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STABILITY INDICATING SPECTROPHOTOMETRIC AND DENSITOMETRIC METHODS FOR THE DETERMINATION OF ENROFLOXACIN AND FLUMEQUINE

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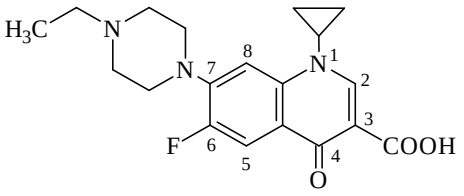
فى هذا البحث تم إستخدام طريقتين لتقييم ثباتية مادتى انروفلوكساسين وفلوميكين وتعتمد الطريقة الأولى على قياس الكثافة الضوئية بعد فصل المركبات عن نواتج التحلل باستخدام كروماتوجرافيا الطبقة الرقيقة. أما الطريقة الثانية فتعتمد على قياس المشتق الأول النسبى الطيفى لتعيين المادتين تحت البحث. وقد أمكن أستخدام الطرق المستحدثة لتقييم المواد تحت البحث فى وجود نواتج تحللهم وبدون تداخل من هذه النواتج وكذلك فى مستحضراته الصيدلية.

Two methods were developed for the determination of intact enrofloxacin (ER) and flumequine (FQ) in presence of their degradation products. In the first method, ER and FQ were determined using first derivative ratio spectrophotometric technique (^{1}DD) at 290 nm and 260 nm in the ranges of 1-12 μg ml^{-1} and 2-14 μg ml^{-1}, respectively. The second method depends on the quantitative densitometric evaluation of thin-layer chromatograms of both drugs. Good linearities were obtained in the range of 1.5-4 μg ml^{-1} and 4-14 μg ml^{-1} for ER and FQ respectively.

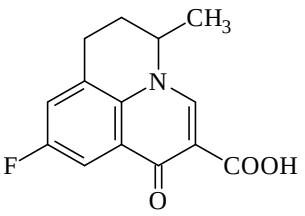
The proposed procedures retained their accuracy in the presence of up to 70% degradation products for the two drugs. The results obtained by applying the proposed methods were statistically analyzed and compared with those obtained by reported methods.

INTRODUCTION

Enrofloxacin and Flumequine are synthetic antimicrobial agents of the 6-fluoroquinolone family, specially designed for use in veterinary medicine.[1]



Enrofloxacin (ER)



Flumequine (FQ)

Several methods have been reported for the determination of ER including, HPLC,[2-5] TLC,[6] colorimetry[7,8] and voltammetry.[9] Methods of analysis of FQ include HPLC,[10-12] TLC,[13] HPTLC,[14] electrophoresis,[15] colorimetry[16] and polarography.[17]

Decarboxylation of ER and FQ decreases the pharmacological activity of both drugs since the carboxylic group is of particular importance in quinolones antibacterials.[18]

The purpose of this study was to determine the two drugs in the presence of their degradation products by simple, rapid and selective stability - indicating assays for quality control and routine analysis.

EXPERIMENTAL

Samples

- Reference standard enrofloxacin, Batch No. 20020326 and flumequine, Batch No. 20020735 (China Jiangsu, International

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Economic Technical Cooperation Corporation, China) were provided by ADWIA, 10th of Ramadan City, Egypt. Their purity was checked by HPLC methods adopted by ADWIA research and development laboratories and found to be 99.30% and 99.62%, respectively.

- Enrotryl 10% injection, product of ADWIA Batch No. 030753: label claim for each ml was 100 mg enrofloxacin.
- Flumequine 10% powder, product of ADWIA Batch No. 031068.
- Stock degraded solutions:[19] prepared by refluxing 0.1 g of pure ER or FQ with 75 ml 2M HCl or 75 ml 4 M HCl solution, respectively, for 36 hours then cooling and neutralizing using 5M NaOH solution and evaporating to dryness under vacuum. Residue was extracted with ethanol for ER or methanol for FQ, then filtered into a 100 ml volumetric flasks, and adjusted to volume with the indicated solvents. 6% undegraded ER or 7% undegraded FQ was found to be present as analyzed by iron (III) chelation procedures.[7,16]

Reagents

- Ethanol and methanol (Fisher, England)
- Hydrochloric acid (Prolabo, France)
- Sodium hydroxide (Prolabo, France)
- Deuterated chloroform (Prolabo, France)
- Chloroform (Prolabo, France)
- Plates for thin-layer chromatography (20x20 cm), precoated with silica gel GF 254, 0.20 mm thickness (Marcherey-Nagel, Germany).

Apparatus

- UV/VIS Spectrophotometer-Shimadzu 1601 PC attached to IBM computer and Hewlett Packard printer (Shimadzu, Japan).
- Densitometer - Dual wavelength Shimadzu flying spot CS-9000 with video display.
- IR spectra were recorded as KBr discs on a Shimadzu IR 200-91527 Spectrophotometer (Japan).
- Hamilton syringe (10 µl).
- UV Lamp: Vilbert Lourmat, France.

Procedures

Preparation of degradate solutions for spectrophotometric and densitometric procedures

A volume of 10 ml of stock degraded solutions was diluted to 100 ml with ethanol or methanol for ER or FQ, respectively. A solution containing 0.094 mg ml^{-1} degraded ER plus 0.006 mg ml^{-1} intact ER or 0.093 mg ml^{-1} degraded FQ plus 0.007 mg ml^{-1} intact FQ, was obtained.

Linearity

Spectrophotometric procedure

Different volumes of standard drug solutions (100 µg ml^{-1}) containing 1-12 µg ER or 2-14 µg FQ, were transferred into a series of 10 ml volumetric flasks, then diluted to volume with ethanol for ER and methanol for FQ. First derivative of the ratio spectra of both drugs were recorded against the respective solvent as blank at $\Delta\lambda$ =2 and ordinate values of (0.005 and –0.095) or (0.005 and –0.1) for ER or FQ, respectively. Peak height at 290 nm (ER) or 260 nm (FQ) as maxima and zero-crossing lines as minima were measured. Calibration curves relating the measured heights in (mm) and drug concentrations in µg ml^{-1} were constructed.

Densitometric procedure

Aliquots of ER or FQ standard solutions (0.1mg ml^{-1}) containing 1.5-4 µg ER or 4-14 µg FQ were transferred into a series of 10 ml volumetric flasks. Ten microlitres of each solution were applied to a TLC plate (20×20 cm) using a 10 µl syringe. Spots were spaced 2 cm apart from each other and 1.5 cm from the edge of the plate. The plate was placed in a chromatographic tank previously saturated for 1 h with mobile phase of chloroform-methanol (4:9, v/v) or (3:9, v/v) for ER or FQ. The plates were developed to 16 cm then removed and air-dried. Spots were detected under UV- lamp and scanned at 240 nm for ER and at 280 nm for FQ (photo mode: reflection; scan mode: zigzag). The calibration curves representing the relationship between the recorded area under the peak and the corresponding concentration were plotted.

Assay of pharmaceutical formulations

Determination of ER in Enrotryl injection

A volume of 0.5 ml of the injection (equivalent to 50 mg ER) was transferred into 50 ml volumetric flask, then diluted to volume with ethanol and mixed (1 mg ml^{-1}). An aliquot of this solution was diluted to obtain a concentration of 0.1 mg ml^{-1}. The obtained solution was analyzed by derivative ratio and densitometric procedures, as described under linearity.

Determination of FQ in flumequine 10% powder

Three preparations were mixed. Powder equivalent to 10 mg FQ was accurately weighed, dissolved in methanol by shaking for 20 min, then diluted to 100 ml. The obtained solution was filtered and the filtrate was analyzed by the spectrophotometric and densitometric procedures, as mentioned under linearity.

RESULTS AND DISCUSSION

Degradation of ER and FQ

The two fluroquinolones under study were expected to undergo decarboxylation reaction when heated with 2M HCl at 100° for 15 hours, as reported for norfloxacin.[20] Only 50% degradation was obtained under these conditions. Maximum degradation (94% for ER and 93% for FQ) was attained by boiling the acid solution for 36 hrs and using 2 M HCl for ER and 4M HCl for FQ.

Spectrophotometric procedure

Zero-order absorption spectra of intact drugs and their degradates were found to be extensively overlapped. (Figs. 1 and 2). Such overlapping was omitted by adopting the derivative of the ratio spectra method. The main advantage of this method may be the chance of doing measurements in correspondence of peaks, hence a potential greater sensitivity and accuracy. While the main disadvantages of the zero crossing method in derivative spectrophotometry for resolving a mixture of components with overlapped spectra are the risk of small drifts of the working wavelengths. This may be particularly dangerous when the slope of the spectrum is very high with consequent loss of accuracy and precision. In this procedure, the UV absorption spectra of intact ER and FQ were divided by a definite spectrum of their degradates, the first derivative was then calculated and measured at 290 nm for ER and at 260 nm for FQ, (Figs. 3 and 4). The corresponding regression parameters are shown in Table (1).

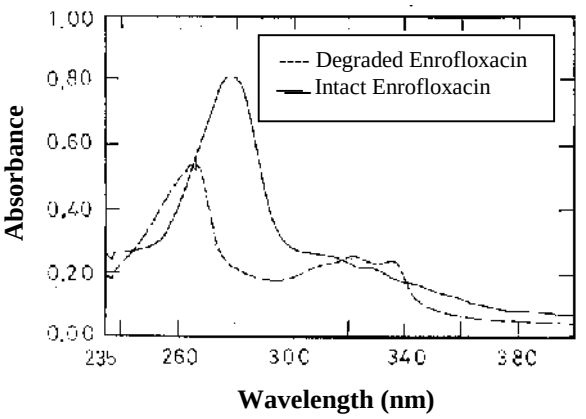

Fig. 1: Absorption spectra of: Intact ER, 6 µg ml^{-1} (—), Degraded ER, 6 µg ml^{-1} (----) in ethanol.

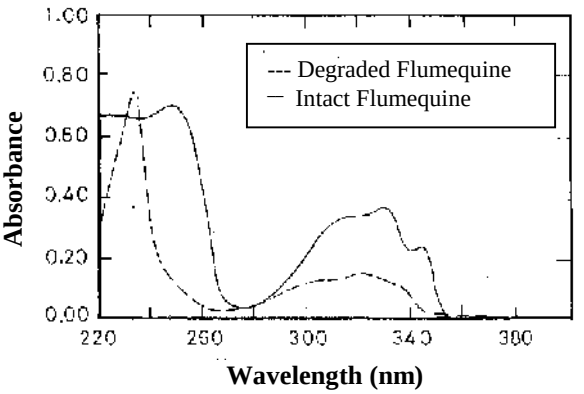

Fig. 2: Absorption spectra of: Intact FQ, 8 µg ml^{-1} (—), Degraded FQ, 6 µg ml^{-1} (----) in methanol.

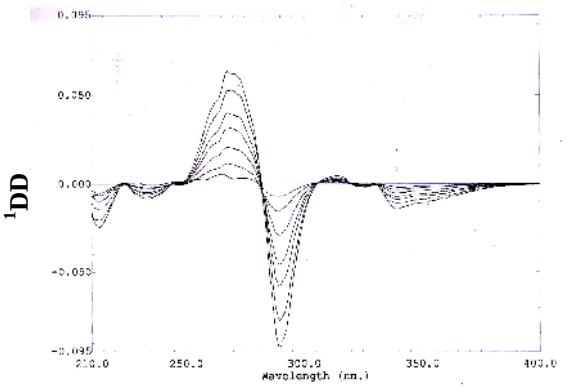

Fig. 3: First derivative ratio spectra of ER in presence of its degradate (using 1-12 µg ml^{-1} ER) in methanol and 6 µg ml^{-1} of its degradate as a divisor.

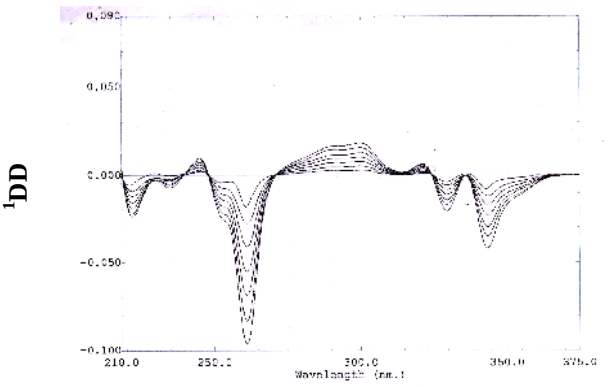

Fig. 4: First derivative ratio spectra of FQ in presence of its degradate (using 2-14 µg ml^{-1} FQ) in methanol and 6 µgml^{-1} of its degradate as a divisor.

Table 1: Selected spectral data for the determination of ER and FQ by the proposed procedures.

Parameters	Enrofloxacin		Flumequine	
	^{1}DD	Densitometric	^{1}DD	Densitometric
Linearity range (µg ml^{-1})	1-12	1.5-4	2-14	4-14
LOD (µg ml^{-1})	0.6	0.5	0.6	2
LOQ (µg ml^{-1})	0.8	1	1	3
Regression parameters				
Slope ± SD (s_b)	0.967 ± 0.027	2.449 ± 0.021	0.805 ± 0.005	1.33 ± 0.021
Intercept ± SD(s_a)	-0.074 ± 0.195	-0.008 ± 0.062	0.057 ± 0.048	0.307 ± 0.20
SD of residual (s_{xy})	0.074	0.002	0.003	0.032
Correlation coefficient	0.9998	0.9998	0.9999	0.9994
Accuracy*				
Intraday R%	99.5 - 101	100.9 - 102.2	101.6 - 102.3	99.9 – 101.6
Interday R%	99.8 - 102.0	98.0 - 101.5	100.8-102.0	98.5 - 102.8
Precision*				
Intraday RSD	1.21- 2.55	0.25 - 0.55	0.81-1.54	0.16 - 0.66
Interday RSD	0.23 - 1.05	0.69 - 1.72	0.19 - 0.31	0.34 - 2.30

*n = 4

Densitometric procedure

The second procedure for eliminating interference due to degradation products is concerned with the application of densitometric technique. The method depends on the difference of the R_f values of the drug and its degradation product. Complete separation of ER (R_f = 0.25) and its degradate (R_f = 0.65) using a mobile phase of chloroform - methanol (4: 9, v/v) while R_f values of intact and degraded FQ were 0.17 and 0.82, respectively, using a mobile phase of chloroform - methanol (3:9, v/v). The chromatograms were scanned quantitatively at 280 and 250 nm for ER and FQ, respectively. The corresponding regression equations were computed and their parameters are illustrated in Table (1). Interday and interday precision (RSD%) were ranged between 0.23-2.55%, which prove that the proposed procedures are accurate and precise, Table (1).

The specificity of the procedures was tested by analyzing laboratory-prepared mixtures of the drugs and their degradates in different ratios, no interferences were found in the presence of degradation products at the levels of ~ 70%. The results are shown in Tables (2 and 3).

The proposed procedures were also applied for the determination of both drugs in their pharmaceutical formulations, results illustrated in Tables (4 and 5) showed no interference from excipients or additives when applying the technique of standard addition.

Table (6) shows that the calculated t and F values are less than the corresponding theoretical values, indicating that there is no significant difference between the proposed methods and reported methods with respect to precision and accuracy at 95% confidence limits.

Confirmation of the degradation products

The degradates were separated on preparative plates using the respective eluting system and extracted with chloroform, the extracts were evaporated to dryness and then confirmed in the residues by IR and ^{1}HNMR. IR spectra of degraded ER and FQ show complete disappearance of the band at 1700 cm^{-1} which characterizes the carbonyl moiety of the carboxylic group in the intact drugs which confirm decarboxylation. The ^{1}HNMR spectra of pure drugs in $CDCL_3$ are characterized by a sharp singlet at ~8.5 ppm due to the CH proton in position 2. While the ^{1}HNMR of the degradation products spectra revealed two new doublets at ~7.5 and 6.0 ppm which correspond to the CH proton at position 2 and the new CH proton in position 3 produced as a result of decarboxylation.

Table 2: Determination of enrofloxacin and flumequine in mixtures with their decarboxylated degradates by the proposed derivative ratio procedure.

Enrofloxacin				Flumequine			
Intact* µg ml^{-1}	Degradate µg ml^{-1}	Degradate** %	Recovery % of intact	Intact* µg ml^{-1}	Degradate µg ml^{-1}	Degradate** %	Recovery % of intact
12.24	3.76	23.5	98.4	14.14	1.86	11.5	102.9
10.36	5.64	35.2	100.4	12.28	3.72	23.2	99.2
8.48	7.52	47.0	102.5	10.42	5.58	34.8	100.2
6.60	9.4	58.7	103.0	8.56	7.44	46.50	102.9
4.72	11.28	70.5	100.0	6.70	9.30	58.10	98.0
2.84***	13.16	82.0	128	4.84	11.16	69.70	99.5
				2.98***	13.02	81.3	128
Mean ± RSD%			100.86±1.88				100.46±2.01

* Taken + undegraded part in the hydrolysed solutions; 6 % for ER and 7 % for FQ.

** of the total weight.

*** Rejected.

Table 3: Determination of enrofloxacin and flumequine in mixtures with their decarboxylated degradates by the proposed densitometric procedure.

Enrofloxacin				Flumequine			
Intact* µg ml^{-1}	Degradate µg ml^{-1}	Degradate** %	Recovery % of intact	Intact* µg ml^{-1}	Degradate µg ml^{-1}	Degradate** %	Recovery% of intact
4.14	1.86	31.0	99.03	12.24	3.76	23.5	99.8
3.67	2.32	38.6	98.63	10.36	5.64	35.2	100.4
3.21	2.79	46.5	99.73	8.48	7.52	47.0	101.2
2.74	3.25	54.1	99.04	6.60	9.40	58.7	101.6
2.28	3.72	62.0	100.32	4.72	11.28	70.5	99.2
1.81	4.18	69.6	99.88	2.84***	13.16	82.2	122.0
1.35***	4.65	77.5	111.70				
Mean ± RSD %			99.4±0.63				100.4±0.96

* Taken + undegraded part in the hydrolysed solutions; 6% for ER and 7% for FQ.
** of the total weight.
*** Rejected.

Table 4: Recovery of ER and FQ in their pharmaceutical formulations by the proposed derivative ratio procedure.

Enrofloxacin					Flumequine				
	Mean* ± RSD%	Standard addition				Mean* ± RSD%	Standard addition		
		Taken µg ml^{-1}	Added µg ml^{-1}	Recovery % of added			Taken µg ml^{-1}	Added µg ml^{-1}	Recovery % of added
Enrotryl 10% inj.	100.8± 0.48	2	2	102.6	Flumequine 10% powder	100.1 ±1.67	2	2	101.70
		2	4	101.10			2	8	98.18
		2	6	100.30			2	10	98.48
		2	10	98.80			2	12	101.37
Mean± RSD%	100.70±1.58				Mean± RSD%	99.93±1.85			

*n = 4

Table 5: Recovery of ER and FQ in their pharmaceutical formulations by the proposed densitometric procedure.

Enrofloxacin					Flumequine				
	Mean ± RSD%	Standard addition				Mean± RSD%	Standard addition		
		Taken µg ml^{-1}	Added µg ml^{-1}	Recovery % of added			Taken µg ml^{-1}	Added µg ml^{-1}	Recovery % of added
Enrotryl 10% inj.	100.9± 0.74	1.5	1.0	100.8	Flumequine 10% powder	99.6 ±0.88	4	2	101.6
		1.5	1.5	101.0			4	4	101.0
		1.5	2.0	100.6			4	6	98.2
		1.5	2.5	100.5			4	8	100.6
Mean± RSD%	100.7±0.22				Mean± RSD%	100.3±1.49			

Table 6: Statistical analysis of the results obtained by the two proposed and reported procedures for the determination of ER and FQ in their pharmaceutical formulations.

Parameters	Enrotryl 10% Injection			Flumequine 10% powder		
	Reported* Procedure	[1]DD Procedure	Densitometric Procedure	Reported** Procedure	[1]DD Procedure	Densitometric Procedure
Linearity range (μgml^{-1})	60-130	1-12	1.5-4	60-130	2-14	4-14
N	5	5	5	5	5	5
Mean, %	100.20	100.8	100.9	99.6	100.1	99.6
S.D, ±	0.54	0.48	0.74	0.78	1.67	0.88
Variance	0.29	0.23	0.54	0.61	2.78	0.77
t	-	1.86	1.75	-	1.04	0.19
F	-	1.26	1.86	-	4.6	1.26

The theoretical value of $F = 6.39$ and $t = 2.31$ at ($p = 0.05$).

* ER was determined by chelation with iron (III) in water and the obtained yellow coloured complex was measured at 434 nm.[7]

** FQ was determined by chelation with iron (III) at pH 3 in dimethyl formamide medium, yellow complex was measured at 384 nm.[16]

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