

VALIDATION OF A FORCED DEGRADATION UPLC METHOD FOR ESTIMATION OF FLUNIXIN

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ABSTRACT

A specific, precise, accurate and forced degradation UPLC method is validated for estimation of Flunixin. The method employed, with Hypersil BDS C18 (100 mm x 2.1 mm, 1.7 μ m) column in an gradient mode, with mobile phase of Methanol, 0.1% Orthophosphoric acid and Acetonitrile in the ratio of 45:35:20 %v/v/v. The flow rate was 0.1 ml/min and effluent was monitored at 239 nm. Retention time was found to be 3.453 \pm 0.08 min. The method was validated in terms of linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ) etc. in accordance with ICH guidelines. Linear regression analysis data for the calibration plot showed that there was good linear relationship between response and concentration in the range of 1- 10 μ g/ml respectively. The LOD and LOQ values for were found to be 0.31607(μ g/ml) and 0.95781(μ g/ml) respectively. No chromatographic interference from parenteral's excipients and degradants were found. The proposed method was successfully used for estimation of (parenterals form).

KEYWORDS: Flunixin, UPLC, Validation, forced degradation method.

1. INTRODUCTION

Flunixin, 2-[[2-Methyl-3-(trifluoromethyl) phenyl] amino] pyridine-3-carboxylic acid (Fig. 1), is a nonsteroidal anti-inflammatory drug (NSAID), analgesic, and antipyretic used in horses, cattle and pigs. It is often formulated as the meglumine salt.^[1,2]

Chemical stability of Flunixin is a matter of great concern as it affects the safety and efficacy of the finished drug product. Forced degradation studies provide data to support identification of possible degradants; degradation pathways and intrinsic stability of the Flunixin molecule and validation of stability indicating analytical procedures.^[4,5,6]

A detailed literature revealed that several analytical methods have been reported for the determination of Flunixin in pharmaceutical dosage forms. In our present knowledge, there is no method reported for the estimation forced degradation studies of Flunixin in pharmaceutical dosage form by UPLC.^[13-17]

Hence, the aim was to develop a specific, rapid, sensitive, and accurate UPLC method which can estimate the forced degradation studies of Flunixin.

The present work aims with the developed method to be only this mobile phase is sufficient for quantification of degraded products under stress.

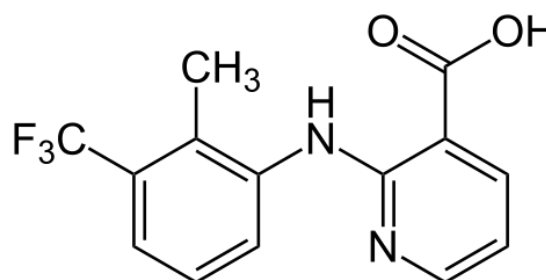


Fig. 1: Molecular Structure of Flunixin, 2-[[2-Methyl-3-(trifluoromethyl) phenyl] amino] pyridine- 3-carboxylic acid.^[12]

2. EXPERIMENTAL

Materials

Flunixin (99.99 % purity) used as analytical standard was procured from Active Pharma Labs (Hyderabad). HPLC grade methanol, Acetonitrile (HPLC grade) was purchased from Qualigens fine chemicals, Mumbai, India. Distilled, 0.45 μ m filtered water used for UPLC quantification and preparation of buffer. Buffers and all other chemicals were analytical grade.

Instrumentation

Acquity, Waters UPLC system consisting of a Water 2695 binary gradient pump, an inbuilt auto sampler, a column oven and Water 2996 wavelength absorbance detector (PDA) was employed throughout the analysis.

The data was collected using Empower 2 software. The column used was Hypersil BDS C18 (100 mm x 2.1 mm, 1.7 μ m). A Band line sonerex sonicator was used for enhancing dissolution of the compounds. A Labindia pH System 362 was used for pH adjustment.

Chromatographic Conditions**Table 1: Chromatographic Conditions of the validating method.**

Parameter	Value
Column	Hypersil BDS C18 (100 mm x 2.1 mm, 1.7 μ m)
Mobile Phase	Methanol, 0.1% Orthophosphoric acid and Acetonitrile in the ratio of 45:35:20 %v/v/v
Flow rate	0.1 mL/min
Run time	12 Min.
Column Temperature	Maintained at ambient temperature
Injection volume	20 μ L
Detection wavelength	239nm
Diluent	Mobile Phase

Preparation of Standard Stock Solution**Preparation of Diluent**

In order to achieve the separation under the optimized conditions after experimental trials that can be summarized. Stationary phase like Hypersil BDS C18 (100 mm x 2.1 mm, 1.7 μ m) column was most suitable one, since it produced symmetrical peaks with high resolution and a very good sensitivity and with good resolution. The flow rate was maintained 0.1mL min⁻¹ shows good resolution. The PDA detector response of Flunixin was studied and the best wavelength was found to be 239 nm showing highest sensitivity.

The mixture of three solutions methanol, 0.1% orthophosphoric acid and acetonitrile in the ratio of 45:35:20 %v/v/v. The buffer used is 2.96 gms of Sodium phosphate in 100 ml with gradient programming was used as mobile phase at 0.1 mL/min was found to be an appropriate mobile phase for separation of Flunixin. The column was maintained at ambient temperature.

Preparation of internal standard solution: (Sol 1)

Weighed accurately about 100 mg of Testosterone propionate working standard and transfer to 1000 ml volumetric flask, add 100 ml Diluent and sonicate to dissolve it completely and then dilute to 1000 ml with diluent. 10 ml of the above solution to 100 ml with diluent and filter out above solution through the 0.45micron filter. (10 μ g/ml Concentration)

Preparation of Flunixin standard solution: (Sol 2)

Weighed accurately about 100 mg of Flunixin and transfer to 1000 ml volumetric flask, add 100 ml Diluent and sonicate to dissolve it completely and then dilute to 1000 ml with diluent.

Preparation of Mixed Standard solution

Pipette out 10 ml of (solution 1) and (solution 2) in to 100 ml volumetric flask, dilute to volume with diluent and mix.

Preparation of Sample solution

Weigh 1000 ml dry volumetric flask into 1000 ml volumetric flask and weigh accurately the sample quantity for calculation add 80 ml diluent and sonicate for 10 minutes, cool to room temperature and diluent to make up 1000 ml. Transfer 100 ml above solution and 300 ml of (solution 1) into 500 ml volumetric flask and make up the volume with diluents. Filter the solution through 0.45 μ .filter (concentration of Flunixin 10 mcg/ml).

Forced Degradation Studies

Forced degradation studies like acid hydrolysis, basic hydrolysis, dry heat degradation, wet heat degradation and oxidative degradation were carried out.

3. RESULTS AND DISCUSSIONS**Validation**

The analytical method was validated with respect to parameters such as linearity, precision, specificity and accuracy, limit of detection (LOD), limit of quantitation (LOQ) and robustness in compliance with ICH guidelines.

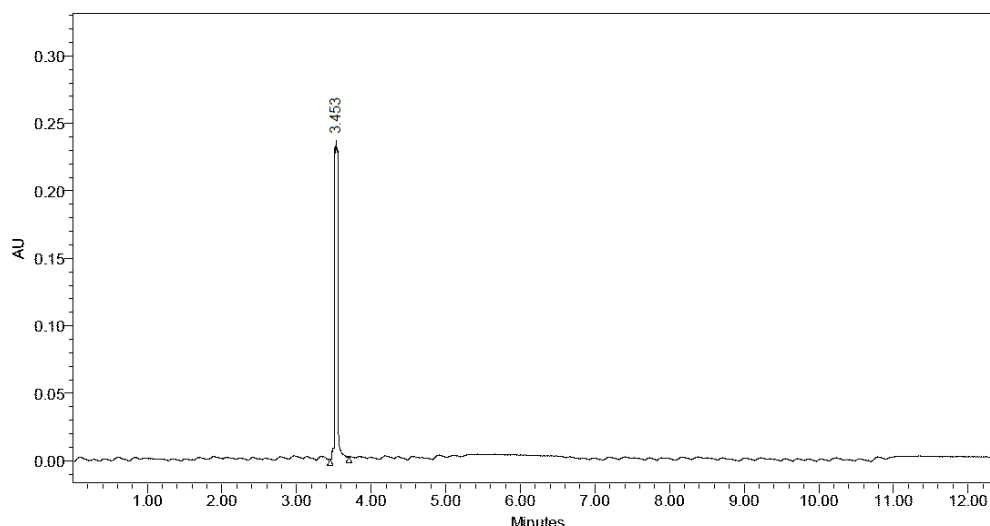


Fig. 2: Standard Chromatogram of Flunixin, using mobile phase of Methanol, 0.1% Orthophosphoric acid and Acetonitrile in the ratio of 45:35:20 % v/v/v.

Linearity and Range

The linearity of an analytical procedure is the ability to obtain test results that are directly proportional to the concentration of an analyte in the sample.

The calibration curve showed good linearity in the range of 1 – 10 µg/ml, for Flunixin(API) with correlation coefficient (r^2) of 0.9996. A typical calibration curve has the regression equation of $y = 51046.38x + 4603.667$ for Flunixin. Results are given in Table 3.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD and LOQ of Flunixin were calculated by mathematical equation. $LOD = 3.3 \times \text{standard deviation} \div \text{slope}$ and $LOQ = 10 \times \text{standard deviation} \div \text{slope}$. The LOD of Flunixin was found to be 0.31607(µg/ml) and the LOQ of Flunixin was found to be 0.95781(µg/ml). Results are given in Table 3.

Table 3: Summary of validation parameters for the proposed method.

Parameter	Flunixin
Linearity	1 – 10 ppm
Intercept (c)	4603.667
Slope (m)	51046.38
Correlation coefficient	0.9996
LOD	0.31607(µg/ml)
LOQ	0.95781(µg/ml)

Precision

The Precision of the method was studied in terms of intraday and interday precision of sample injections (5µg/ml). Intraday precision was investigated by injecting six replicate samples of each of the sample on the same day. The % RSD was found to be 0.534. Interday precision was assessed by analysis of the 6

solutions on three consecutive days. The % RSD obtained was found to be 0.925. Low % RSD values indicate that the method is precise. The results are given in table 4.

Robustness

Small deliberate changes in chromatographic conditions such as change in temperature ($\pm 2^\circ\text{C}$), flow rate ($\pm 0.1\text{ml/min}$) and wavelength of detection ($\pm 2\text{nm}$) were studied to determine the robustness of the method. The results were in favor of (% RSD < 2%) the developed UPLC method for the analysis of Flunixin. The results are given in table 6.

Accuracy

To study the accuracy of method, recovery studies were carried out by spiking of standard drug solution to pre-analyzed sample at three different levels i.e., at 50, 100, and 150%. The resultant solutions were then reanalyzed by the proposed method. At each level of the amount, six determinations were performed. From the data obtained, the method was found to be accurate. The % recovery and %RSD were calculated and presented in Table 5.

Table 4: Results of Precision Studies.

Flunixin			
Precision Studies			
Parameter	Peak Area	% RSD	%LC
Intraday precision	512738	0.02%	99.98%
	512619		99.99%
	512803		99.99%
Inter day precisio	513583	0.02%	100.14%
	513691		100.16%
	513830		100.19%
Instrument:1 Acquity UPLC Waters, 2695H	512637	0.03%	99.96%
	512764		99.98%
	512906		100.01%
Instrument:2 Agilent Technologies,1290	512639	0.03%	99.96%
	512772		99.98%
	512908		100.01%
Average			100.02
Std. Dev			0.083
%RSD			0.08%

Table 5: Results of accuracy study.

Flunixin						
Level %	Amount added (µg/ml)	Amount found (µg/ml)	% Recovery	Mean recovery (%)	Std. Dev	% RSD
50	16.51	16.29	98.66	98.90	0.552	0.56%
100	33.03	32.88	99.54			
150	49.54	48.81	98.52			

Table 6: Results of Robustness Studies.

Robustness Studies			
Parameter	Value	Peak Area	% RSD
Flow Rate	Low	512939	0.06%
	Actual	513272	
	Plus	513508	
Temperature	Low	512948	0.06%
	Actual	513265	
	Plus	513523	
Wavelength	Low	512604	0.13%
	Actual	513442	
	Plus	513897	

Forced Degradation Studies

According to Singh and Bakshi, the stress testing suggests a target degradation of 20-80 % for establishing stability indicating nature of the method. UPLC study of samples obtained on stress testing of Flunixin under different conditions using mixture Methanol, 0.1% Orthophosphoric acid and Acetonitrile in the ratio of 45:35:20 %v/v/v as a mobile solvent system suggested the following degradation behaviour.

a. Acid hydrolysis

An accurate 10 ml of pure drug sample solution was transferred to a clean and dry round bottom flask (RBF). 30 ml of 0.1 N HCl was added to it. It was refluxed in a

water bath at 60°C for 4 hours. Drug became soluble after reflux which was insoluble initially. Allowed to cool at room temperature. The sample was then neutralized using 2N NaOH solution and final volume of the sample was made up to 100ml with water to prepare 100ppm solution. It was injected into the UPLC system against a blank of Methanol, 0.1% Orthophosphoric acid and Acetonitrile in the ratio of 45:35:20 %v/v/v after optimizing the mobile phase composition, chromatogram was recorded and shown in Fig. 3.

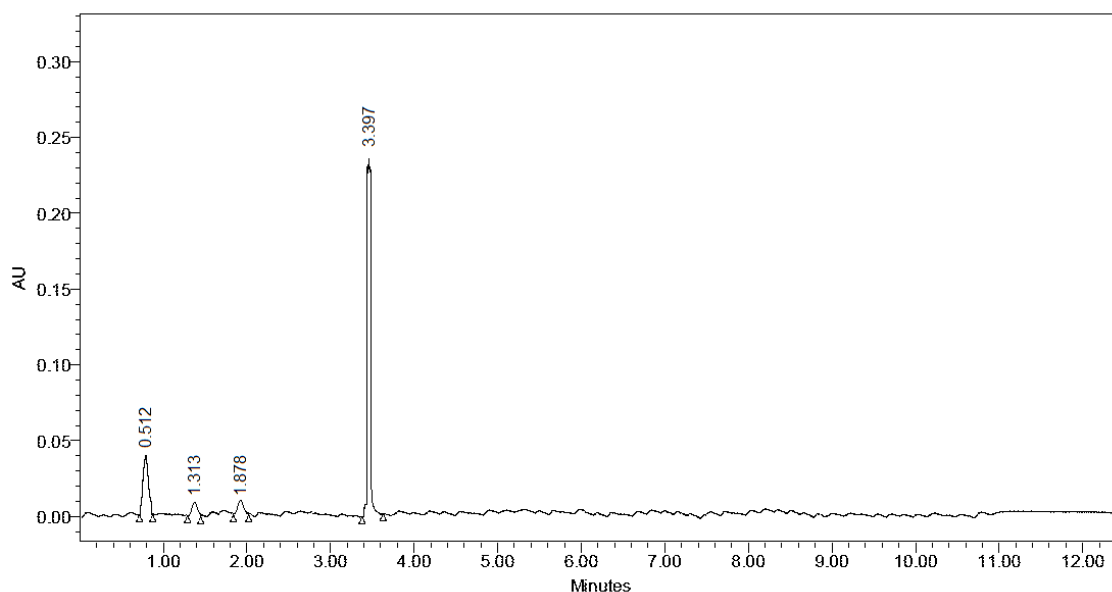


Fig. 3: Chromatogram showing the degraded products in Acidic degradation.

b. Basic hydrolysis

An accurate 10 ml of pure drug sample solution was transferred to a clean and dry RBF. 30 ml of 0.1N NaOH was added to it. It was refluxed in a water bath at 60°C for 4 hours. Drug became soluble after reflux which was insoluble initially. It was allowed to cool at room temperature. The sample was then neutralized using 2N

HCl solution and final volume of the sample was made up to 100ml with water to prepare 100ppm solution. It was injected into the UPLC system against a blank of Methanol, 0.1% Orthophosphoric acid and Acetonitrile in the ratio of 45:35:20 %v/v/v after optimizing the mobile phase composition, chromatogram was recorded and shown in Fig. 4.

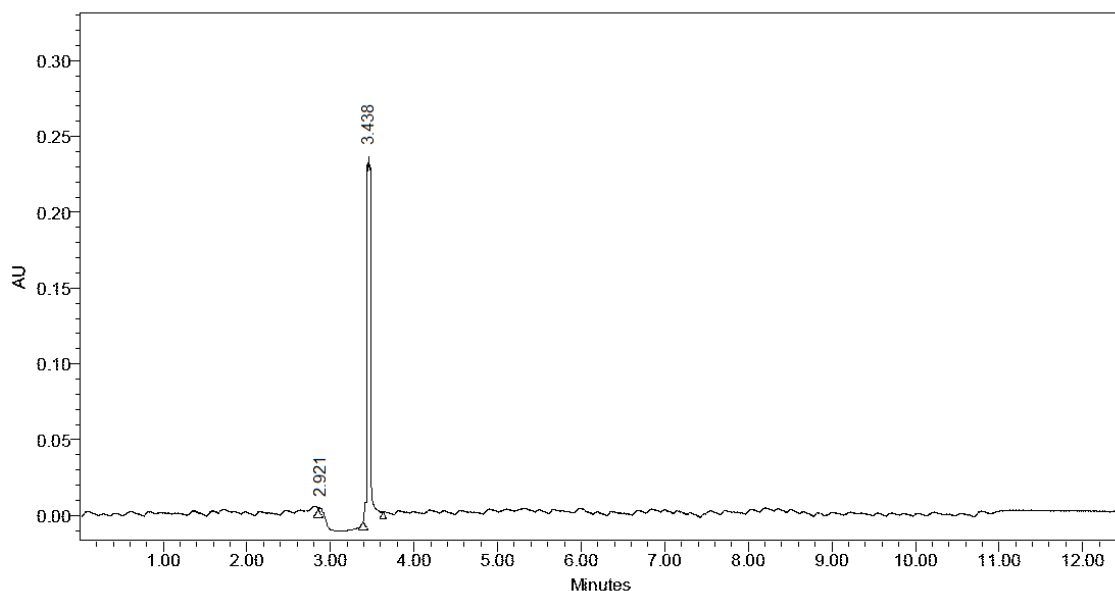


Fig. 4: Chromatogram showing the degraded products in Basic degradation.

c. Wet heat degradation

Accurate 10 ml of pure drug sample was transferred to a clean and dry RBF. 30 ml of HPLC grade water was added to it. Then, it was refluxed in a water bath at 60°C for 6 hours uninterruptedly. After the completion of reflux, the drug became soluble and the mixture of drug and water was allowed to cool at room temperature.

Final volume was made up to 100 ml with HPLC grade water to prepare 100 ppm solution. It was injected into the UPLC system against a blank of Methanol, 0.1% Orthophosphoric acid and Acetonitrile in the ratio of 45:35:20 %v/v/v after optimizing the mobile phase composition, chromatogram was recorded and shown in Fig. 5.

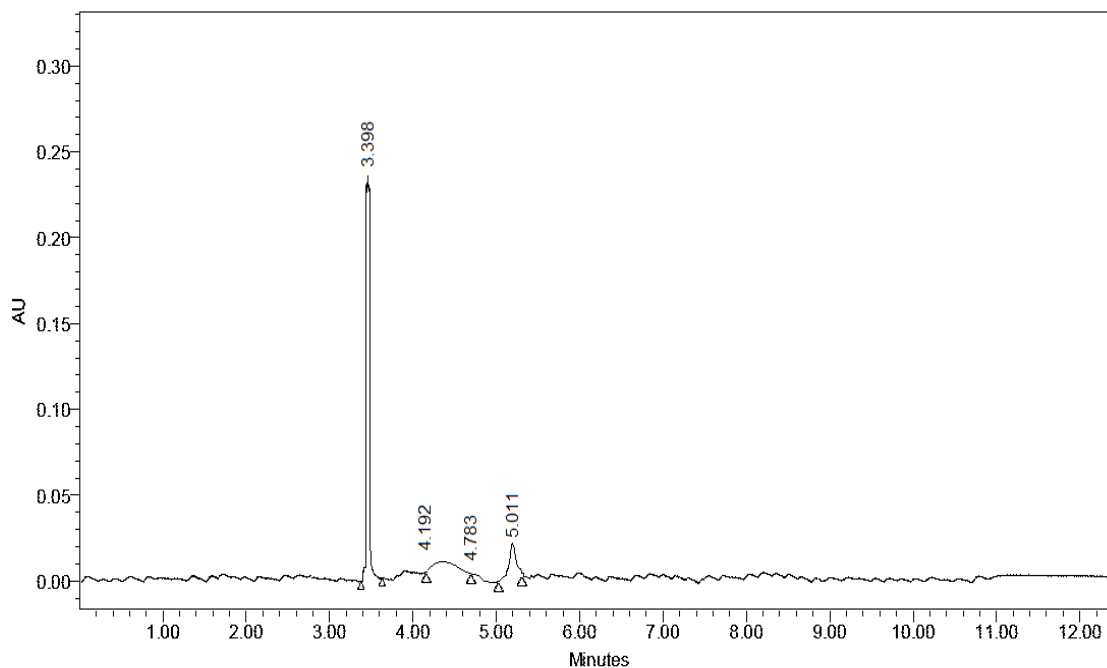


Fig. 5: Chromatogram showing the degraded products in Wet heat degradation.

d. Oxidation with (3%) H₂O₂

Approximately 10 ml of pure drug sample was transferred in a clean and dry 100 ml volumetric flask. 30 ml of 3% H₂O₂ and a little methanol was added to it to make it soluble and then kept as such in dark for 24

hours. Final volume was made up to 100 ml using water to prepare 100 ppm solution. The above sample was injected into the UPLC system. The chromatogram was recorded and shown in Fig. 6.

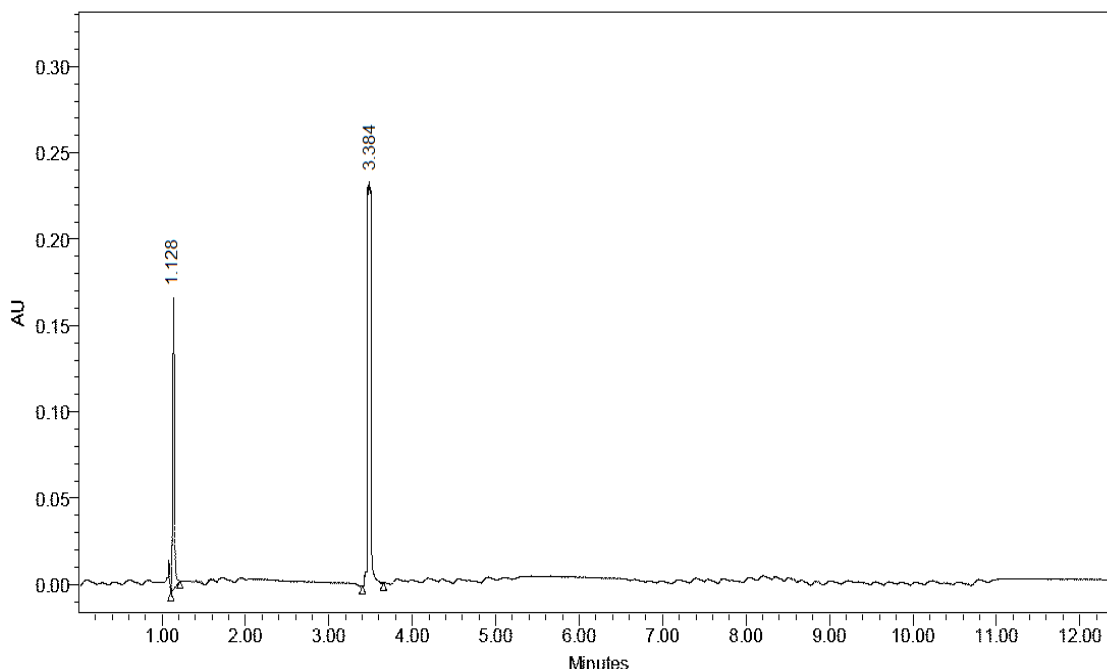


Fig. 6: Chromatogram showing the degraded products in H₂O₂.

In all degradation studies, there was a significant formation of degradation products when compared to that of a standard. This indicates that, the drug may be

degraded to low molecular weight non-chromophoric compounds.

Table 7: Forced Degradation study for the developed method.

Nature of Stress	Degradation condition	Time(h)	Number of degradation products (Rt)
Acidic	60°C	3	3 (0.512, 1.313, 1.878)
Basic	60°C	9	1 (2.921)
Oxidative	RT	48	1 (1.128)
Wet Heat	105°C	24	3 (4.192, 4.783, 5.011)

4. CONCLUSION

A specific, sensitive and selective forced degradation UPLC method has been validated for the analysis of Flunixinin bulk dug and parenteral dosage form. Based on peak purity results, obtained from the analysis of force degradation samples using described method, it can be concluded that the absence of co-eluting peak along with the main peak of Flunixin indicated that the developed method is specific for the estimation of Flunixinin presence of degradation products. Further the proposed UPLC method has excellent precision, sensitivity and reproducibility. Even though no attempt has been made to identify the degraded products, proposed method can be used as forced degradation method for assay of Flunixinin commercial formulations.

5. ACKNOWLEDGEMENTS

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