sensitive and precise RP-HPLC method with photo diode array detector has been developed and validated for the simultaneous estimation of three commonly available anti-dengue, anti-cancer and
anti-inflammatory phytochemical markers. The chromatographic separation was achieved using C18 column (250 mm×4.0 mm, 5 μm) with 0.2% v/v formic acid: acetonitrile (50:50; v/v) as mobile phase at the flow rate of 0.7 mL/min. The dual wavelength (280 and 360 nm) was selected for the identification and quantification of rutin, gallic acid and quercetin (Rt 2.56, 2.95 and 4.60 min). The method was validated as per ICH guidelines in terms of specificity, linearity, precision, and accuracy, LOD and LOQ, respectively [9].

Result and discussion

Development and optimization of the HPLC method

Proper selection of the methods depends upon the nature of the sample (ionic or ionisable or neutral molecule), its molecular weight and solubility. Warfarin sodium is soluble in polar solvent therefore reversed phase mode of HPLC was chosen. To develop a rugged and suitable HPLC method for the quantitative determination of warfarin sodium, the analytical conditions were selected after testing the different parameters such as diluents, buffer, buffer concentration, organic solvent for mobile phase and mobile phase composition and other chromatographic conditions. Our preliminary trials using different composition of mobile phases consisting of water with methanol or acetonitrile, did not give good peak shape. By using 50 mM disodium hydrogen phosphate dihydrate buffer, adjusted to pH 3.0 with orthophosphoric acid and keeping mobile phase composition as acetonitrile 50 mM sodium hydrogen phosphate dibasic dihydrate buffer pH 3.0 (50:50, v/v), best peak shape was obtained. For the selection of organic constituent of mobile phase, acetonitrile was chosen to reduce back pressure, the longer retention time and to achieve good peak shape. Figure 2 and 3. represents wavelength selection and PDA scan of standard preparation. Figure 4 and 5. represents the chromatograms of standard and test preparation respectively.

![Figure 2. Wavelength selection for standard preparation](image-url)
Figure 3. PDA scan for standard preparation

Figure 4. Chromatogram of standard preparation

Figure 5. Chromatogram of test preparation
Degradation study
The degradation samples were prepared by transferring powdered tablets, equivalent to 10 mg warfarin sodium into a 250 mL round bottom flask. Then, the prepared samples were employed for acidic, alkaline and oxidant media and also for thermal and photolytic conditions. After the degradation treatments were completed, the stress content solutions were allowed to equilibrate to ambient temperature and diluted with mobile phase to attain 100 μg/mL concentrations. Specific conditions were described as follows.

Acidic condition
Acidic degradation study was performed by heating the drug content in 0.1 N HCl at 80 °C for 1.5 hours and mixture was neutralized. Warfarin sodium was found stable in acidic condition. (Figure 6).

![Figure 6. Chromatogram of acidic forced degradation study](image_url)

Alkaline condition
Alkaline degradation study was performed by keeping the drug content in 1 N NaOH at ambient temperature for 2 hours and then, mixture was neutralized. In alkali degradation, it was found that around 2% of the drug degraded (Figure 7).

![Figure 7. Chromatogram of alkali forced degradation study](image_url)
Oxidative condition

Oxidation degradation study was performed by keeping the drug content in 3% v/v H₂O₂ at ambient temperature for 1 hour. Minor degradation was found in oxidative condition that 5% of the drug was degraded. The major impurity peaks was found at 2.6 and 3.8 min (Figure 8).

![Figure 8. Chromatogram of oxidative forced degradation study](image)

Thermal condition

Thermal degradation was performed by exposing formulation at 80 °C for 72 hours. Warfarin sodium is found to be stable under thermal degradation condition. There is no degradation observed in above specific thermal condition (Figure 9).

![Figure 9. Chromatogram of thermal degradation study](image)

Photolytic condition

Photolytic degradation study was performed by exposing the drug content in UV-light for 72 hours. In photolytic degradation, it was found that around 6.2% of the drug degraded (Figure 10).
Method validation

Specificity
The evaluation of the specificity of the method was determined against placebo and stress (forced degradation) application. The interference of the excipients of the claimed placebo presence in the pharmaceutical dosage form was derived from placebo solution. Further, the specificity of the method toward the drug was established by means of the interference of the degradation products against drug during the forced degradation study.

Linearity
Seven points calibration curve was obtained in a concentration range from 40-160 μg/mL for warfarin sodium. The response of the drug was found to be linear in the investigation concentration range and the linear regression equation was $y = 49503136.60714x + 158762.26786$ with correlation coefficient 0.9995 (Figure 11). Chromatogram obtained during linearity study were shown in Figure 12-18.
Figure 12. Linearity study chromatogram of level-1 (40%)

Figure 13. Linearity study chromatogram of level-2 (60%)

Figure 14. Linearity study chromatogram of level-3 (80%)
Figure 15. Linearity study chromatogram of level-4 (100%)

Figure 16. Linearity study chromatogram of level-5 (120%)

Figure 17. Linearity study chromatogram of level-6 (140%)
LOD and LOQ
The limit of detection and limit of quantification were evaluated by serial dilutions of warfarin sodium stock solution in order to obtain signal to noise ratio of 3:1 for LOD and 10:1 for LOQ. The LOD value for warfarin sodium was found to be 0.25 µg/mL and the LOQ value 0.5 µg/mL. Chromatogram of LOD and LOQ study were shown in Figure 19-20.
**Precision**

The result of repeatability and intermediate precision study are shown in Table 1. The developed method was found to be precise as the % RSD values for the repeatability and intermediate precision studies were 0.22% and 0.70%, respectively, which confirm that method was precise.

**Table 1. Evaluation data of precision study**

<table>
<thead>
<tr>
<th>Set</th>
<th>Intraday (n = 6)</th>
<th>Interday (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100.8</td>
<td>101.1</td>
</tr>
<tr>
<td>2</td>
<td>100.4</td>
<td>99.9</td>
</tr>
<tr>
<td>3</td>
<td>100.3</td>
<td>100.9</td>
</tr>
<tr>
<td>4</td>
<td>100.3</td>
<td>100.5</td>
</tr>
<tr>
<td>5</td>
<td>100.7</td>
<td>99.5</td>
</tr>
<tr>
<td>6</td>
<td>100.3</td>
<td>99.5</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td>100.5</td>
<td>100.2</td>
</tr>
<tr>
<td><strong>Standard deviation</strong></td>
<td>0.23</td>
<td>0.70</td>
</tr>
<tr>
<td><strong>% RSD</strong></td>
<td>0.22</td>
<td>0.70</td>
</tr>
</tbody>
</table>

**Accuracy**

The HPLC area responses for accuracy determination are depicted in Table 2. The result shows the best recoveries (98.95-99.15%) of the spiked drug were obtained at each added concentration, indicating that the method was accurate. Chromatogram obtain during accuracy study were shown in Figure 21-23.

**Table 2. Evaluation data of accuracy study**

<table>
<thead>
<tr>
<th>Level (%)</th>
<th>Amount added concentration a (mg/mL)</th>
<th>Amount found concentration a (mg/mL)</th>
<th>% Recovery</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>0.05240</td>
<td>0.05196</td>
<td>99.15</td>
<td>0.42</td>
</tr>
<tr>
<td>100</td>
<td>0.10168</td>
<td>0.10076</td>
<td>99.09</td>
<td>0.17</td>
</tr>
<tr>
<td>150</td>
<td>0.15067</td>
<td>0.14908</td>
<td>98.95</td>
<td>0.28</td>
</tr>
</tbody>
</table>

a Each value corresponds to the mean of three determinations

**Figure 21. Accuracy study chromatogram of level-1 (50%)**
Solution stability study

Table 3. shows the results obtain in the solution stability study at different time intervals for test preparation. It was concluded that the test preparation solution was found stable up to 48 h at 2-8 °C and ambient temperature, as during this time the result was not decrease below the minimum percentage.

<table>
<thead>
<tr>
<th>Intervals</th>
<th>% Assay for test preparation solution stored at 2-8 °C</th>
<th>% Assay for test preparation solution stored at ambient temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>--</td>
<td>100.3</td>
</tr>
<tr>
<td>12 h</td>
<td>99.5</td>
<td>99.4</td>
</tr>
<tr>
<td>24 h</td>
<td>98.9</td>
<td>100.1</td>
</tr>
<tr>
<td>36 h</td>
<td>100.3</td>
<td>99.2</td>
</tr>
<tr>
<td>48 h</td>
<td>99.6</td>
<td>99.7</td>
</tr>
</tbody>
</table>
Robustness

The results of robustness study of the developed assay method are presented in Table 4. The result shows that during all variance conditions, assay value of the test preparation solution was slightly affected and it was in accordance with that of actual. System suitability parameters were also found satisfactory; hence, the analytical method would be concluded as robust. Chromatogram obtain during robustness study were shown in Figure 22-28.

<table>
<thead>
<tr>
<th>Robust conditions</th>
<th>% Assay</th>
<th>System suitability parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Theoretical plates</td>
</tr>
<tr>
<td>Flow 0.9 mL/min</td>
<td>100.9</td>
<td>1958.38</td>
</tr>
<tr>
<td>Flow 1.1 mL/min</td>
<td>99.7</td>
<td>1959.38</td>
</tr>
<tr>
<td>Buffer pH 2.5</td>
<td>97.0</td>
<td>2467.5</td>
</tr>
<tr>
<td>Buffer pH 3.5</td>
<td>98.4</td>
<td>2485.28</td>
</tr>
<tr>
<td>Buffer-ACN (52: 48, v/v)</td>
<td>102.9</td>
<td>2075.1</td>
</tr>
<tr>
<td>Buffer-ACN (48: 52, v/v)</td>
<td>95.5</td>
<td>2063.39</td>
</tr>
<tr>
<td>Column change</td>
<td>98.6</td>
<td>2526.77</td>
</tr>
</tbody>
</table>

Figure 22. Standard chromatogram (0.9 mL/min flow rate)

Figure 23. Standard chromatogram (1.1 mL/min flow rate)
Figure 24. Standard chromatogram (Buffer-ACN (52:48, v/v))

Figure 25. Standard chromatogram (Buffer-ACN (48:52, v/v))

Figure 26. Standard chromatogram (pH 3.5)
System suitability

A system suitability test of the chromatographic system was performed before each validation run. Five replicate injections of standard preparation were injected and asymmetry, theoretical plate and % RSD of peak area were determined for the same. Acceptance criteria for system suitability, asymmetry not more than 2.0, theoretical plates not less than 1500 and % RSD of peak area not more than 2.0, were full fill during all validation parameter.

Conclusion

This LC method for assay and determination of content uniformity of warfarin in a tablet formulation was successfully developed and validated for its intended purpose. The method was shown to specific, linear, precise, accurate, and robust. Because the method separates warfarin and all the degradation products formed under variety of stress conditions, it can be regarded as
stability indicating. This method is recommended to the industry for quality control of drug content in pharmaceutical preparations.

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**References**

[1] Link K.P. *Circulation*, 1959, **19**:97