AMERICAN JOURNAL OF PHARMTECH RESEARCH
Journal home page: http://www.ajptr.com/

Development and Validation of Stability-Indicating RP-HPLC Method for Determination of Fluindione

Mrinalini C. Damle*, Mahesh G. Thakare
1.AISSMS College of Pharmacy, Kennedy Road, Near RTO, Pune-411001.

ABSTRACT

Fluindione is an oral anticoagulant drug. A simple and rapid and was validated stability indicating HPLC method for Fluindione was successfully developed and was validated. This method is based on HPLC separation followed by UV detection at 285 nm. HPLC method was developed on a Symmetry thermo C_{18} (4.6 x 250 mm) column with a mobile phase consisting of 10mM di-sodium hydrogen phosphate buffer pH 3.5: methanol 15:85 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 eluted compound was detected by using PDA detector. Fluindione was eluted at 2.4 ± 0.2 min. Stress degradation study shows that sample degraded with acid and base hydrolysis, under oxidation, thermal and photolytic stress conditions. The method was validated in accordance with requirement of ICH guidelines.

Keywords: Fluindione, Stability indicating RP-HPLC, Forced degradation, validation

*Corresponding Author Email: mcdamle@rediffmail.com
Received 21 July 2016, Accepted 18 August 2016

Please cite this article as: Damle MC *et al*., Development and Validation of Stability-Indicating RP-HPLC Method for Determination of Fluindione. American Journal of PharmTech Research 2016.

INTRODUCTION

Fluindione is an oral anticoagulant that works by inhibition of synthesis of vitamin K-dependent clotting factors. Fluindione is used in various cardiologic diseases for the prevention of thromboembolism. 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. Chemically it is 2-(4-fluorophenyl) indene-1, 3-dione [1-3].

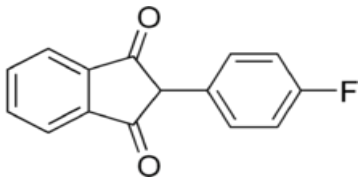


Figure 1: Chemical structure of Fluindione

Literature survey reveals that very few analytical methods have been reported for Fluindione, by UV-spectrophotometer and HPLC. But, reported HPLC method does not mentioned about degradation product obtained for acid hydrolysis and used expensive solvents in their mobile phase. Hence, need to redevelop with economic solvents. [4-5]

MATERIALS AND METHOD

Chemicals and reagents

Analytically pure sample of Fluindione was kindly supplied by Mylan Laboratories Ltd. (Hyderabad) India. Methanol (HPLC grade) was purchased from Merck specialties Pvt. Ltd. (Mumbai, India).

Instruments

Quantitative HPLC was performed using isocratic high performance liquid chromatography (Jasco HPLC system) with a LC-PU 2080 Plus pump, manual injector with loop volume of 20µL (Rheodyne), programmable MD 2010 PDA detector and Thermo C_{18} 0860953Q (250 x 4.6 mm i.d, 5 µm particle size). The HPLC system was equipped with Borwin-PDA software (version1.5). An electronic balance (Shimadzu AY-120), UV-Visible (Jasco model V-550) spectrophotometer, ElgaLab water (PURELAB UHQ-II) water purification system were used in this study.

Selection of analytical wavelength

From the standard stock solution further dilutions were done using methanol and scanned over the range of 200–400 nm.

Selection of mobile phase

The standard solution of Fluindione (30µg/ml) was injected into the HPLC system and run in different solvent systems. Different mobile phases like acetonitrile and water, methanol and acetate

buffer, acetonitrile and phosphate buffer, methanol and phosphate buffer in varying proportion of mobile phase components, varying conditions of pH were tried in order to obtain the desired system suitability parameters for the Fluindione

Optimized chromatographic conditions

The mobile phase consisted of methanol and 10mM di-sodium hydrogen phosphate buffer (pH 3.5) in the ratio of 85: 15 v/v. It was then filtered through 0.45 µ membrane filter paper using vacuum filtration assembly and then sonicated on ultrasonic water bath for 15 min. The flow rate of mobile phase was maintained at 1ml/min. The column and the HPLC systems were kept in ambient temperature.

Preparation of solutions

Preparation of standard stock solution

Prepared standard stock solution of Fluindione was prepared in methanol (1000 µg/ml). Further dilution was made in methanol to get final solution of Fluindione 10, 20, 30, 40, 50µg/ml.

Preparation of buffer

90 mg of di-sodium hydrogen phosphate was dissolved in100ml of HPLC grade water and the pH was adjusted to 3.5 using10% ortho-phosphoric acid.

Preparation of 0.1 N Sodium hydroxide solution

Sodium hydroxide powder (40mg) was dissolved in methanol in 10 ml volumetric flask the volume was made up with methanol to get 0.1N sodium hydroxide solution.

Preparation of 0.1 N hydrochloric acid solution

Hydrochloric acid (0.1N) was prepared by diluting 0.085 ml of concentrated hydrochloric acid solution with methanol up to 10 ml.

Preparation of 3 % v/v hydrogen peroxide solution

Hydrogen peroxide (3 % v/v) was prepared by appropriately diluting 1 ml of 30 % v/v hydrogen peroxide to 10 ml with methanol.

Preparation of sample solution of blend

Strength of marketed tablets is 20 mg of Fluindione per tablet since marketed formulation was not available in Indian market; the excipient blend was spiked with Fluindione for assay and accuracy.

Preparation of blend

Blend containing 250 mg Fluindione was prepared by spiking drug into blank blend 1.125 gm Starch, 1.125 gm Lactose. Geometric mixing method was used. Blend equivalent to 20 mg of Fluindione was used for studies.

Forced degradation studies

In order to determine whether the method is stability indicating, Forced degradation studies were carried under condition of acid, alkaline and neutral hydrolysis, oxidation, dry heat and photolysis as per ICH Q1A (R2) and Q1B. The analysis was carried out by HPLC with a DAD detector. For each study, two samples were prepared: the blank subjected to stress in the same manner as the drug solution and working standard solution of Fluindione subjected to degradation condition. Dry heat and photolytic degradation were carried out in solid state [6-8].

Acid hydrolysis

Acid induced degradation was performed by adding 1 ml of 0.1N Hydrochloric acid (HCl) to volumetric flask containing 1ml of Fluindione standard solution (300µg/ml). The volume was made up to 10 ml with methanol & kept for 3 days in dark place. The solution was neutralized with 0.1ml of Sodium hydroxide (NaOH) solution. Final solution (30µg/ml) was injected.

Alkaline hydrolysis

Alkali induced forced degradation was performed by adding 1 ml of 0.01N sodium hydroxide (NaOH) to volumetric flask containing 1ml of Fluindione standard solution (300µg/ml). The volume was made up to 10 ml with methanol & kept for 1 hr in dark place. The solution was neutralized with 0.1 ml of Hydrochloric acid (HCl) solution. Final solution (30µg/ml) was injected.

Neutral Hydrolysis

Neutral hydrolysis was performed by adding 1ml of Fluindione standard solution (300µg/ml) was mixed with 1ml of water in 10ml of volumetric flask and the volume was made upto the mark with methanol. Solution was kept for 1 hr dark place. Final solution (30µg/ml) was injected.

Oxidative Hydrolysis

Oxidative degradation was performed by adding 1ml of Hydrogen peroxide (H_2O_2, 0.3% v/v) to volumetric flask containing 1ml of Fluindione standard solution (300µg/ml). The volume was made up to 10 ml with methanol & kept for 1 hr protected from light.

Degradation under dry heat

Dry heat study was performed by exposing Fluindione in oven (60^0C) for a period of 6 hours. A sample was withdrawn after 6 hours, weighed and dissolved in methanol to get solution of 1000 µg/ ml and further dilutions were made with methanol to get final concentration (30 µg/ ml)

Photo-degradation studies

Photolytic degradation studies were carried out by exposure of drug to UV light up to 200 watt hours /square meter and subsequently to fluorescence light illumination not less than 1.2 million lux hours. Sample was weighed, dissolved in methanol to get concentration of 1000 µg/ml. and further dilutions were made with methanol to get final concentration (30 µg/ ml).

LC-MS Conditions

For the identification of unknown impurity during forced degradation study under acid stress conditions LC-MS system was used. Column: Thermo scientific-50 x 2.1 micron size was used as stationary phase. The flow rate was 350µl/min and the column temperature was maintained at 23°C. Mobile phase contains a mixture of methanol: water (60:40 v/v) pH 3.0 adjusted with formic acid. The analysis was performed in positive electrospray ionization mode.

RESULTS AND DISCUSSION

Selection of analytical wavelength

The UV spectrum was scanned over range of 200-400 nm. The drug showed considerable absorbance at 285 nm (Figure 2).

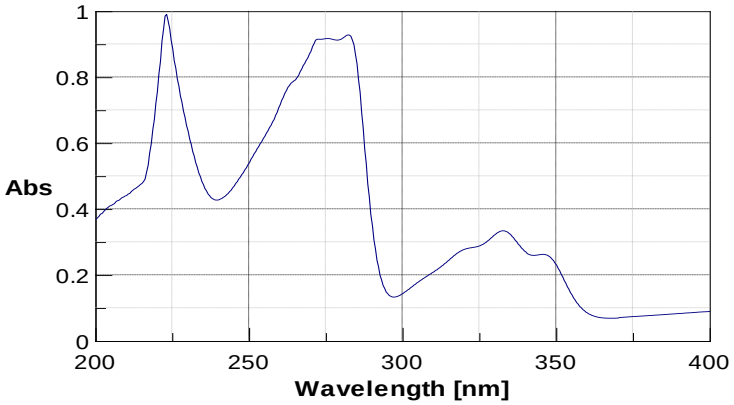


Figure 2: UV Spectrum of Fluindione (10µg/ml)

Selection of mobile phase

After several trials, methanol and 10 mM di-sodium hydrogen phosphate pH 3.5 in the ratio of 85: 15 v/v was chosen as the mobile phase, which gave good resolution and acceptable peak parameters.

Chromatogram and system suitability parameter of drug

The column was equilibrated with the mobile phase (indicated by constant back pressure at desired flow rate). Working standard solution of drug (30µg/ml) was injected into the system. The retention time for the drug was found to be 2.4 ± 0.2 min. System suitability parameters of Fluindione are summarized in (Table 1).

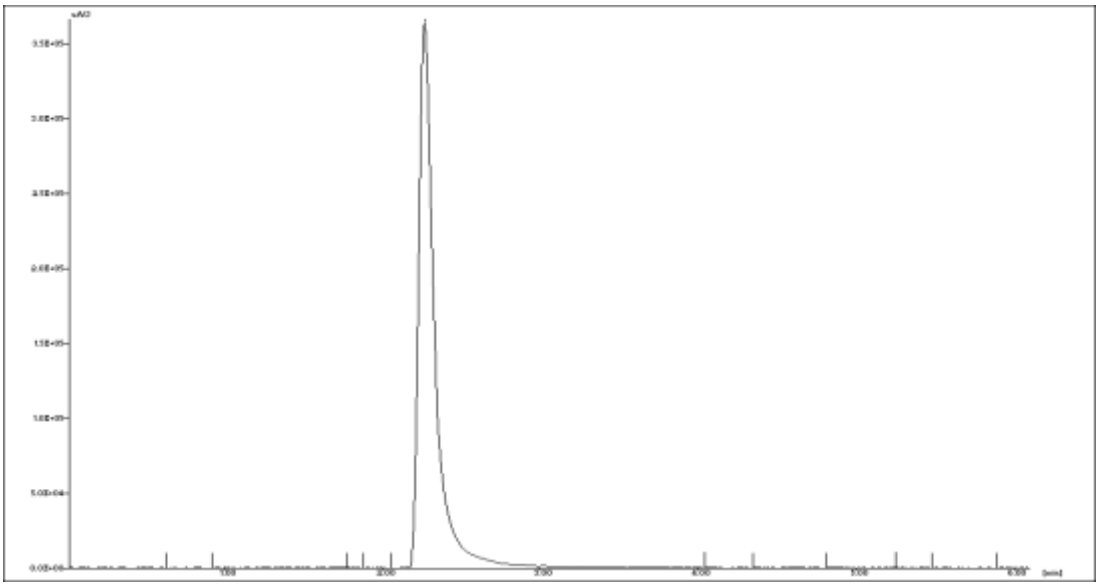


Figure 3: Chromatogram of standard solution of Fluindione (RT = 2.4 ± 0.2 min)

Table 1: System suitability parameter

Name	RT (Min)	Conc. (µg/ml)	Area	Theoretical Plates	Asymmetry
Fluindione	2.42	30	4548923.6	3470.26	1.68

Stress degradation studies of bulk drug

Acid hydrolysis

Under acid hydrolysis, 83.06 % Fluindione was recovered with the product of degradation at RT 3.2 min. The chromatogram was showed in Figure 4 with degradation product.

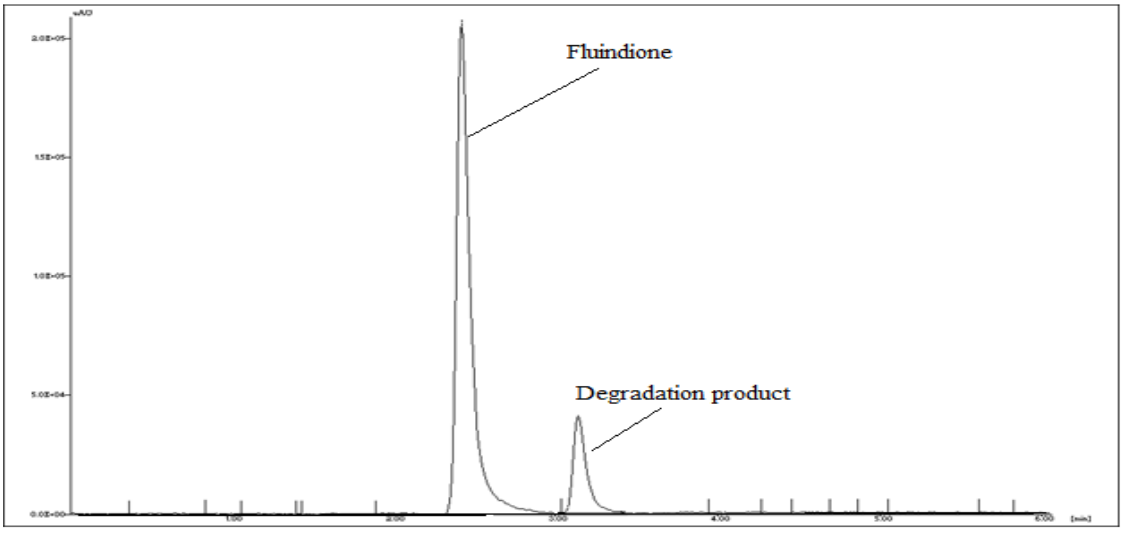
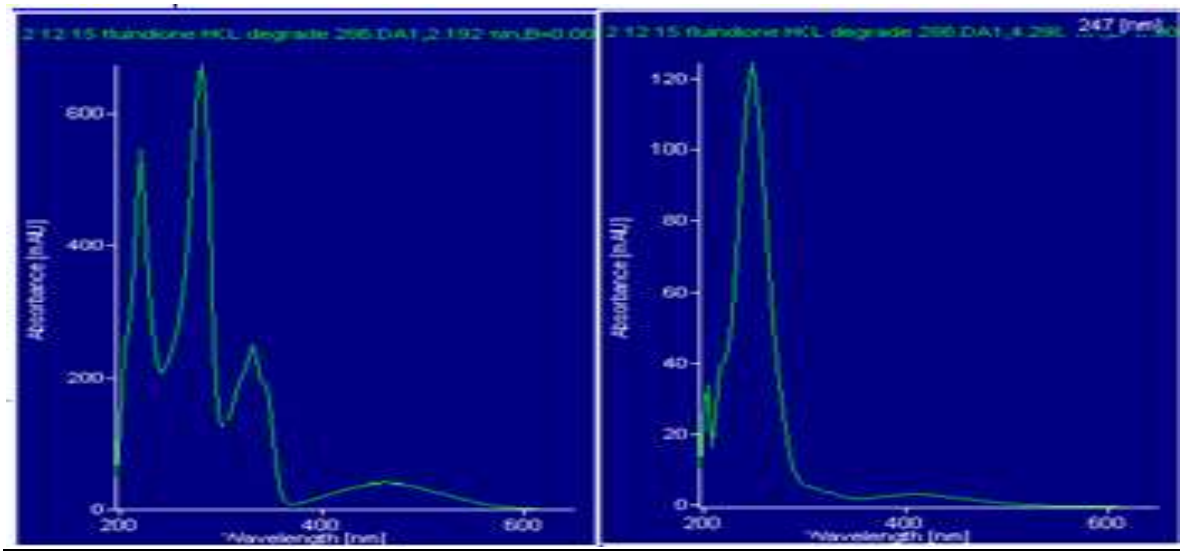


Figure 4: Chromatogram of acid treated Fluindione (30µg/ml)

Degradation product Confirmed by HPTLC at λmax 247nm and HPLC at λmax 247nm are same in UV spectrum.



A) **B)**

Figure 5: A) UV spectrum of Fluindione at RT- 2.4 min. B) UV spectrum of degradation product at RT- 3.2 min

Alkaline hydrolysis

After alkaline hydrolysis, 84.74 % Fluindione was recovered with no peak of degradation.

Neutral Hydrolysis

Neutral hydrolysis studies showed about 90.86 % Fluindione was recovered with no peak of degradation

Oxidative Hydrolysis

Oxidative hydrolysis studies showed about 89.57 % Fluindione was recovered with no peak of degradation

Degradation under dry heat

After the dry heat degradation, 83.46% Fluindione was recovered with no peaks of degradation.

Photo-degradation studies

After the photo degradation study under UV light 80.47% and Fluorescence light 79.52% Fluindione was recovered with no peak of degradation.

Characterization of degradation product by LC-MS

Acid hydrolysis

From LC-MS study it was observed that, for the chromatographic condition used for LC-MS, Fluindione RT 1.16 min and the acid catalyzed degradation product eluted at 3.04 min. Mass of Fluindione peak eluted at 1.16 min was m/z 240 where as mass of the degradation product eluting

at 3.04 min was m/z 239.04. The probable structure of degradation product m/z 239.04 is shown in (Figure 6).

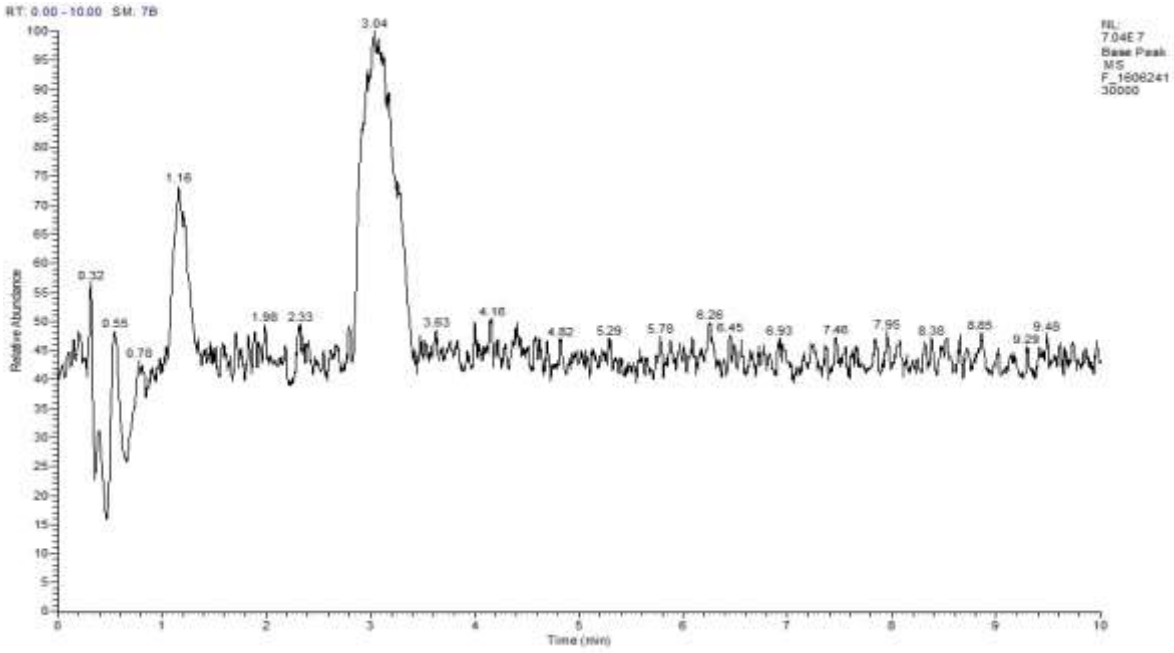


Figure 6: Chromatogram obtained from sample subjected to acid degradation

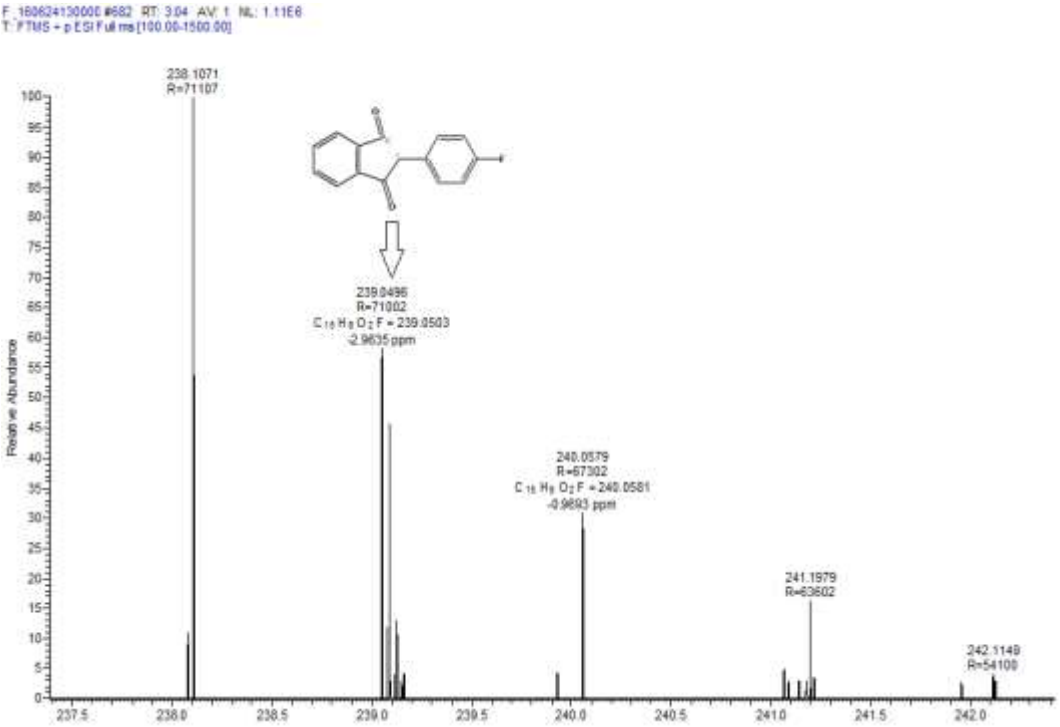
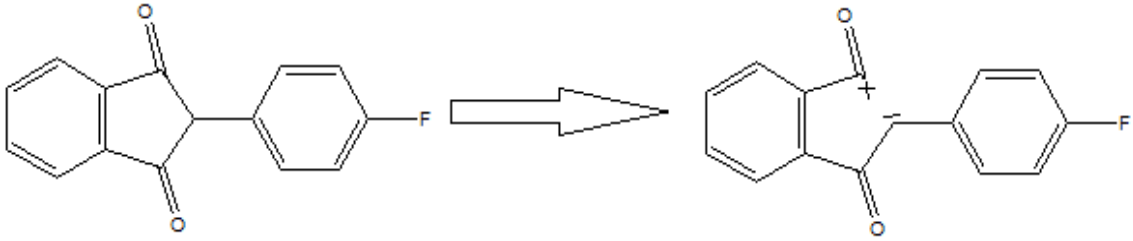


Figure 7: LC-MC spectra of acid degradation product



Fluindione cleavage of cyclopentadione ring

Figure 8: Structure Fluindione and acid degradation product

Probable reaction mechanism

A) Cleavage of ring

Cleavage of cyclopentadione ring

Table 2: Summary of stress degradation study of Fluindione

Sr. No	Stress Degradation Conditions	%Recovery	RT of Degradation product	Pear purity	
				Peak front	Peak tail
1	Acid hydrolysis (0.1N HCl for 3 days)	83.06	3.2	990.2	994.2
2	Base hydrolysis (0.01N NaOH for 1 hrs)	84.74	-	981.3	987.25
3	Oxidation (0.3 % H_2O_2 for 1 hr)	89.57	-	987.3	989.2
4	Neutral Hydrolysis (1 ml water for 48 hrs)	90.86	-	992.1	995.6
5	Dry Heat (60^0c in oven for 6 hrs)	83.46	-	996.3	993.8
6	UV (200 watt hours./square meter)	80.47	-	998.2	998.5
	Florescent light, (1.2 million Lux. Hrs)	79.52		997.2	998.1

Validation of Analytical Method

The method was validated as per ICH Q2 (R1) guidelines [9].

Specificity

The specificity was carried out by determining the peak purity values which were found to be more than 990, indicating that there is non interference of any impurities or degradation product in retention time of analytical peak. The study was performed by injecting blank.

Table 3: specificity

Drug	Purity tail	Purity front
Fluindione	994.8	997.8

Linearity

Linearity was tested for the range of concentrations 10-50µg/ml. Each sample in Five replicates was analyzed and peak areas were recorded. The response factors were plotted against the

corresponding concentrations of Fluindione to obtain the calibration curve. Figure 9 and 10 represents the linearity and calibration curve for Fluindione respectively.

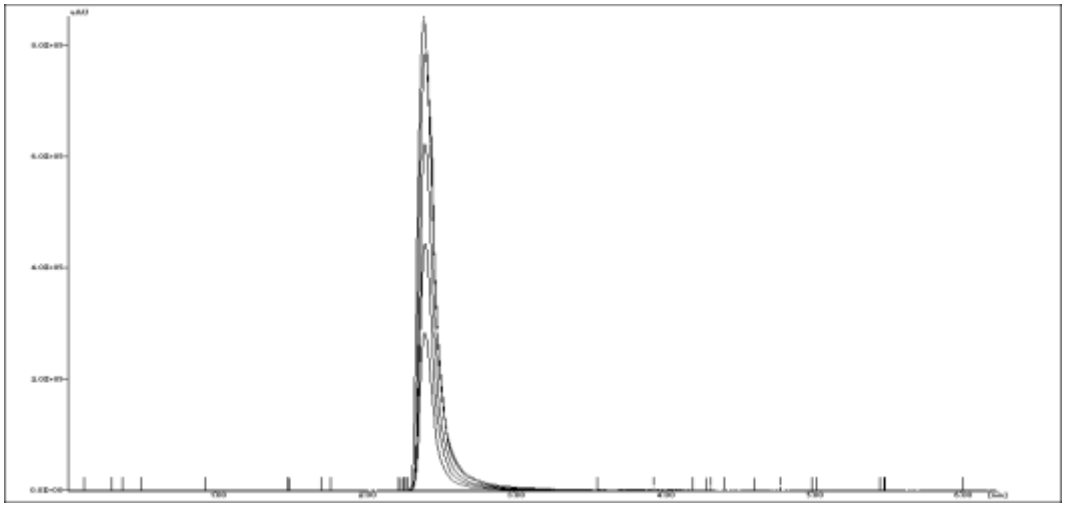


Figure 9: Chromatogram of linearity of Fluindione (10-50µg/ml)

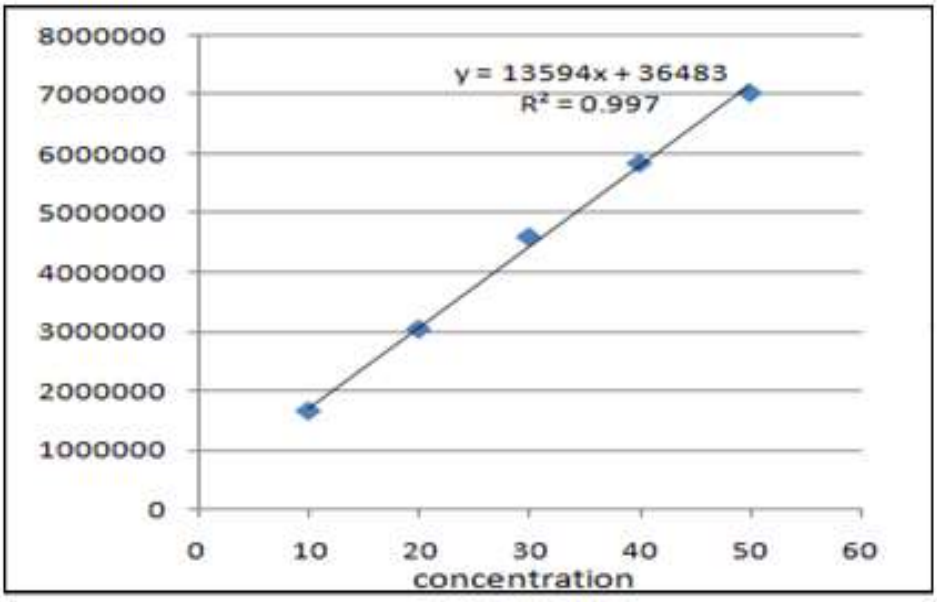


Figure 10: Calibration curve for Fluindione

Range

The linearity range of was found to be 10-50µg/ml.

Assay

Blend containing 250 mg Fluindione was prepared by spiking drug into blank blend 1.125 gm Starch, 1.125 gm Lactose. Geometric mixing method was used. Blend equivalent to 20 mg of Fluindione used for assay. It was repeated for six times. The sample solution was injected and area was recorded. Concentration and % purity was determined from linearity equation. The results obtained are shown in (Table 4).

Table 4: Assay of Spiked Blend

Sr. No.	Peak area Of Fluindione (20µg/ml)	Amount Recovered(µg/ml)	% Recovery
1	3044260	19.70	98.54828
2	3076179	19.94	99.72224
3	3104984	20.15	100.7817
4	3095786	20.08	100.4434
5	3178834	20.69	103.4979
6	3098656	20.10	100.5489
Mean	**3099783.15**	**20.11**	**100.59**
SD	**44572.11**	**0.327**	**1.63**
%RSD	**1.43**	**1.62**	**1.62**

Accuracy

The accuracy study was performed recovery studies were carried out by adding standard drug to Blend (Excipient) at three different levels 80%, 100% and 120 %. Each level The accuracy study was performed for % recovery of Fluindione. Concentration of sample chosen was 20 µg/ml of standard. These solutions were injected into HPLC system in triplicate to obtain the chromatogram. The drug concentrations were calculated by using linearity equation of Fluindione. The results obtained are shown in (Table 5)

Table 5: Recovery studies of Fluindione

Level	Amount taken (µg/ml)	Amount added (µg/ml)	Area	Recovered Conc.(µg/ml)	% Recovery
80%	20	16	5340841	36.60311	101.67
100%	20	20	5912080	40.8051	102.03
120%	20	24	6433975	44.64411	101.46

Precision

The precision study was performed the method was demonstrated by intra-day and inter-day studies. In the intra-day studies, 3 replicates of 3 standard solutions (10, 20 and 30µg/ml) were analyzed in a same day and percentage RSD was calculated (Table 6). For the inter-day variation studies, 9 replicates of 3 standard solutions (10, 20 and 30µg/ml) were analyzed on 3 consecutive days and percentage RSD was calculated (Table 7).

Table 6: Intra-day precision study of Fluindione

Concentration(µg/ml)	Area	Mean Area	SD	% RSD
10	3096699 3110603 3184508	3130603	1.735913	1.706494
20	4423546 4462583	4462611	0.958199	0.953656

	4501703			
30	5878095	5908298	0.910272	0.892921
	5881375			
	5965423			

Table 7: Inter-day precision of Fluindione

Concentration(µg/ml)	Area	Mean Area	SD	% RSD
10	3110603	3113549	1.334237	1.319764
	3078834			
	3151208			
20	4396471	4456614	1.40723	1.40261
	4510788			
	4462583			
30	5895786	5853096	1.243332	1.2319
	5775144			
	5888357			

Limit of Detection (LOD)

LOD is calculated from the formula: -

$$LOD = \frac{3.3\ \sigma}{S}$$

Where,

σ = standard deviation of peak area of drug for the lowest conc. in the range

S = slope of the calibration curve.

LOD of Fluindione = 1.246 µg/ ml

Limit of Quantification (LOQ)

The Quantitation limit is expressed as:

$$LOQ = \frac{10\,\sigma}{S}$$

LOQ of Fluindione was found to be 3.7779µg/ ml.

Robustness

Robustness of the method was determined by carrying out the analysis under conditions during which flow rate, concentration (strength) of buffer, mobile phase ratio were altered and the effects on the peak area were noted (Table 8).

Table 8: Robustness study

Drug	% RSD Found For Robustness Study(peak area)					
	Flow Rate (1 ml/min)		pH. of Buffer (pH 3.5)		Mobile phase ratio (85:15 v/v)	
	0.9	1.1	3.4	3.6	83:17	87:13
Fluindione	1.286	1.644	1.051	1.411	1.60	1.47

Summary of validation study

Table 9: Summary of validation parameter

Sr. No.	Validation parameters	Fluindione
1	Linearity Equation	y= 135945x + 364831
	(r2)	r^2 = 0.9974
	Range	10-30µg/ml
2	Precision (% RSD)	
	Interday	1.318
	Intraday	1.184
3	Accuracy	% Recovery
	80	101.67
	100	102.0
	120	101.46
4	Limit of Detection	1.246 µg/ml
5	Limit of Quantitation	3.779 µg/ml
6	Specificity	Specific
7	Robustness	Robust

DISCUSSION

The percent degradation under various stress conditions in our study, match fairly with reported method but this literature report does not mention the product of degradation, hence there is a need to investigate acid catalysed hydrolysis and characterization of degradation product by LC-MS.This probable structure does not match the bathochromic shift observed to UV λmax of 247 nm obtained by HPTLC and HPLC.

CONCLUSION

The developed method is stability indicating, since the drug peak was found to be pure as confirmed by peak purity profiling studies. In the forced degradation studies, there was interference of a degradation product in the acidic condition which was confirmed by LC-MS studies. The developed method is specific, accurate, precise, and robust and can be used for routine quality control as well as assessing the stability of Fluindione.

ACKNOWLEDGEMENT

The authors are thankful to principal and management of AISSMS college of Pharmacy, Pune for providing the research facilities. We also thankful to Mylan Laboratories Ltd. Hyderabad (India) for providing us Fluindione as gift sample.

REFERENCES

1. https://www.google.co.in/fluindione (accessed on 21/10/2015).

2. https://en.wikipedia.org/wiki/fluindione (accessed on 22/10/2015).

3. Imming P, Sinning C, Meyer A. Drugs, their targets and the nature and number of drug targets. Nat Rev Drug Discov 2006;5(10):821-34.

4. Rao NM,Gowrisankar D. Development and validation of Stability-Indicating RP-HPLC and UV-Spectrophotometric method for the Estimation of Fluindionein Bulk and Tablet Dosage Form. Eurasian J ournal of Analytical Chemistry 2015; 10(2): 127-136.
5. Thimmaraju M, Hemanth K. Development and validation of UV spectrophotometric method for Fluindione. Der Pharmacia Lettre, 2015; 7(2): 237-242.
6. International Conference on Harmonization (ICH), Stability testing of new drug substances and products, Q1A (R2), (2003).
7. International Conference on Harmonization (ICH), Photo stability testing of new drug substances and products, Q1B.
8. International Conference on Harmonization (ICH), Impurities in new drug product Q3B, (2006).
9. International Conference on Harmonization (ICH) harmonized tripartite guideline Validation of analytical procedures: text and methodology Q2 (R1) ICH, Geneva, Nov (2005).