EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 5
Issue: 10
01-08
Year: 2018

VALIDATED STABILITY- INDICATING HPLC METHOD FOR DETERMINATION OF RAFOXANIDE IN PURE FORM AND PHARMACEUTICAL FORMULATION

Mohammad Wafaa I. Nassar[a], Khalid Abdel-Salam M. Attia[a], Ahmed Abdelhalim A. Mohamad[b], Ragab Ahmad M. Said[b] and Rady Fathy A. Gaber*

[1]Analytical Chemistry Department, Faculty of Pharmacy, Al-Azhar University, 11751, Nasr City, Cairo, Egypt.
[2]Analytical Chemistry Department, Faculty of Pharmacy, Heliopolis University, 11785, El-Salam City, Cairo, Egypt.

***Corresponding Author: Rady Fathy A. Gaber**
Analytical Chemistry Department, Faculty of Pharmacy, Al-Azhar University, 11751, Nasr City, Cairo, Egypt.

Article Received on 23/07/2018 **Article Revised on 13/08/2018** **Article Accepted on 03/09/2018**

ABSTRACT
A simple, sensitive and accurate stability-indicating HPLC method was developed and validated for the determination of rafoxanide in presence of its degradation products. Analysis was carried out on an BDS Hypersil C_{18} column (250mm, 4.6mm, 5µm particle size) using a mobile phase consists of acetonitrile: 0.1M ammonium acetate (70:30, v/v). The analysis was performed at ambient temperature with a flow rate of 1 mL/min and UV detection at 240 nm. Drug was exposed to different forced degradation conditions like hydrolysis, oxidation, thermal and photolysis. The method has been linear for the range of 10 – 70 µg/mL with r^2 0.9996. This work focused on the stability of rafoxanide and its degradation behaviour under different stress conditions. The method was validated according to the ICH guidelines and successfully applied to the determination of the drug in pure form and in its pharmaceutical preparation without interference from its degradation products.

KEYWORDS: Degradation products, HPLC, rafoxanide, stability indicating.

1. INTRODUCTION
Rafoxanide, **figure (1)**, is 3′-chloro-4′-(4-chlorophenoxy)-3,5-di-iodosalicyl-anilide, it is an anthelmintic drug used in veterinary medicine for the treatment of fascioliasis in cattle and sheep.[1] Literature survey reveals that rafoxanide was determined by several techniques including colorimetry[2,3], UV-spectrophotometry[3,4], TLC[5], GC[6], UPLC[5] and HPLC.[6,10]

In this work, we aimed to perform a validated stability-indicating HPLC method for the determination of rafoxanide under different stress conditions. Also this work was conducted for the quantitative analysis of the studied drug in its pharmaceutical dosage form.

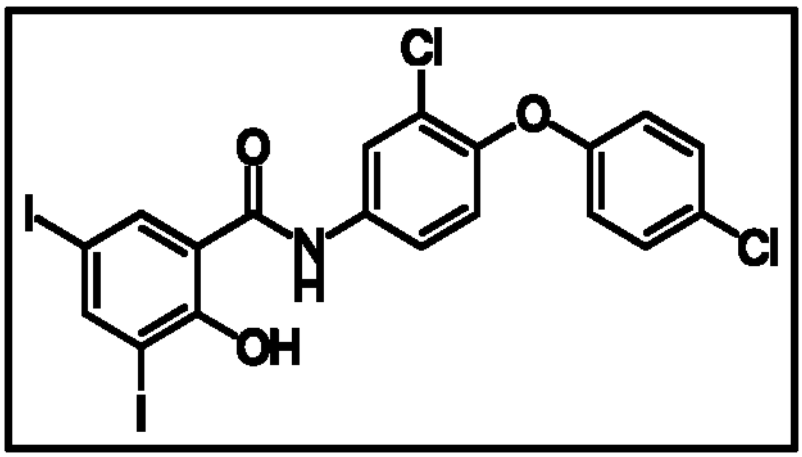

Figure (1): Structural formula of rafoxanide.

2. EXPERIMENTAL

2.1. Materials

2.1.1. Pure sample

Pure rafoxanide (99.8%) (B. No. RF/110315), was kindly supplied by Pharma-Swede, Egypt. 10th of Ramadan city, Egypt.

2.1.2. Pharmaceutical preparation

Flukanil® injection (B. No. 1509104), nominally containing 75 mg of rafoxanide per mL was kindly supplied by Pharma-Swede, Egypt. 10th of Ramadan city, Egypt.

2.2. Chemicals and reagents

All reagents used were of analytical grade, solvents were of HPLC grade, water used throughout the procedure was freshly distilled.

- Acetonitrile and methanol HPLC grade (Sigma-Aldrich, Germany).
- Ammonium acetate, prepared as 0.1M aqueous solution and potassium dihydrogen orthophosphate (Oxford®, India) prepared as 0.005M and adjusted at pH with orthophosphoric acid of analytical grade (Biotech®, Egypt).
- Hydrochloric acid and sodium hydroxide (El-Nasr Company, Egypt), prepared as 0.1 N and 1N aqueous solutions.
- Hydrogen peroxide (El-Nasr Company, Egypt), prepared as 3% aqueous solutions.

2.3. Apparatus

- HPLC, LDC Analytical (Milton Roy, USA), equipped with diode-array UV-visible detector and auto sampler injector. The chromatographic analysis was carried out using (Thermo ChromQuest 4.2.34, version 3.1.6) data analysis program.
- Hot plate (Torrey pines Scientific, USA).

2.4. Standard solution

Standard stock solution of rafoxanide (400 µg/mL) was prepared by dissolving 10 mg of the drug powder in 25-mL measuring flask using methanol as a solvent. This standard solution was stable for at least 4 and 9 days when stored at room temperature and in refrigerator, respectively.

2.5. Preparation of forced degradation solutions

- **Hydrolytic degradation**

(i) Acidic and basic hydrolysis

One mL of rafoxanide stock solution (400µg/mL) was treated with 1 mL of 1 N HCl or 1 N NaOH using 10-mL volumetric flasks. Then the sample was kept at room temperature for 6 h. Each sample was neutralized with alkali or acid before dilution and then completed to 10 mL with the mobile phase.

(ii) Neutral hydrolysis

It was done using 1 mL deionized water instead of acid or base. Then the sample was refluxed for 6 h at 80 °C, and then completed to 10 mL with the mobile phase.

(iii) Oxidative degradation

One mL of rafoxanide stock solution (400µg/mL) was diluted with 1 mL of 3% H_2O_2 using a 10-mL volumetric flask. It was kept aside for 4 h at room temperature. Then the sample was evaporated to expel the remaining H_2O_2 and completed to volume with the mobile phase.

(iv) Thermal degradation

The standard drug powder was subjected to dry heat at 80ºC for 6h. Appropriate dilution was made with the mobile phase to get (40µg/mL) of rafoxanide.

(v) Photo-degradation

One mL of rafoxanide stock solution (400µg/mL) was transferred to10-mL volumetric flask and exposed to sunlight for 24 h. Then the sample was diluted with the mobile phase to get (40µg/mL) of rafoxanide.

2.6. Procedures

2.6.1. Chromatographic conditions

At room temperature, the chromatographic separation process was performed using isocratic elution on BDS Hypersil C_{18} column (250 X 4.6mm, 5µm particle size). The eluted mobile phase consists of acetonitrile: 0.1M ammonium acetate (70:30, v/v). The mobile phase was degassed by a degasser then pumped at flow rate of 1 mL/min. UV detection was carried out at 240 nm, and the injection volume was 20 µL.

2.6.2. General procedure

Aliquots of standard rafoxanide (400 µg/mL) containing (100–700 µg) were transferred into a series of 10-ml volumetric flasks and adjusted to volume with the mobile phase. Into HPLC column, 20 µL were injected form rafoxanide solution and eluted with the mobile phase under the previously described chromatographic conditions.

2.6.3. Validation of the proposed procedure[11,12]

- **Linearity and range (construction of the calibration graph)**

The general procedure of the method under **"**2.6.2**"** was repeated. Calibration graph was constructed by plotting the peak area values of rafoxanide versus its concentrations in µg/mL, alternatively, the regression equation was derived.

- **Limit of detection (LOD) and limit of quantitation (LOQ)**

LOD and LOQ values were calculated according to ICH guidelines from the following equations:

$LOD = 3.3\ \sigma / S$

$LOQ = 10\ \sigma / S$

Where σ is the residual standard deviation of regression lines and S is the slope of the calibration curve.

- **Accuracy and precision**

Accuracy of the method, calculated as the mean percent recovery (%R), was assessed by applying the described procedure for triplicate determination of three

concentration levels covering the linearity range of rafoxanide (20, 40, 60 µg/mL).

Precision of the method, calculated as the percent of relative standard deviation (% RSD), was assessed by applying the described procedure for triplicate determination of three concentration levels covering the linearity range of rafoxanide (20, 40, 60µg/mL) within one day for repeatability and on three successive days for intermediate precision.

- **Specificity**

The specificity of the method was assessed by applying the proposed procedure for determination of rafoxanide in the presence of its possible degradation products where 20 µL from each forced degradation sample were injected and eluted with the mobile phase.

Also, the specificity of the method was determined by applying the standard addition technique through adding known quantities of rafoxanide in its pure form to already analyzed pharmaceutical preparation and the percent recovery (%R) of the pure added concentrations was calculated.

- **System suitability**

System suitability test was applied to a representative chromatogram to check various parameters such as the number of theoretical plates (N), resolution factor (Rs), capacity factor (k^{-}) and tailing factor (T). These parameters were calculated according to FDA regulations.[12]

- **Robustness**

The robustness of the method was evaluated by slight changes in the chromatographic conditions such as flow rate (±0.1 mL/min), and mobile phase contents ratio (±2%). In each case only one parameter was changed while other parameters were kept constant.

2.6.4. Procedure for pharmaceutical preparation

One mL of **Flukanil®** injection (75 mg/mL Rafoxanide) equivalent to 75 mg of rafoxanide was accurately taken and transferred into 100- mL volumetric flask and the volume was made up to 50 mL with methanol. The solution was shaken vigorously for 20 min. The volume was completed to 75 mL with methanol to produce a stock solution labeled to contain 1mg/mL of rafoxanide. The concentrations label claimed were analyzed using the general procedure of the described method.

2.6.5. Reported method[4]

Dual wavelength method, where the difference in absorbance were calculated at 242 and 281 nm.

3. RESULTS AND DISCUSSIONS

In the present study, a stability-indicating HPLC method was suggested for the determination of rafoxanide in the presence of its possible degradation products. Also, the work was conducted to the determination of the drug in bulk powder and pharmaceutical preparation.

3.1. Optimization of the experimental conditions

The conditions affecting the chromatographic performance were carefully checked to recognize the most optimum chromatographic parameters required for the best separation of the cited compound from its forced degradation products.

Several experiments were carried out using acetonitrile, methanol, water, 0.1M ammonium acetate and 0.005M potassium dihydrogen orthophosphate buffer, adjusted at pH 3.5 with orthophosphoric acid in different ratios. Initially the mobile phase consisting of acetonitrile: water (50: 50, v/v) was tried, rafoxanide was retained on the column and the peak of the drug appeared after a long period of time with longer analytical run time. Moreover, the produced peak was very broad with fronting and tailing. So we replaced acetonitrile with methanol, the peak shape was improved with good peak symmetry and less broadening behavior. But the retention time of rafoxanide was relatively close to the degradation products which led to poor resolution. Furthermore, 0.005M potassium dihydrogen orthophosphate buffer, adjusted at pH 3.5 with orthophosphoric acid and acetonitrile as a mobile phase was tried to make rafoxanide in an unionized form and increasing its retention on the column, but no elution was observed. Finally when replacement of that buffer with 0.1M ammonium acetate was occured, the chromatographic resolution and the retention conditions of rafoxanide was enhanced to be in the acceptable region, After extensive trials of ratios in that mobile phase, acetonitrile: 0.1M ammonium acetate (70: 30, v/v) proved to be the best mobile phase since it allowed the separation of rafoxanide from its degradation products within appropriate analytical run time and excellent sensitivity. The chromatographic peaks were better defined and resolved without tailing.

Several wavelengths were tried, namely 210, 220, 240 and 280 nm, and 240 nm was chosen to compromise between the sensitivity of the drug and its degradation products and enabled better visualization of the separation process as shown in **figure (2)**.

The effect of flow rate of the mobile phase on the separation of the studied drug was investigated and a flow rate of 1 mL/min was found to be the optimal one for good separation within a reasonable time.

After optimization of the chromatographic conditions, a well-defined symmetrical peak was obtained, as shown in **figure (2)**. From this chromatogram, rafoxanide was clearly eluted and its corresponding peak was sharply produced at retention time of 4.906 min± 0.036 min with minimum fronting and reasonable analysis time.

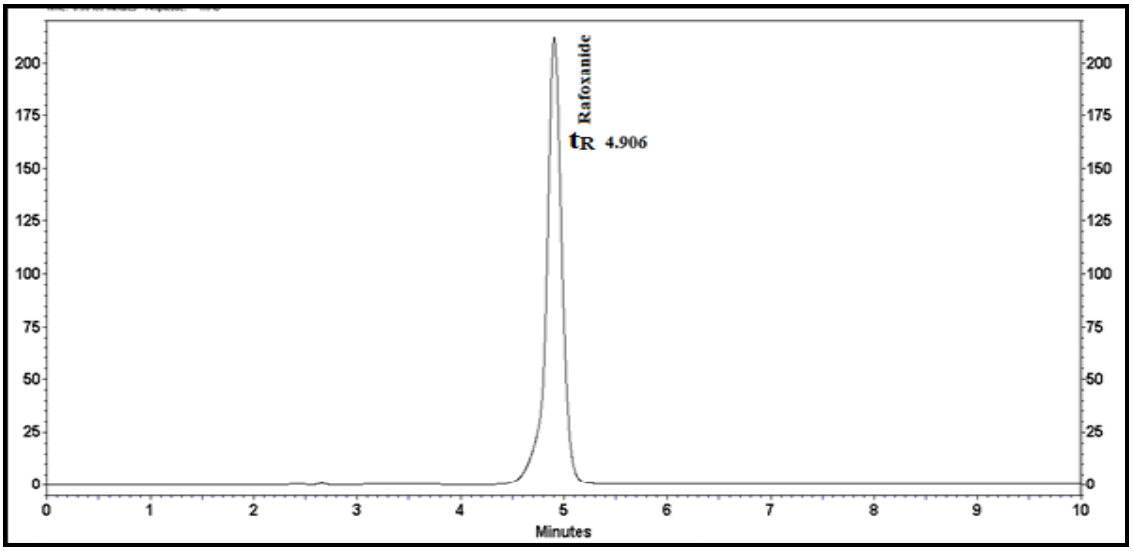

Figure (2): HPLC chromatogram of rafoxanide (40 µg/mL).

3.2. Forced degradation studies

Under various stress conditions the degradation behavior of rafoxanide was studied using the described HPLC method. It was evaluated by monitoring the obtained chromatograms of rafoxanide after subjecting to different stress conditions.

Acidic and basic hydrolysis of rafoxanide was tested under different conditions as different strengths of HCl and NaOH and different exposure times. These conditions were applied either at room temperature or reflux for different time intervals. rafoxanide was found to be stable upon using 0.1 N HCL and NaOH, and no degradation products were observed either at room temperature or after reflux for 6 h at 80°C. So we applied more stress conditions and the results indicated that rafoxanide was found to be to be sensitive to hydrolysis using 1 N HCL or 1 N NaOH when kept in room temperature for 6 h. Two degradation products were appeared in acidic hydrolytic condition at 2.492 and 6.657 min, and in basic hydrolytic condition at 2.741 and 6.770 min with different degree in the percent of degradation, **figures (3, 4)**. Moreover upon applying the neutral hydrolysis of rafoxanide using deionized water for 1 to 4 h at room temperature or after reflux at 80 °C, no degradation products were found.

Oxidative degradation was checked using 3% H_2O_2 at room temperature for 1 to 4 h. Evaporation of the samples was done to expel the remaining H_2O_2. The drug was degraded after 4 h to yield one degradation product at 2.362 min, **figure (5)**.

No degradation products under thermal and photolytic stress conditions. There was no reduction in the peak area of rafoxanide even after subjecting the drug to dry heat at 80ºC for 6 h or exposing to sunlight for 24 h. The results of rafoxanide stability studies were given in **table (1)**.

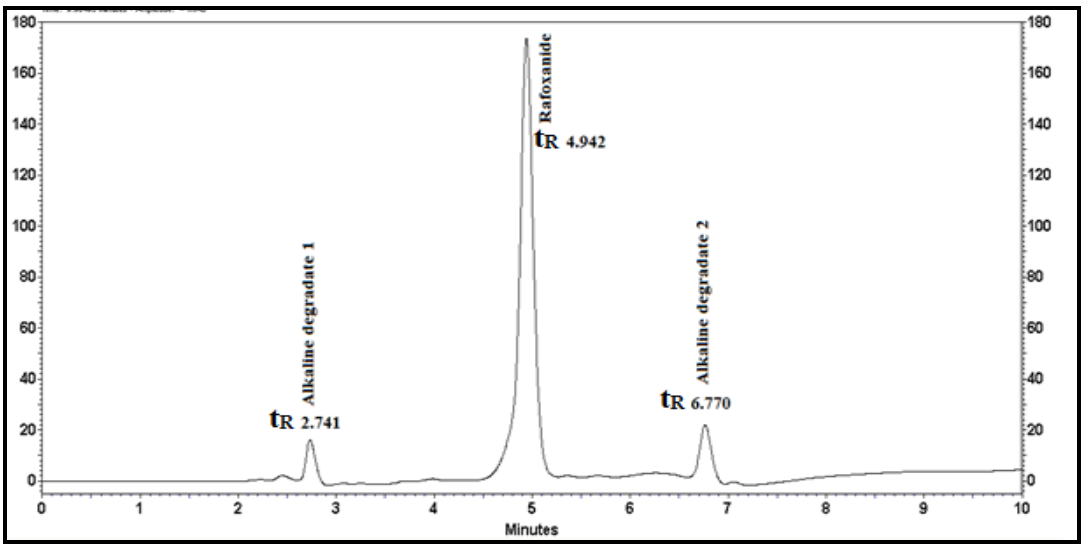

Figure (3): HPLC chromatogram of rafoxanide (40 µg/mL) and its alkaline degradation products using 1 N NaOH for 6 h at room temperature.

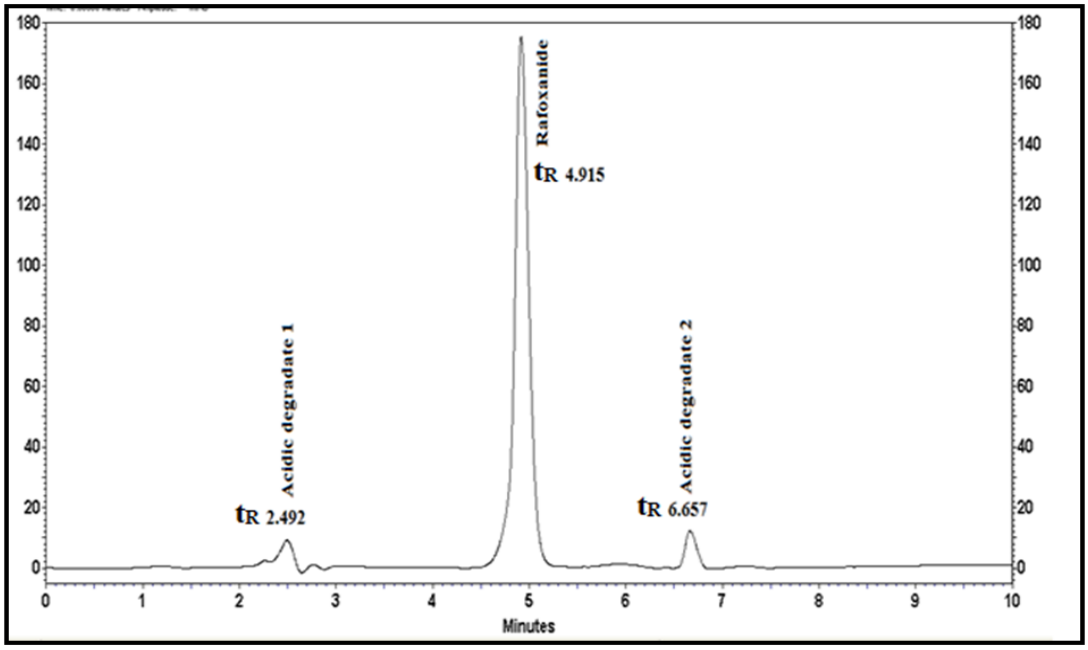

Figure (4): HPLC chromatogram of rafoxanide (40 µg/mL) and its acidic degradation products using 1 N HCl for 6 h at room temperature.

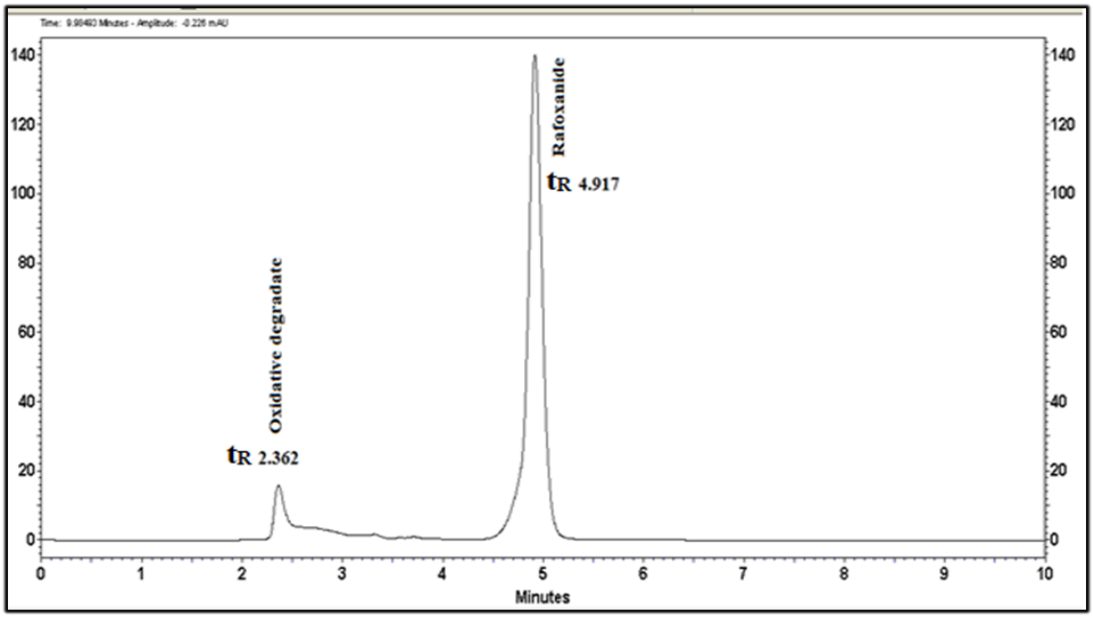

Figure (5): HPLC chromatogram of rafoxanide (40 µg/mL) and its oxidative degradation product using 3% H_2O_2 for 4 h at room temperature.

3.3. Method validation

- **Linearity and range**

Under the described experimental conditions, the calibration graph for the method was constructed by plotting the peak area values of rafoxanide versus its concentrations in µg/mL. The regression plot was found to be linear over the range of 10-70 µg/mL. The regression data was presented in **table (2)**. The high values of coefficient of determination and the small values of slope and intercept indicated the linearity of the calibration graph.

- **Limits of detection and quantitation**

LOD and LOQ values were calculated and the obtained results indicated the sensitivity of the proposed method for the analysis of the studied drug as shown in **table (2)**.

- **Accuracy and precision**

Accuracy of the proposed method was indicated by the obtained good % R as shown in **table (1)**. While, the small values of % RSD indicated high precision of the method as shown in **table (2)**.

- **Specificity**

The specificity of the method was confirmed by perfect determination of rafoxanide in the presence of its related degradation products. The results revealed that rafoxanide and its degradation products were well-separated, and the obtained peaks were neat, symmetric, and well separated from each other, thus confirming the selectivity and specificity of the method. Moreover, the standard addition technique was also applied to check the effect of the parenteral excipients on the separation of rafoxanide. The obtained results, **table (3)**, proved that the proposed method could selectively analyze the drug without any interference from any excipients.

- **System suitability tests**

To confirm that, the chromatographic system was working correctly during the analysis process, various parameters such as the number of theoretical plates (N), resolution factor (Rs), retention factor ($\bar{k}$) and tailing factor (T) were evaluated. The results of system suitability test parameters for the method were presented in **table (4)**.

- **Robustness**

The described minor changes did not affect the separation and resolution of rafoxanide confirming the robustness of the proposed method. The results were given in **table (5)**.

3.4. Pharmaceutical application

The proposed HPLC method was applied for the determination of rafoxanide in Flukanil ® injection (7.5% Rafoxanide) Satisfactory results were obtained in good agreement with the label claimed, indicating no interference from excipients and additives which was confirmed by the results of the standard addition technique. The obtained results were statistically compared to those obtained by the reported method.[4] No significant differences were found by applying student's *t*-test and *F* value at 95% confidence level[13], indicating good accuracy and precision of the proposed method for the analysis of the studied drug in its pharmaceutical dosage form, as shown in **table (6)**.

Table (1): Summary of forced degradation studies of rafoxanide.

Stress conditions	Number of degradation products and retention times	% Degradation
1 N HCl at room temperature for 6 h.	2 (2.492 & 6.657 min.)	17.74
1 N NaOH at room temperature for 6 h.	2 (2.741 & 6.770 min.)	19.06
Neutral hydrolytic degradation at 80 °C for 6 h.	-	-
3% H_2O_2 at room temperature for 4 h.	1 (2.362 min.)	33.66
Sunlight for 24 hours	-	-
Thermal at 80 °C for 6 hours	-	-

Table (2): Regression and validation parameters for determination of rafoxanide by the proposed HPLC method.

Parameters		HPLC method
Wavelength (nm)		240
Linearity range (µg/mL)		10 ─ 70
- Regression equation - Slope (b) - Intercept (a)		$y^{*} = b\,x^{**} + a$ 55637.111 665.286
Coefficient of determination (r^2)		0.9996
LOD (µg/mL)		1.400
LOQ (µg/mL)		4.241
Accuracy (%R)***		100.27
Precision (%RSD)***	Repeatability	1.479
	Intermediate precision	1.713

y^{*} is the peak area of rafoxanide.
x^{**} is the concentration of rafoxanide in µg/mL
*** Values for 3 determinations of 3 different concentrations

Table (3): Recovery study of rafoxanide by applying standard addition technique.

Pharmaceutical taken(µg/mL)	Pharmaceutical found (µg/mL)	Pure added (µg/mL)	Pure found (µg/mL)	%Recovery
10	9.98*	20	20.24	101.21
		30	29.89	99.63
		40	40.53	101.32
Mean± %RSD				100.72 ± 0.940

*Average of five determinations.

Table (4): System suitability results for the determination of rafoxanide by the proposed HPLC method.

Parameters		Obtained value	Reference value[12]
Retention time (t_R)		4.906 min	---
Retention factor (K´)		3.905	1-10
Theoretical Plates (N)		5892	>2000
Height equivalent to theoretical plates (H in cm)		0.004	The smaller the value the higher the column efficiency
Tailing factor (T)		0.863	< 2
Resolution (Rs)	1N HCl	3.209 and 2.354	>2
	1N NaOH	3.412 and 2.649	
	3%H_2O_2	3.059	

Table (5): Robustness results for the determination of rafoxanide by the proposed HPLC method.

Parameters		Retention time (t_R)	Tailing factor (T)	Resolution (Rs)*
Flow rate (mL/min)	0.9	4.929	0.834	3.029
	1	4.906	0.863	3.059
	1.1	4.871	0.866	3.079
Mobile phase ratio (acetonitrile:0.1 M ammonium acetate)	72:28	4.877	0.852	2.999
	70:30	4.906	0.863	3.059
	68:32	4.932	0.846	3.039

* The robustness results of the drug was related to its oxidative degradation product

Table (6): Determination of rafoxanide in Flukanil® injection by the proposed HPLC and reported methods.

Parameters	Proposed method	Reported method[4]
n*	5	5
Average (% recovery)	101.34	99.66
%RSD	1.541	1.494
Student's *t*-test (2.306)**	1.743	——
F value (6.388)**	1.100	——

* Number of samples

** The values in parenthesis are tabulated values of "t "and "F" at (P = 0.05)

4. CONCLUSION

This study described a simple, precise, selective and stability indicating RP-HPLC method for the estimation of rafoxanide. This work focused on the stability of the studied compound and its degradation behaviour under different stress conditions. The method was validated according to the ICH guidelines and can be used for the analysis and for checking quality during stability studies of pharmaceutical preparations containing the cited compound. Moreover, the proposed stability-indicating method uses simple reagents, with minimal preparation procedures and short run time encouraging application in routine analysis and quality control.

REFERENCES

1. Sweetman S. Martindale: the complete drug reference. 36th ed. London: The Pharmaceutical Press, 2009.
2. Fink DW. *Spectrophotometric quantification of the salicylanilide anthelmintic rafoxanide based on the charge-transfer absorbance of its iron(III) complex.* Anal Chimica Acta., 1981; 131: 281-285.
3. Devadasu CH, Vedith aK, Babu PS.*Quantitative estimation of rafoxanide in powder for suspension by spectrophotometric methods.* Int J Institut Pharm Life Sci., 2015; 5(2): 376-387.

4. Nassar MWI, Attia KAM, Mohamed AA, Said RAM, Abdel-Monem AH. *Quantitative Estimation and Validation of Rafoxanide in Presence of its Alkali-Induced Degradation Product by Different Spectrophotometric Methods.* J Adv Pharm Res., 2018; 2(1): 242-249.
5. Saad AS, Hamdy AM, Salama FM, Abdelkawy M. *Validated UPLC and TLC-densitometry stability indicating methods for the determination of rafoxanide in the presence of its degradation products.* J Chromatogr Sci., 2016; 54(9): 1661-1669.
6. Abdel-Aziz, O, El kosasy, AM, Ahmed N. *Novel chromatographic methods for simultaneous determination of some anthelmintic drugs in bulk powder and pharmaceutical formulation*. American J Pharm Health Res., 2015; 3(9): 81-95.
7. Benchaoui, HA, McKellar QA. *Determination of rafoxanide and closantel in ovine plasma by high performance liquid chromatography*. Biomed Chromatogr, 1993; 7: 181-183.
8. Yeung H. Lee W. Wong Y. *Screening of closantel and rafoxanide in animal muscles by HPLC with fluorescence detection and confirmation using MS*. J Separation Sci., 2010; 33: 206-211.
9. Blanchflower WJ, Kennedy DG, Taylor SM. *Determination of rafoxanide in plasma using high performance liquid chromatography (HPLC) and in tissue using HPLC-thermospray mass spectrometry*. J Liq Chromatogr, 1990; 13: 1595-1609.
10. Talley CP, Trenner NR, Downing GV. VandenHeuvel WJA. *Gaschromatographic determination of rafoxanide(3'-chloro-4'-(4-chloro-phenoxy)-3,5-diiodosalicylanilide) in plasma by electron capture detection of its trimethylsilyl derivative*. Anal Chem., 1971; 43: 1379-1382.
11. International Conference on Harmonization, ICH Harmonised Tripartite Guideline. *Validation of analytical procedure: text and methodology, Q2 (R1).* Geneva: International Conference on Harmonization, 2005.
12. Food and Drug Administration, Center for Drug Evaluation and Research. *Reviewer guidance: validation of chromatographic methods.* Silver Spring (MD): Center for Drug Evaluation and Research, 1994.
13. Armitage P, Berry G. *Statistical methods in medical research.* 3rd ed. Oxford (UK): Blackwell, 1994.