

**A COMPARATIVE STUDY OF DIFFERENT APPROACHES FOR
STABILITY-INDICATING DETERMINATION OF FLUNIXIN
MEGLUMINE IN PRESENCE OF ITS ALKALINE-INDUCED
PRODUCT**

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ABSTRACT

Four Simple, rapid, sensitive, accurate and precise stability-indicating spectrophotometric methods were developed for the determination of flunixin meglumine (flunixin-M) in pure and dosage forms. The first method is the second derivative technique; is based on measuring derivatized spectra at 226.8 nm in the range of 5 –50 $\mu\text{g ml}^{-1}$ with LOD of 0.763 $\mu\text{g ml}^{-1}$ and LOQ of 2.312 $\mu\text{g ml}^{-1}$. The second method is the derivative ratio method (1DD); is used for the determination of flunixin-M in presence of its degradation product at 272 nm in the range of 5 –50 $\mu\text{g ml}^{-1}$ with LOD of 0.672 $\mu\text{g ml}^{-1}$ and LOQ of 2.038 $\mu\text{g ml}^{-1}$. The third method is the ratio difference method; is based on measuring the difference in the amplitude of flunixin-M in presence of its degradation product at two different wavelengths ,this is done at 270 nm and 287 nm in the range of 5 – 50 $\mu\text{g ml}^{-1}$ with LOD of 0.273 $\mu\text{g ml}^{-1}$ and LOQ of 0.828 $\mu\text{g ml}^{-1}$. The fourth method is the mean centering method; the method was applied for analysis of flunixin-M in presence of its degradation product at 288 nm in the range of 5 – 50 $\mu\text{g ml}^{-1}$ with LOD of 0.260 $\mu\text{g ml}^{-1}$ and LOQ of 0.790 $\mu\text{g ml}^{-1}$. The obtained results were statistically compared with those of the reported method by applying t-test and F-test at 95% confidence level and no significant difference was observed regarding accuracy and precision.

KEYWORDS: Flunixin-M, second derivative, derivative ratio, ratio difference, mean centering, pure form, dosage forms, stability-indicating methods

1. INTRODUCTION

Flunixin, 2-[[2-methyl-3-(trifluoro-methyl) phenyl] amino]-3- pyridine carboxylic acid **figure (1)**^[1], is usually found as its meglumine salt. Its actions are related to its ability to inhibit cyclooxygenase. It is used in horses for the alleviation of inflammation, pain associated with musculoskeletal disorders and visceral pain associated with colic. In cattle, it is indicated for the control of pyrexia associated with bovine respiratory diseases, endotoxemia and acute bovine mastitis.^[2] Various methods were reported for the determination of flunixin-M including electrochemical^[3], gas chromatography^[4-6], thin layer chromatography^[7], spectrophotometric^[8] and liquid chromatographic (LC) methods.^[7,9-14] The aim of the present study is to develop simple, sensitive, rapid, reliable and precise UV spectrophotometric methods for analysis of flunixin-M in pure form and in pharmaceutical formulations in presence of its degradation product without previous separation by UV-VIS spectrophotometry. Suitable statistical tests were performed on validation data.^[15,16]

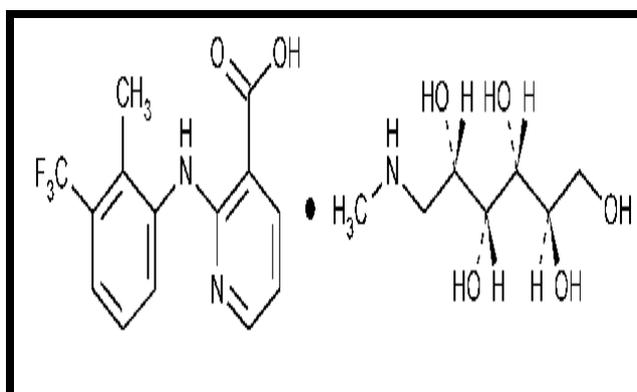


Figure 1: chemical structure of flunixin-M.

2. EXPERIMENTAL

2.1. Instruments

- Shimadzu UV-Vis. 1650 Spectrophotometer (Japan).
- Hot plate (Torrey pines Scientific, USA).
- Jenway, 3510 pH meter (Jenway, USA).
- Rota-Vapor SCI-Logics (RE-100-PRO) with Buchi pump.

2.2. Materials

2.2.1. Pure samples

Pure flunixin-M (99.75%) was kindly supplied by Adwiya Pharmaceutical Industries, Cairo, Egypt.

2.2.2. Pharmaceutical preparation

Flunidyne[®] injections: each (1 ml) claimed to contain 83 mg flunixin-M (B.No. 1256/13, manufactured by Arab company for medical products, Egypt), purchased from local market.

2.3. Chemicals and reagents

- All reagents used were of analytical grade, water used throughout the procedure was freshly distilled.
- Methanol, hydrochloric acid and sodium hydroxide (El-Nasr Company, Egypt).

2.4. Standard Solution

2.4.1. Standard solution of intact flunixin-M

A standard solution of flunixin-M (100 µg/ml) was prepared by dissolving 10 mg of the drug powder in 50 ml of methanol and complete to 100 ml with methanol.

2.4.2. Standard solution of degraded sample

100 mg of pure flunixin-M powder was dissolved in 45 ml distilled water and transferred to a 100-ml round bottomed flask to which 10 ml of 5 N NaOH were added. The solution was heated under reflux for 7 hours, filter and the filtrate was adjusted to pH 1.7 using 5 N HCl where the carboxylic acid degradation product was precipitated. The obtained residue was extracted with methanol (2×10 ml), filtered into a 100-ml volumetric flask and diluted to volume with methanol to obtain a stock solution labeled to contain degradation product derived from 1 mg/ml of flunixin-M. Working solution of degradation product (100 µg/ml) was obtained by further dilution of the stock solution with methanol.

3. Confirmation of the carboxylic acid degradation product

3.1. Confirmation of the degradation product using TLC technique

Complete degradation was exactly determined by spotting on TLC plates every 30 minutes using mobile phase system consists of dichloromethane - methanol (3:2 v/v), complete degradation of flunixin-M was confirmed by absence of spot in the region of the degradation product corresponds to the spot of the intact drug.

3.2. Confirmation of the degradation product using IR technique

Confirming degradation using IR technique for both flunixin-M and its degradation product was achieved. IR spectrum of flunixin-M **figure (2)**, shows a peak of carboxylic acid (-OH) at 3174.24 cm^{-1} and peak of secondary amine (-NH-) at 3362.77 cm^{-1} . However, IR spectrum of its degradation product **figure (3)**, shows disappearance of secondary amine and appearance of (C=O) stretch of carboxylic acid at 1710.47 and peak of carboxylic acid (-OH) still present.

3.3. Confirmation of the degradation product using ^1H NMR techniques

The ^1H NMR of flunixin-M using deuterated methanol as a solvent **figure (4)**, shows signals of six aromatic protons at $7.314 - 8.323$ ppm, 2 peaks of secondary amines at $3.305 - 3.316$ ppm and appearance of 2 peaks of 2 (-CH₃) groups at $2.434 - 2.701$ ppm and peaks of 12 protons of 6 (-CH₂-) at $3.679 - 3.698$ ppm. However, ^1H NMR of its degradation product using deuterated methanol as a solvent **figure (5)**, shows disappearance of the 6 (-CH₂-) and 2 (-CH₃) peaks. ^1H NMR of the degradation product only shows appearance of aromatic protons at $7.540 - 9.123$ ppm and carboxylic acid (-OH) at 10.925 ppm. The expected equation of degradation pathway showed in **figure (6)**.

4. Procedures

4.1. Construction of the Calibration Curves (linearity)

4.1.1. Method A (second derivative method)

Different aliquots of flunixin-M standard solution ($100\text{ }\mu\text{g/ml}$) ranging from ($50-500$) μg were transferred to 10-ml volumetric flasks and completed to volume with methanol. The absorption spectra (from 200 to 400 nm) of these solutions were recorded using methanol as a blank. The second derivative corresponding to each absorption spectrum was recorded, using $\Delta\lambda = 4$ nm. The amplitude values were measured at 226.8 nm. The measured amplitude values versus the final drug concentrations in $\mu\text{g/ml}$ were plotted to get the calibration graph and the regression equation was derived.

4.1.2. Method B (Derivative ratio method)

Different aliquots of flunixin-M standard solution ($100\text{ }\mu\text{g/ml}$) ranging from ($50-500$) μg were transferred to 10-ml volumetric flasks and completed to volume with methanol. The absorption spectra (from 200 to 400 nm) of these solutions were recorded using methanol as a blank and then divided by the spectrum of flunixin-M degradation product solution ($25\text{ }\mu\text{g/ml}$). The first derivative corresponding to each ratio spectrum was recorded, using $\Delta\lambda = 4$

nm. The amplitude values were measured at 272 nm. The measured amplitudes versus the final drug concentrations in $\mu\text{g/ml}$ were plotted to get the calibration graph and the regression equation was derived.

4.1.3. Method C (Ratio difference method)

To the ratio spectra obtained as before, the difference in the peak amplitudes (ΔP) in the ratio spectra was measured at 270 and 287 nm (ΔP 270-287 nm). The measured difference values in peak amplitudes (ΔP) versus the final drug concentrations in $\mu\text{g/ml}$ were plotted to get the calibration graph and the regression equation was derived.

4.1.4. Method D (Mean Centering method)

The ratio spectra obtained as before were mean centered and the mean centered values were measured at 288 nm. The measured mean centered values versus the final drug concentrations in $\mu\text{g/ml}$ were plotted to get the calibration graph and the regression equation was derived.

4.2. Specificity (Procedure for synthetic mixture)

The general procedures under each method was repeated using aliquots of flunixin-M solution (100 $\mu\text{g/ml}$) together with aliquots of flunixin-M degradation product solution (100 $\mu\text{g/ml}$). Flunixin-M concentrations were calculated from the corresponding regression equation.

4.3. Procedure for pharmaceutical preparation

Contents of 5 **Flunidyne**[®] injections (83 mg/ml) were mixed well. A volume equivalent to 10 mg of flunixin-M was transferred into 100-ml volumetric flask and completed to volume with methanol to obtain a solution labeled to contain 100 $\mu\text{g/ml}$ of flunixin-M. Repeat the general procedure under each method using aliquots covering the working concentration range. Determine flunixin-M content of the injections from the corresponding regression equation.

5. RESULTS AND DISCUSSION

5.1. Method A (Second derivative method)^[17]

The zero-order absorption spectra of flunixin-M and its alkaline degradation product show severe overlap **figure (7)**, which does not permit direct determination of flunixin-M in presence of its carboxylic acid degradation product.

In this method, the second derivatives of the absorption spectra were obtained to increase the selectivity of the method and the amplitudes at 226.8 nm are proportional to the

concentrations of the drug without interference from its degradation product, as shown in **figure (8)**.

Linearity range, regression equation, intercept, slope and squared correlation coefficient for the calibration data were presented in **table (1)**.

5.2. Method B (Derivative ratio method)^[18-21]

The zero-order absorption spectra of flunixin-M and its alkaline degradation product show severe overlap **figure (7)**, which does not permit direct determination of flunixin-M in presence of its degradation product.

In this method, the absorption spectra of the drug were divided by the absorption spectrum of the degradation product (25 µg/ml), as a divisor, to get the ratio spectra, as shown in **figure (9)**. The amplitudes of the first derivative of the ratio spectra at 272 nm are proportional to the concentrations of the drug without interference from its degradation product (divisor), as shown in **figure (10)**.

Linearity range, regression equation, intercept, slope and squared correlation coefficient for the calibration data were presented in **table (1)**.

5.3. Method C (Ratio difference method)^[22]

The zero-order absorption spectra of flunixin-M and its alkaline degradation product show severe overlap **figure (7)**, which does not permit direct determination of flunixin-M in presence of its degradation product.

In this method, the absorption spectra of the drug were divided by the absorption spectrum of the degradation product (25 µg/ml), as a divisor, to get the ratio spectra, as shown in **figure (9)**. The difference in peak amplitudes between 270 and 287 nm in the ratio spectra is proportional to the concentration of the drug without interference from its degradation product (divisor).

Linearity range, regression equation, intercept, slope and squared correlation coefficient for the calibration data were presented in **table (1)**.

5.4. Method D (Mean centering method)^[23]

The zero-order absorption spectra of flunixin-M and its alkaline degradation product show severe overlap **figure (7)**, which does not permit direct determination of flunixin-M in presence of its degradation product.

In this method, the absorption spectra of the drug were divided by the absorption spectrum of the degradation product (25 µg/ml), as a divisor, to get the ratio spectra, as shown in **figure (9)**. The obtained ratio spectra were mean centered. The mean centered values at 288 nm are proportional to the concentrations of the drug without interference from its degradation product (divisor), as shown in **figure (11)**.

Linearity range, regression equation, intercept, slope and squared correlation coefficient for the calibration data were presented in **table (1)**.

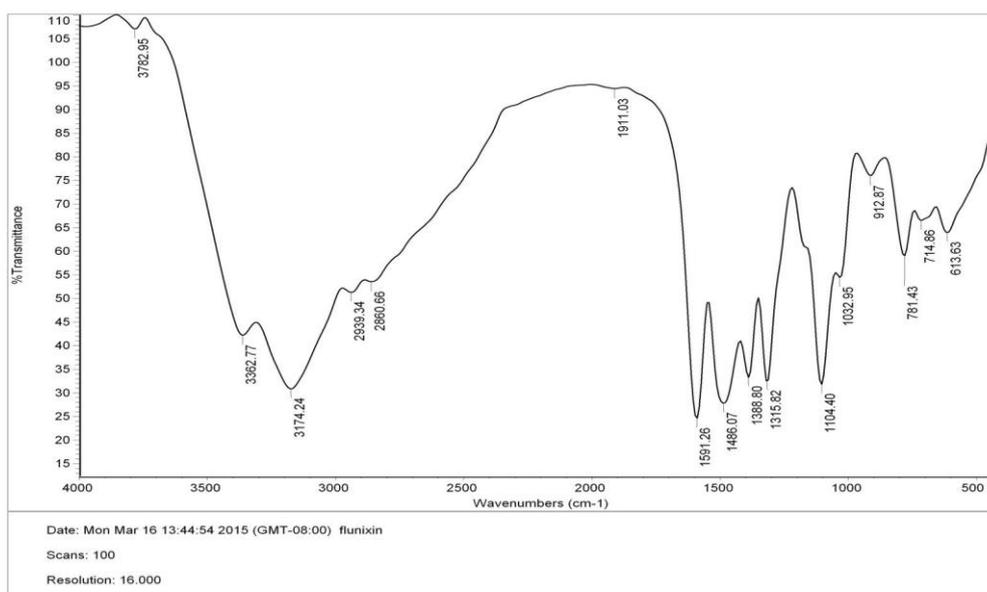


Figure (2): IR spectrum of intact flunixin-M.

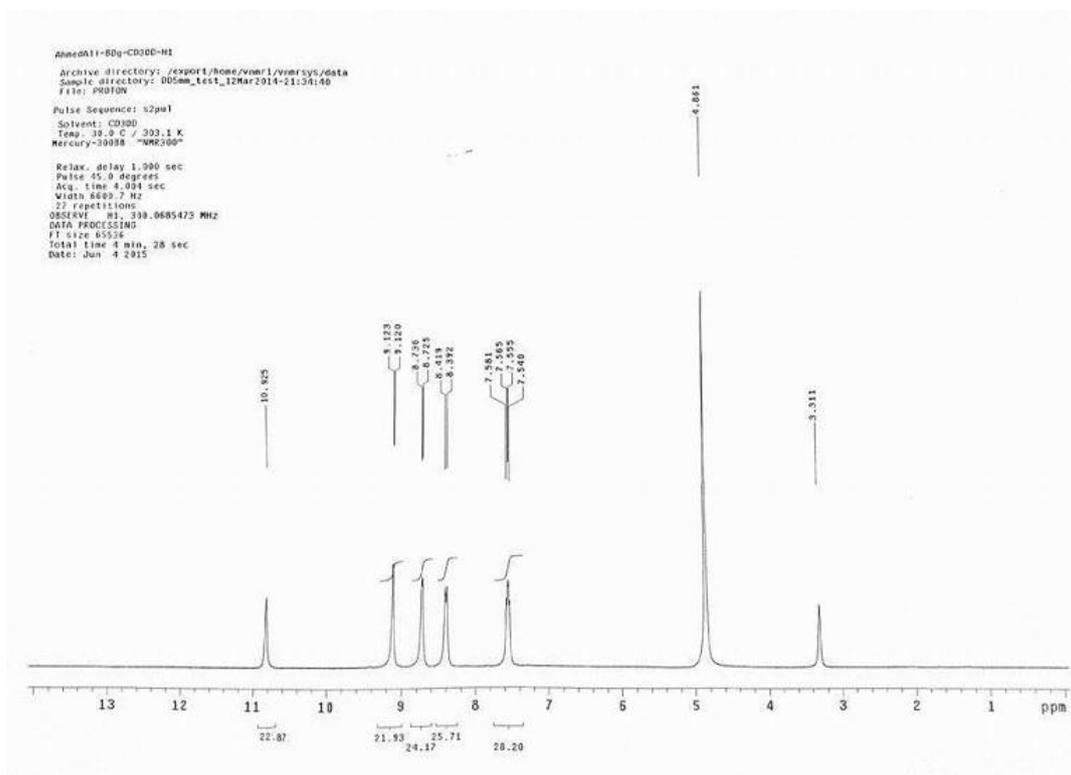


Figure (5): ^1H NMR spectrum of alkaline degradation product using methanol as a solvent.

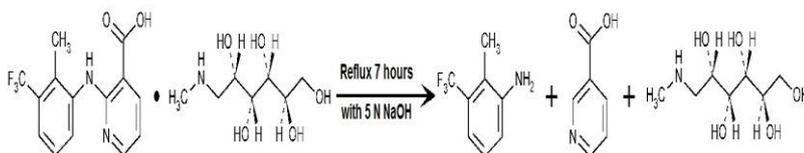


Figure (6): The expected equation of the degradation pathway.

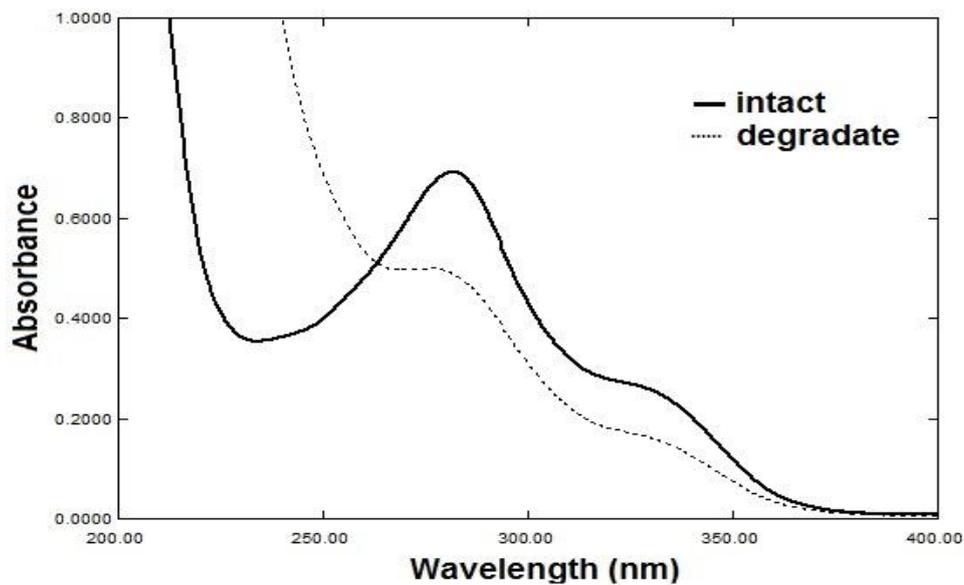


Figure (7): Absorption spectra of flunixin-M (25 $\mu\text{g/ml}$) and its alkaline degradation product (25 $\mu\text{g/ml}$) in methanol.

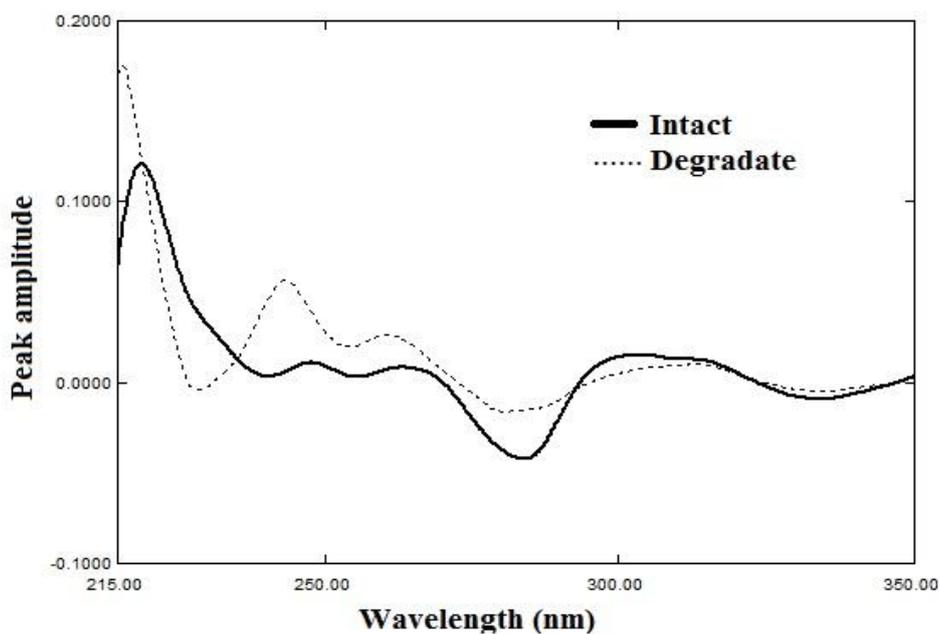


Figure (8): Second derivative of absorption spectra of flunixin-M (25 $\mu\text{g/ml}$) and its degradation product (25 $\mu\text{g/ml}$) in methanol.

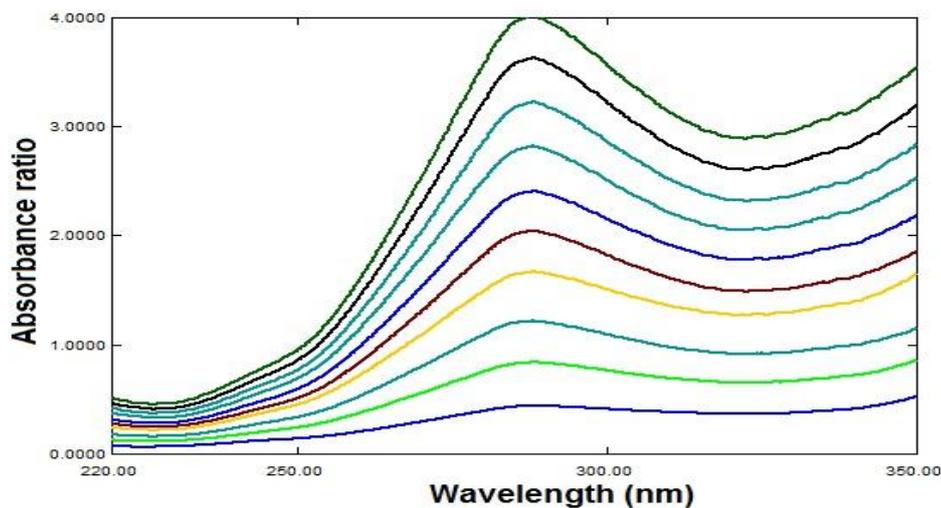


Figure (9): Ratio spectra of flunixin-M (5-50 $\mu\text{g/ml}$) using 25 $\mu\text{g/ml}$ of its degradation product as a divisor.

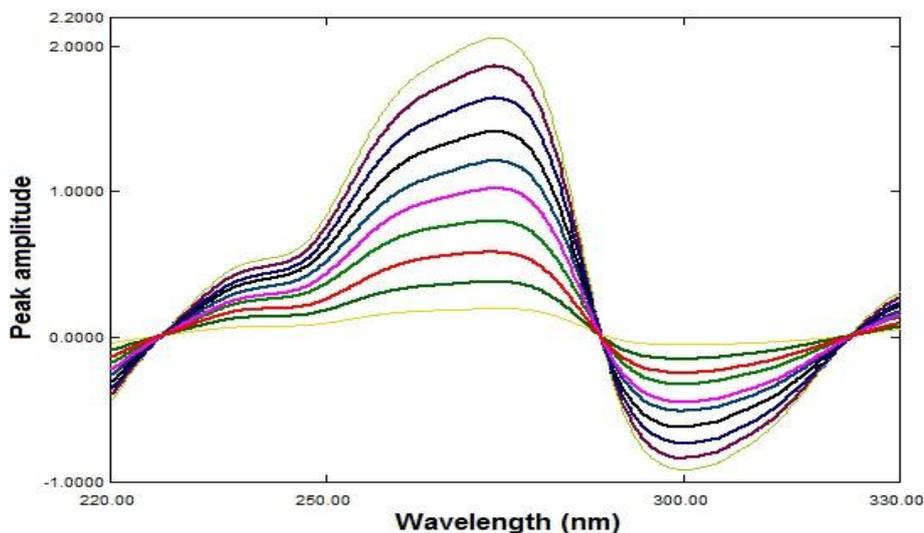


Figure (10): First derivative of the ratio spectra of flunixin-M (5-50 $\mu\text{g/ml}$) using 25 $\mu\text{g/ml}$ of its degradation product as a divisor.

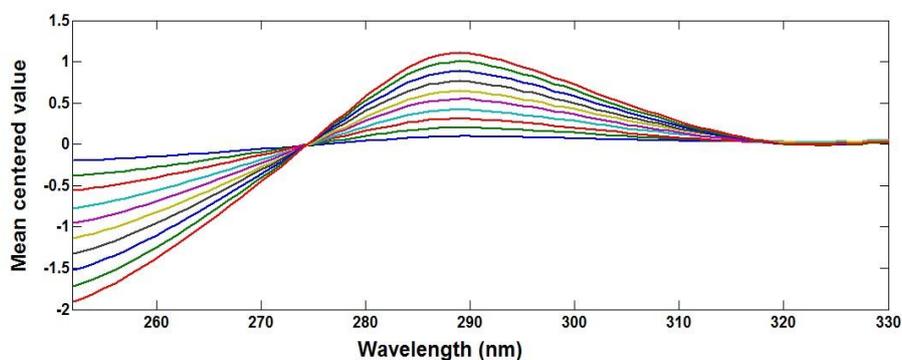


Figure (11): Mean centering of the ratio spectra of flunixin-M (5-50 $\mu\text{g/ml}$) using 25 $\mu\text{g/ml}$ of its degradation product as a divisor.

6. Methods validation

The proposed methods were validated in compliance with the ICH guidelines.^[15] **table (2)** shows the accuracy and precision of the proposed methods while **table (3)** shows the specificity; recovery of the laboratory prepared mixtures of the drug with its alkaline degradation product. LOD, LOQ, linearity and range were shown earlier in **table (1)**.

7. Pharmaceutical applications

The proposed procedures were applied to the determination of flunixin-M in **Flunidyne[®]** injections. Satisfactory results were obtained in good agreement with the label claim, indicating no interference from excipients and additives. The obtained results were statistically compared to those obtained by the reported method.^[8] No significant differences were found by applying t-test and F-test at 95% confidence level,^[16] indicating good accuracy and precision of the proposed methods for the analysis of the studied drug in its pharmaceutical dosage form, as shown in **table (4)**.

Table (1): Spectral data for the determination of the studied drug by the proposed methods.

| Parameters | Second derivative | Derivative Ratio | Ratio difference | Mean Centering |
|---|-------------------|------------------|------------------|----------------|
| Wavelength (nm) | 226.8 | 272 | 270&287 | 288 |
| Linearity range (µg/ml) | 5-50 | 5-50 | 5-50 | 5-50 |
| LOD (µg/ml) | 0.763 | 0.672 | 0.273 | 0.260 |
| LOQ (µg/ml) | 2.312 | 2.038 | 0.828 | 0.790 |
| Regression equation * | | | | |
| Slope (<i>b</i>) | 0.0018 | 0.0415 | 0.0295 | 0.0225 |
| Intercept (<i>a</i>) | 0.0043 | -0.0317 | -0.0201 | -0.0257 |
| Correlation coefficient (<i>r</i> ²) | 0.9997 | 0.9997 | 0.9996 | 0.9997 |

* $y = a + bx$ where *y* is the response and *x* is the concentration.

Table (2): Intra-day and inter-day accuracy and precision for the determination of the flunixin-M by the proposed methods.

| Method | Conc. µg.ml ⁻¹ | Intra-day | | | Inter-day | | |
|-------------------|---------------------------|------------------|---------------|------------------|------------------|---------------|------------------|
| | | Found Conc. ± SD | Accuracy (R%) | Precision (RSD%) | Found Conc. ± SD | Accuracy (R%) | Precision (RSD%) |
| Second derivative | 10 | 9.81± 0.011 | 98.16 | 0.113 | 9.84± 0.037 | 98.46 | 0.384 |
| | 30 | 29.66±0.254 | 98.88 | 0.858 | 29.74± 0.115 | 99.13 | 0.388 |
| | 45 | 45.33±0.309 | 100.74 | 0.682 | 45.12± 0.224 | 100.28 | 0.497 |

| | | | | | | | |
|------------------|----|-------------|--------|-------|--------------|--------|-------|
| Derivative ratio | 10 | 10.03±0.097 | 100.32 | 0.970 | 9.94±0.086 | 99.44 | 0.873 |
| | 30 | 29.79±0.177 | 99.30 | 0.596 | 29.72±0.169 | 99.06 | 0.569 |
| | 45 | 45.38±0.404 | 100.85 | 0.892 | 44.30±0.158 | 98.45 | 0.356 |
| Ratio difference | 10 | 10.01±0.071 | 100.14 | 0.704 | 10.17±0.089 | 101.72 | 0.881 |
| | 30 | 29.67±0.186 | 98.91 | 0.629 | 30.12±0.039 | 100.42 | 0.129 |
| | 45 | 44.86±0.447 | 99.69 | 0.997 | 45.51±0.103 | 101.15 | 0.227 |
| Mean centering | 10 | 9.84± 0.045 | 98.48 | 0.458 | 10.07± 0.038 | 100.76 | 0.386 |
| | 30 | 29.86±0.033 | 99.54 | 0.112 | 29.62 ±0.191 | 98.76 | 0.647 |
| | 45 | 44.74±0.244 | 99.42 | 0.545 | 45.08± 0.160 | 100.18 | 0.356 |

Table (3): Determination of flunixin-M and its degradation product in their laboratory mixtures by the proposed methods.

| | Intact in ($\mu\text{g ml}^{-1}$) | Degradate in ($\mu\text{g ml}^{-1}$) | Percent of degradate | Intact found in ($\mu\text{ ml}^{-1}$) | Recovery % of intact |
|-------------------|-------------------------------------|--|----------------------|--|----------------------|
| Second derivative | 45 | 5 | 10 | 45.27 | 100.61 |
| | 25 | 25 | 50 | 24.83 | 99.33 |
| | 20 | 30 | 60 | 20.05 | 100.27 |
| | 10 | 40 | 80 | 9.94 | 99.44 |
| | 5 | 45 | 90 | 4.94 | 98.88 |
| | Mean ± SD | | | | |
| Derivative ratio | 45 | 5 | 10 | 45.02 | 100.06 |
| | 40 | 10 | 20 | 39.46 | 98.65 |
| | 25 | 25 | 50 | 24.64 | 98.57 |
| | 15 | 35 | 70 | 14.86 | 99.06 |
| | 5 | 45 | 90 | 4.98 | 99.61 |
| | Mean ± SD | | | | |
| Ratio difference | 30 | 20 | 40 | 30.07 | 100.24 |
| | 20 | 30 | 60 | 20.01 | 100.02 |
| | 15 | 35 | 70 | 14.88 | 99.23 |
| | 10 | 40 | 80 | 9.80 | 98.00 |
| | 5 | 45 | 90 | 4.91 | 98.37 |
| | Mean ± SD | | | | |
| Mean centering | 30 | 20 | 40 | 30.11 | 100.37 |
| | 20 | 30 | 60 | 20.15 | 100.75 |
| | 15 | 35 | 70 | 15.04 | 100.27 |
| | 10 | 40 | 80 | 9.96 | 99.65 |
| | 5 | 45 | 90 | 5.01 | 100.21 |
| | Mean ± SD | | | | |

Table (4): Determination of flunixin-M in Flunidyne[®] injection by the proposed and reported methods.

| | Second derivative | Derivative Ratio | Ratio difference | Mean centering | Reported method ^[8] |
|--------------|-------------------|------------------|------------------|------------------|--------------------------------|
| <i>N</i> * | 5 | 5 | 5 | 5 | 5 |
| \bar{X} | 98.91 | 99.17 | 99.97 | 98.62 | 99.29 |
| <i>SD</i> | 0.625 | 0.486 | 0.661 | 0.766 | 0.960 |
| <i>RSD</i> % | 0.632 | 0.490 | 0.662 | 0.777 | 0.966 |
| <i>t</i> ** | 0.751 (2.306) | 0.262 (2.306) | 1.317 (2.306) | 1.219 (2.306) | — |
| <i>F</i> ** | 2.442 (6.388) | 3.910 (6.388) | 2.123 (6.388) | 1.561 (6.388) | — |

*No. of experimental.

**The values in the parenthesis are tabulated values of *t* and *F* at (*p*= 0.05).

8. CONCLUSION

This work introduced a comparative study of four techniques applied for the determination of flunixin-M in presence of its alkaline-induced degradation product. They are also sensitive, selective and can be used for the routine analysis of flunixin-M in its dosage form.

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