Development and Validation of a Stability-Indicating RP-HPLC and UV Spectrophotometric methods for the Estimation of Fluindione in Bulk and Tablet Dosage Forms

N. Mallikarjuna Rao¹, D. Gowri Sankarb

¹Research & Development, Department of Pharmaceutical Sciences, Jawaharlal Nehru Technological University, Kakinada. Andhra Pradesh, India.
²Department of Pharmaceutical Analysis and Quality Assurance, University College of Pharmaceutical Sciences, Andhra University, Visakhapatnam, Andhra Pradesh, India.

Abstract

Fluindione is an oral anticoagulant. The aim of the present study was to develop a stability-indicating HPLC and UV Spectrophotometric methods for the determination of fluindione in bulk and its solid dosage forms. HPLC method was developed on a Symmetry (4.6 x 150 mm, 5 µm, Make: ODS) column with a mobile phase consisting of sodium phosphate buffer pH 3.5: acetonitrile 50:50 v/v, pumped at 1.0 ml min⁻¹ flow rate. The pH of buffer was adjusted to 3.5 with ortho phosphoric acid. The column was maintained at ambient temperature and 20µL of solutions were injected. The analyte was quantified spectrophotometrically at 285 nm. Fluindione eluted at 3.5 min. The method was validated reaching satisfactory results for selectivity, precision and accuracy. Forced degradation samples could be simultaneously evaluated, without interferences in the quantitative analysis. For the spectrophotometric analysis, methanol was used as solvent and the wavelength of 285 nm was selected for the detection. Both methods were found to quantify fluindione in bulk and its tablets accurately. Statistical analysis by Student's t-test showed no significant difference between the results obtained by the two methods. Therefore HPLC and UV methods presented the most reliable results for the analyses of fluindione tablets.

Keywords:
Fluindione, Stability indicating RP-HPLC and UV Spectrophotometry, Development and validation, Bulk and Dosage forms

1. Introduction

Fluindione is an oral anticoagulant with a long half-life that inhibits the synthesis of vitamin K-dependent clotting factors. It is used in various cardiologic diseases for the prevention of thromboembolism [1]. Chemically it is 2-(4-fluorophenyl) indene-1, 3-dione as shown in Fig. 1. Fluindione (FLU) a vitamin K antagonist has been the essential key in deep venous thromboembolism treatment. It acts as a vitamin K antagonist to antagonize the effect of vitamin K required for the synthesis of active clotting factors II, VII, IX, and anticoagulant proteins C and S. Antagonism of vitamin K reduces the amount of these clotting factors, thereby producing anticoagulation [2].
Very few analytical methods were reported on Fluindione, two methods have been published for assaying fluindione: Isabelle Fourel et al. [3] developed a Liquid Chromatography– Tandem Mass Spectrometry Ion-Trap Technique for the determination of fluindione in human plasma using a reversed-phase column (C₁₈) and Lofti et al. [4] described a HPLC method for the determination of thirteen anticoagulants, including fluindione, in human plasma using a reversed-phase column (C₁₈) with a diode array detector. Aymard et al. [5] developed new high-performance liquid chromatographic (HPLC) assay method without any extraction procedure was developed for the quantification of fluindione in plasma using a C₁₈ column and a UV detector set at 280 nm. Saravanan et al. [6] developed a RP-HPLC method for simultaneous quantification method for aspirin and fluindione in tablet formulation using Thermo Hypersil BDS C₁₈ column with water and acetonitrile with 1% TFA as mobile phase and UV detection at 236 nm. A UV method has been reported by Manish Kumar Thimmaraju et al. [7] which demonstrates that the drug was dissolved in the mixture of 10 % methanol and hydrochloric acid. The $\lambda_{\text{max}}$ was found to be 230 nm. This method suffers with poor analytical linearity range (1 to 5 $\mu$g/ml), which is very narrow and difficult to acquire in case of dosage forms. Addition of hydrochloric acid to the solvent or diluent may cause the degradation of the drug.

As far as the knowledge of the authors were concerned that no methods were reported on stability-indicating HPLC and UV Spectrophotometric methods for the determination of fluindione in bulk and its solid dosage form. The purpose of this study was to develop and validate analytical methods to quantify fluindione in bulk and its tablets, using HPLC and UV spectrometry. The results obtained by these methods were statistically compared, by using analysis of variance (ANOVA). In addition, the reliability and feasibility of them were evaluated focusing on routine quality control analysis.

2. Experimental

2.1. Materials and Reagents

Fluindione was provided by Toronto Research Chemicals Inc., India. All HPLC-grade solvents were obtained from Merck, Mumbai, India. Sodium dihydrogen phosphate, methanol, hydrochloric acid, sodium hydroxide and hydrogen peroxide (30%) were obtained from Merck, India. Water (18.2 MΩ.cm) was obtained using a Milli-Q system (Millipore, USA).

2.2. Chromatographic system and conditions

Waters Alliance e2695 separation module (Waters Corporation, Milford, USA) equipped with 2489 UV/Vis detector or diode array detector (DAD) with empower-2 software was used for the analysis. Symmetry (4.6 x 150 mm, 5 µm, Make: ODS) column with sodium phosphate buffer pH 3.5: acetonitrile 50:50 v/v, ratios was used as isocratic mobile phase. Mobile phase was used as diluent. 1.0 mL min⁻¹ flow rate was maintained. The eluted compounds were monitored at 285 nm. The column oven and auto sampler temperatures were maintained at ambient room temperatures. An injection volume of 20 µL was used. Ultraviolet spectrophotometric analysis was carried out on a UV-Vis Shimadzu UV-mini 1240 (Shimadzu, Kyoto, Japan) spectrophotometer, in a 1 cm quartz cuvette. The wavelength
of 285 nm was selected for the quantitation of fluindione and the measurements were obtained against methanol as a blank.

2.3. Standard and Test solutions

2.3.1. Preparation of Standard solution

A stock solution of fluindione (0.4 mg mL\(^{-1}\)) was prepared by dissolving 40 mg of fluindione in final volume of 100 mL diluent. Working solutions of 16 µg mL\(^{-1}\) in mobile phase were prepared from the above stock solution for assay determination.

2.3.2 Preparation of Test Solution

Twenty fluindione tablets were weighed, transferred to a clean and dry mortar and ground into a fine powder. Tablet powder equivalent to 40 mg (400 mg) drug was then transferred to a 100 mL volumetric flask, 70 mL of diluent was added, and the flask was attached to a rotary shaker for 10 min to disperse the material completely. The mixture was then sonicated for 10 min and diluted to volume with diluent to give a solution containing 0.4 mg/mL. 4 mL of this solution was further diluted to 100 mL with methanol for spectrophotometric analysis or mobile phase for chromatographic analysis, to obtain a solution at 16 µg mL\(^{-1}\) of fluindione. This solution was filtered through a 0.22 µm pore size PVDF membrane filter.

2.4. Method Validation

The optimized spectrophotometric and chromatographic methods were completely validated according to the procedures described in ICH guidelines Q2 (R1) and Q1 A (R2) for the validation of analytical methods and Stability Testing of New Drug respectively [8-9].

2.4.1. Linearity

Standard solutions containing 1000 µg mL\(^{-1}\) of fluindione in water were prepared, in triplicate. Aliquots of these solutions were diluted in methanol for UV analysis and with mobile phase for HPLC analysis, to six different concentrations, corresponding to 4, 8, 12, 16, 20 and 24 µg mL\(^{-1}\) of fluindione. Calibration curves with concentration versus peak area or absorbance were plotted for each method and the obtained data were subjected to regression analysis using the least squares method.

2.4.2. Precision

The intra-day precision was evaluated by analyzing six samples (n = 6), at the test concentration of 16 µg mL\(^{-1}\), using the UV and the HPLC methods on the same day. Fluindione contents and the relative standard deviations (RSD) were calculated. The inter-day precision or ruggedness of the method was evaluated by analyzing six samples (n = 6), at the test concentration of 16 µg mL\(^{-1}\), using the UV and the HPLC methods on second day (second analyst, HPLC column of same make with different lot number and another HPLC system). Fluindione contents and the relative standard deviations (RSD) were calculated

2.4.3. Accuracy

Fluindione reference standard was accurately weighed and added, at three different concentrations. At each concentration, samples were prepared in triplicate and the recovery percentage was determined by UV and HPLC methods.

2.4.4 Ruggedness

The Ruggedness of the method was determined by the variation of the flow rate (± 0.1 mL min\(^{-1}\)) and wave length (±2). The flow rate checked was 0.9 ml/min and 1.1 mL min\(^{-1}\) and wave length was checked at 283 nm to 287nm.
2.4.5. Analysis of Fluindione tablets

Tablets of fluindione were analyzed by the validated HPLC and UV methods. The sample solutions for the HPLC and UV analysis were prepared as described previously. The fluindione contents were determined by using the two methods and the obtained results were statistically compared by using ANOVA test applied at 0.05 significance level.

2.4.6. Forced degradation conditions

In order to determine whether the method is stability indicating, forced degradation studies were conducted on fluindione API powder. The analysis was carried out by HPLC with a DAD detector and a double beam UV-Visible spectrophotometer. 20µl of each of forced degradation samples were injected into HPLC system at regular intervals and the final stress conditions were established in such a way that 10–20% degradation of fluindione was occurred.

2.4.6.1. Acid degradation

100 mg of fluindione sample was taken into a 100 mL round bottom flask, 10 mL of 0.1 mol L⁻¹ hydrochloric acid solution was added, and make up the volume up to the mark with diluent (Mobile phase for HPLC and Methanol for UV). The contents were mixed well and kept for constant stirring for 3 h at room temperature. 1.0 ml of this solution was taken in 10 mL volumetric flask and neutralized with 1.0 mL of 0.1 mol L⁻¹ sodium hydroxide and then diluted to 10 mL with diluent (Mobile phase for HPLC and Methanol for UV).

2.4.6.2. Base degradation

100 mg of fluindione sample was taken into a 100 mL round bottom flask, 10 mL of 0.1 mol L⁻¹ sodium hydroxide solution was added, and diluted to volume with diluent (Mobile phase for HPLC and Methanol for UV). The contents were mixed well and kept for constant stirring for 3 h at room temperature. 1.0 mL of this solution was taken in 10 mL volumetric flask and neutralized with 1.0 mL of 0.1 mol L⁻¹ hydrochloric acid and then diluted to 10 mL with diluent (Mobile phase for HPLC and Methanol for UV).

2.4.6.3 Oxidative degradation

100 mg of fluindione was taken into a 100 mL round bottom flask, 10 mL of 10% hydrogen peroxide solution was added, and contents were mixed well and kept for constant stirring for 6 h at room temperature. 1.0 mL of this solution was diluted to 10 mL with diluent (Mobile phase for HPLC and Methanol for UV).

2.4.6.3 Thermal degradation

100 mg of fluindione sample was taken in to a petri dish and kept in oven at 80°C for 6 h. 10.0 mg of this sample was taken in to a 10 mL volumetric flask, dissolved in diluent and final volume was made up with the diluent (Mobile phase for HPLC and Methanol for UV).

3. Results and Discussion

3.1 HPLC method development

Attempts were made by using different stationary phases like C₁₈ and C₈ and using a combination of different buffers with different pH and organic modifiers like acetonitrile, methanol and ethanol in the mobile phase. Selection of suitable HPLC column was also of major concern. A Symmetry C₁₈ is high purity base deactivated silica, which consists of a unique C₁₈ multi-alkyl bonding and exhaustive end capping. The chromatographic separation was achieved using a mobile phase containing a mixture of sodium phosphate buffer pH 3.5: acetonitrile 50:50 v/v, pH of the buffer is adjusted to 3.5 with ortho phosphoric acid using
Symmetry (4.6 x 150mm, 5 µm, Make: ODS). A sharp peak with good symmetry factor of fluindione noticed when symmetry C_{18} column employed. In the optimized conditions fluindione and its possible degradants were well separated. This shows that the degradants doesn’t have any effect on the elution of fluindione. The typical retention time of fluindione was about 3.5 min. A representative chromatogram of fluindione standard and test was shown in Fig. 2a and 2b. The system suitability results were found to be satisfactory and the developed LC method was found to be specific for fluindione.

4. UV spectrophotometric method development

After the evaluation of the fluindione UV spectrum in various solvents (water, phosphate buffer pH 6.0, methanol, 0.1 mol L\(^{-1}\) hydrochloric acid and sodium hydroxide), and in the range of 200–400 nm (Fig. 3a and 3b), the wavelength of 285 nm was chosen due to the adequate molar absorptivity of fluindione in this region and to minimize possible interference from other compounds and solvent in the samples.
Fig. 3. UV spectrum of (a) fluindione standard substance (b) fluindione sample substance

4.1 Method Validation

A linear relationship was found between the fluindione concentrations and the response of both HPLC and UV methods. The regression analysis data was presented in Table 1. For HPLC and UV methods high regression coefficient ($r^2$) values were obtained, 0.999 and 0.993, respectively.

Table 1. Overview of the Linearity Data Obtained for fluindione by the Chromatographic and Spectrophotometric Methods

<table>
<thead>
<tr>
<th>Regression parameters</th>
<th>HPLC</th>
<th>UV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression equation</td>
<td>$y = 255438x - 30988$</td>
<td>$y = 0.0371x - 0.0061$</td>
</tr>
<tr>
<td>Regression coefficient ($r^2$)</td>
<td>0.999</td>
<td>0.993</td>
</tr>
<tr>
<td>Slope &amp; standard error</td>
<td>$255438 \pm 0.24$</td>
<td>$0.0371 \pm 0.0027$</td>
</tr>
<tr>
<td>Intercept &amp; standard error</td>
<td>$30988 \pm 0.15$</td>
<td>$0.0061 \pm 0.0019$</td>
</tr>
<tr>
<td>Concentration range ($\mu g/mL$)</td>
<td>4 - 24</td>
<td>4 - 24</td>
</tr>
<tr>
<td>Number of points</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

The precision data obtained for the evaluated methods are demonstrated in Table 2. Both methods presented RSD values lower than 2.0%, assuring a good precision. Accuracy (Table 2) was investigated by means of a standard addition experiment. Both chromatographic and UV spectrophotometric methods exhibited mean recoveries (n=9) close to 100% demonstrating an adequate accuracy.

Table 2. Validation Parameters of the Evaluated Methods for fluindione

<table>
<thead>
<tr>
<th>Validation parameters</th>
<th>HPLC (RSD %)</th>
<th>UV (RSD %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-day precision, n = 6</td>
<td>0.28</td>
<td>0.57</td>
</tr>
<tr>
<td>Inter-day precision, n = 6</td>
<td>0.37</td>
<td>0.23</td>
</tr>
</tbody>
</table>

Accuracy

<table>
<thead>
<tr>
<th>Level (%)</th>
<th>HPLC (%)</th>
<th>UV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 %</td>
<td>100.0</td>
<td>98.6</td>
</tr>
<tr>
<td>100 %</td>
<td>98.0</td>
<td>99.3</td>
</tr>
<tr>
<td>150 %</td>
<td>97.7</td>
<td>98.2</td>
</tr>
</tbody>
</table>
The difference in the retention time, the peak area and the analyst (for a given fluindione concentration) caused by the aforementioned minor alterations were insignificant.

4.2 Analysis of fluindione tablets

The validated LC method was successfully applied for the assay of fluindione in drug substance and tablets formulation. Assay results of fluindione tablets, expressed as the percentage of the label claim, were found to be 99.3 and 100.1% (n = 6), for HPLC and UV spectrophotometric methods respectively (Table 3), showing that the content of fluindione in the tablet formulation conformed to the content requirements (90–110% of the label claim). The above results demonstrated that the developed LC and UV methods achieved rapid and accurate determination of fluindione and can be used for the determination of fluindione in drug substance and pharmaceutical formulations.

Table 3. Fluindione Contents in tablet Samples Obtained by HPLC and UV

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Assay</th>
<th>HPLC</th>
<th>UV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluindione Tablets</td>
<td></td>
<td>98.5</td>
<td>98.1</td>
</tr>
</tbody>
</table>

5. Comparison between HPLC method and UV method

The proposed analytical methods were compared using statistical analysis. The Student's $t$-test was applied and does not reveal significant difference between the experimental values obtained in the sample analysis by the two methods. The calculated $t$-value ($t_{\text{calc}}=1.327$) was found to be less than the critical $t$-value ($t_{\text{crit}}=2.228$) at 5% significance level.

5.1 Forced degradation studies

Significant degradation of Fluindione was observed in acid, base, oxidative and base degradation conditions (Fig. 4a-d and 5a-d). Diode array detector was employed to check and ensure the homogeneity and purity of fluindione peak in all the stressed sample solutions. Assay studies were carried out for stress samples against fluindione qualified working standard (Table 4). The purity and assay of fluindione was unaffected by the presence of its impurities and degradation products and thus confirms the stability-indicating power of the developed method. The summary of forced degradation studies by HPLC and UV methods has been given in table 4.
Fig. 4. HPLC chromatogram and their peak purity plots of working standard solution of Fluindione after (a) Acid hydrolysis (0.1 N HCl at room temperature) (b) Alkali hydrolysis (0.1 N NaOH at room temperature) and (c) Oxidative degradation with 3% H₂O₂ hydrolysis (d) Thermal degradation (80°C for 6 hr.)
Fig. 5. UV Spectra’s of working standard solution of Fluindione after (a) Acid hydrolysis (0.1 N HCl at room temperature) (b) Alkali hydrolysis (0.1 N NaOH at room temperature) and (c) Oxidative degradation (10 % H₂O₂) (d) Thermal degradation (80°C for 6 hr.)

Table 4. Summary of forced degradation studies

<table>
<thead>
<tr>
<th>Stress condition</th>
<th>HPLC</th>
<th>UV Spectrophotometer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Assay of Fluindione</td>
<td>% Degradation</td>
</tr>
<tr>
<td>Acid</td>
<td>90.1</td>
<td>8.4</td>
</tr>
<tr>
<td>Base</td>
<td>91.3</td>
<td>7.2</td>
</tr>
<tr>
<td>Peroxide</td>
<td>86.4</td>
<td>12.1</td>
</tr>
<tr>
<td>Thermal</td>
<td>88.2</td>
<td>10.3</td>
</tr>
</tbody>
</table>

Chromatographic analysis showed to be the most sensitive and selective method, and might be applied successfully for Fluindione trace analysis and quantitation in pharmaceutical dosage forms. We cannot discharge, however, the analyses time and cost. The spectrophotometric method is clearly less expensive and requires shorter analysis time, besides the ease of handling and lower residues generation. Since the use of Fluindione as a potent oral anticoagulant drug is widespread, the development and validation of simple and reliable methods are essential to assure the quality of the raw materials and marketed formulations. A simple method to identify and precisely quantify these drugs may be an important tool to avoid treatment inefficacy.

6. Conclusion

HPLC and UV spectrophotometry methods were found to be adequate methods to quantify Fluindione in bulk and tablet dosage forms; the chromatographic and
spectrophotometric methods presented the most reliable results. Since these methods are fast and simple, they may be successfully applied to quality control analyses, with the aim of quantifying and identifying Fluindione in pharmaceutical products.

References


