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Simultaneous determination of norfloxacin and sulfadiazine by first-derivative spectrophotometry

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ABSTRACT

Norfloxacin (NF) and sulfadiazine (SDZ) are antibacterial agents of synthetic origin. NF is a second-generation fluoroquinolone clinically successful to treat urinary tract infections, which exhibits rapid bactericidal action, broad-spectrum efficacy, good tissue penetration, acceptable bioavailability, and tolerable adverse effects. SDZ is a systemic antibacterial sulfonamide used for the treatment of urinary and genitourinary tract infections, burns, sinusitis, and meningococcal meningitis. In the present study, we set up and validated a spectrophotometric technique because of the need to determine and quantify both substances simultaneously. Since zero-order UV absorption spectra of both drugs overlap in the region between 240 and 290 nm, we selected the first-order derivative strategy, measured as "zero-crossing". This technique has the advantage of being simple, low-cost, and fast. The guidelines of the International Conference on Harmonization were followed. NF was determined at a λ_{max} of 257 nm, and it showed linearity in the range 3.2 10^{-6} – 3.2 10^{-5}, whereas SDZ was determined at a λ_{max} of 272 nm, and it showed linearity in the range of 3.0 10^{-6} – 3.0 10^{-5}. In both cases, the correlation coefficient was 0.999. The methodology was statistically validated by evaluating the following parameters: limit of detection and quantification, precision (repeatability and intermediate precision), specificity, and accuracy. The results obtained indicate that the methodology proposed is suitable for the simultaneous determination of NF and SDZ in solution.

1. Introduction

Norfloxacin (NF), 1-ethyl-6-fluoro-4-oxo-7-piperazine-1-yl- 1 H quinoline-3-carboxylic acid (Fig 1), whose chemical formula is $C_{16}H_{18}FN_3O_3$ and whose molecular weight is 319.33, is a second-generation fluoroquinolone approved by the Food and Drug Administration (FDA) in 1986, which belongs to one of the most important families of antibacterial agents. It is currently used to treat urinary tract (cystitis and prostatitis), bladder, and gynecological infections. [1] On account of the therapeutic importance of NF, several analytical methods have been validated for its determination in bulk and pharmaceutical formulations, and/or in biological fluids. Among them, the spectrophotometric technique is the most widely used in pharmaceutical analysis. [2-6] Other analytical methods such as spectrofluorometry, [2,6] high-performance liquid chromatography (HPLC), [2,8] electrochemical analysis, [2,9] and capillary electrophoresis have also been used. [2, 10] For the quantitative analysis of NF raw material, the Argentine Pharmacopoeia (FA), [11] British Pharmacopoeia (BP), [12] and United States Pharmacopoeia (USP) [13] recommend nonaqueous titration, whereas, for the quantification of NF in tablets, they recommend HPLC. Sulfadiazine (SDZ), 4-amino-N-(2-pyrimidinyl)benzene-sulfonamide (Fig 2), whose chemical formula is $C_{10}H_{10}N_4O_2S$ and whose molecular weight is 250.28, is an antibacterial sulfonamide with a broad spectrum of action, used in prophylaxis and treatment of infections in the eyes, skin, and mucous membrane, principally in burn patients. [14] SDZ is also

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an official drug encoded in the FA, [11] BP, [12] USP, [13] and European Pharmacopoeia (EP) [15].

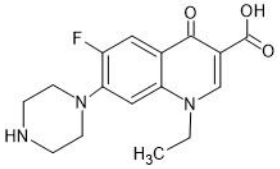

Fig 1. Chemical structure of norfloxacin

The FA [11] and USP [13] describe an HPLC methodology for the quantification of SDZ, whereas the BP [12] and EP [15] recommend the reaction of primary aromatic amines by diazotization with nitrous acid and the determination of the end-point electrometrically. Other analytical methods reported are fluorescence, spectrophotometry, immunochemical methods, capillary electrophoresis, and HPLC-MS or HPLC-MS/MS. [16]

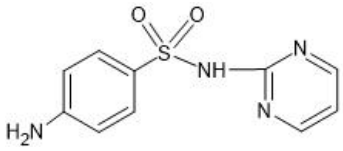

Fig 2. Chemical structure of sulfadiazine

Both drugs, of synthetic origin, are used to treat human and animal bacterial infections. The main problem regarding the simultaneous determination of these drugs is the overlapping of their UV absorption bands from 240 to 290 nm (Fig 3). This restricts their direct measurement when using UV-spectrophotometry.

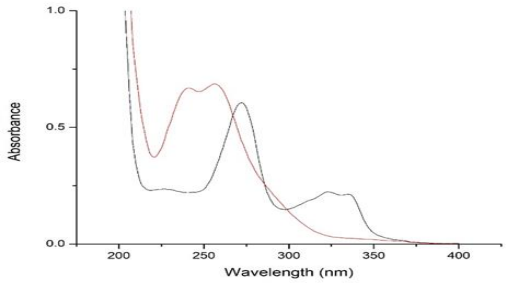

Fig 3. Spectra of NF 1.6 10^{-5} M (black line) and SDZ 1.5 10^{-5} M (red line)

In the last two decades, derivative spectrophotometry has shown an important development in the determination of mixtures of two or more components with overlapping bands and has allowed elimination interferences or matrix background by using, for example, the zero-crossing technique. [17] This method is especially useful to resolve the overlapping spectra because of its inherent simplicity, low-cost, satisfying speed, adequate precision, and wide availability.

Since, in the literature, we found no other simple, low-cost, and rapid analytical method to quantify the drug content of combined solutions of NF and SDZ, this work aimed to simultaneously determine NF and SDZ by using first-derivative spectrophotometry, and to demonstrate that this method can be a very useful tool to determine these drugs in a mixture, without time-consuming separation procedures.

2. Results and Discussion

2.1. Method development

Derivative spectrophotometry is a useful technique to identify and quantify a combination of drugs with overlapping spectra and to eliminate interference from the formulation matrix.[18] Here, the first-derivative technique was able to enhance the resolution of overlapping absorption bands through the application of the zero-crossing technique (Figs 4-5).

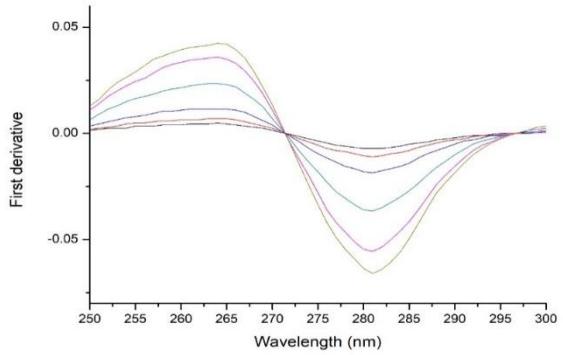

Fig 4. First derivative spectrum of NF (1.6 10^{-6} – 3.18 10^{-5}M)

The upper derivative orders were discarded because they resulted in an increase in noise and a decrease in sensitivity.

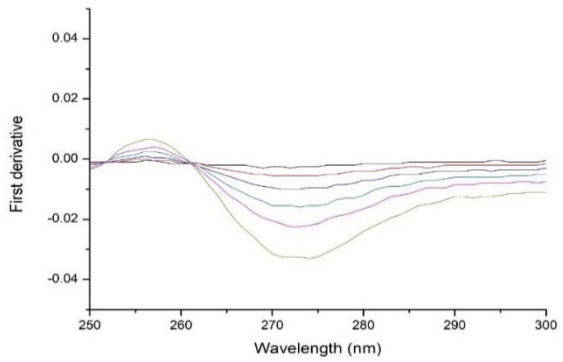

Fig 5. First derivative spectrum of SDZ (1.5 10^{-6} – 3.0 10^{-5} M).

2.2. Method validation

The method was validated following the guidelines of the International Conference on Harmonization (ICH) [18]. Linearity, detection limit (DL), quantitation limit (QL), range, precision (repeatability and intermediate precision), specificity, and accuracy were determined. Satisfactory results were obtained and the values are given in Table 1.

2.2.1. Linearity and range

The high values of the correlation coefficients and the acceptable values of Y-intercepts indicate the good linearity of the calibration curves of NF and SDZ in the ranges between 3.2 10^{-6} and 3.2 10^{-5} M and between 3.0 10^{-6} and 3.0 10^{-5} M respectively. Also, the Student's test for linearity indicates that the intercept is different from zero, because $T_{exp} > T_{tab} = 2.78$, and that the alternative hypothesis can be accepted.

2.2.2. Detection limit (DL) and quantitation limit (QL)

The sensitivity of the method proposed can be confirmed by the low DL and QL values obtained.

2.2.3. Precision

To evaluate the precision of the method, the repeatability of the equipment and that of the method itself were evaluated. Regarding the former, the coefficient of variation found was less than the maximum percentage established in the literature, which is around 1.0%, [18] whereas regarding the latter, the coefficient of variation found was within the maximum allowed intervals, which range between 2.0 and 3.0%. [18]

To analyze the intermediate precision of the method, a concentration of 1.5 10^{-5} M was evaluated, for which an experimental design was developed, varying the analyst and analysis day factors. The analysis of variance (ANOVA), using a model of effects or fixed categories for the calculation of F, showed that F_{exp} values are greater than F_{tab}, indicating that the results do not present a statistically significant difference when the test is performed by a different analyst or on different days.

2.2.4. Specificity

The specificity of the method was also determined by applying the method proposed for the determination of laboratory-prepared mixtures containing different ratios of NF and SDZ. Good results were obtained, indicating good specificity of the method (Table 2).

Table 1. Parameters used to validate the method used for the determination of NF and SDZ

Parameters		**NF (λ_{max} at 272 nm)**				**SDZ (λ_{max} at 257 nm)**			
			CV[a]	T_{tab}[b]	t_{est}		CV[a]	T_{tab}[b]	t_{est}
Linearity	Intercept	0.03	-	2.78	4.65	0.03	-	2.78	6.14
	Slope	34875.33	1.01	-	-	21523.33	1.43	-	-
	Correlation coefficient	0.9999	-	-	-	0.9996	-	-	-
Range		5.0 – 10.5 ug/ml				3.7 – 7.5 ug/ml			
DL		1.09E-07				5.14E-07			
QL		3.31E-07				1.56E-06			
		Precision							
Repeatability of the equipment	Average	6.48E-01				3.68E-01			
	DESVEST	3.00E-04				3.00E-04			
	CV (%)	0.05				0.08			
Repeatability of the method	Average	1.57E-05				1.50E-05			
	DESVEST	1.66E-07				2.36E-07			
	CV (%)	1.06				1.57			
Intermediate precision	Average	1.57E-05				1.53E-05			
	DESVEST	2.18E-07				3.38E-07			
	CV (%)	1.38				2.21			
	$CV_{rep\ met}$ x 2	2.77				4.43			
	Test F	$F_{exp} > F_{tab}$				$F_{exp} > F_{tab}$			

[a] %; [b] degrees of freedom=16 α=0.05

Table 2. Specificity of the method

	NF (dA/dλ at 257 nm)				SDZ (dA/dλ at 272 nm)			
	NF	NF $_{in\ mix}$	T_{tab}[a]	t_{exp}	SDZ	SDZ $_{in\ mix}$	T_{tab}[a]	t_{exp}
Intercept	0.0003	0.0003	2.78	0.70	0.0004	0.0006	2.78	0.56
Slope	1023.60	1000.43	2.78	1.83	626.43	593.42	2.78	0.91

[a] degrees of freedom=4; α=0.05

2.2.5. Accuracy (Recovery)

Finally, assays were carried out at three levels: 80, 100, and 120% to determine accuracy. The percentages of recovery of both drugs in the mixture were calculated according to the corresponding regression equation. The percentages recovered at the three different levels mentioned above for NF and SDZ were found to range from 98.91 % to 102.42 %. The results are given in Table 3.

Table 3. Results of recovery determination

Amount of drug added (%)	Recovery (%)	
	NF	SDZ
80	99.59 ± 0.05	99.01 ± 1.81
100	98.91 ± 2.20	100.42 ± 1.63
120	99.44 ± 1.64	102.42 ± 1.48

3. Experimental

3.1. Materials

NF was obtained commercially from Parafarm Laboratory Argentina (L130666). SDZ was obtained as a gift sample from Vannier Laboratory, Argentina. Distilled water was used to prepare 0.1 M Sodium Hydroxide and buffer pH 7.4.

3.2. Spectrophotometric measurements

The spectrophotometric measurements were performed on a double-beam Varian Cary 50 spectrophotometer connected to a compatible computer loaded with the software Win UV 3.00 (Agilent) used for all the absorbance measurements and data manipulation. All the solutions were scanned at a wavelength range of 200-400 nm and a medium scanning speed (0.9 nm/min), using 1-cm matched quartz cells.

3.3. Preparation of standard solutions

Standard solutions of NF and SDZ at a concentration of $6x10^{-3}$ M were prepared by dissolution of the drug powder in a phosphate buffer of pH 7.4. Aliquots from this standard solution were diluted to 10 ml with buffer to obtain working solutions in the range of 1.50 10^{-6} to 3.18 10^{-5} M. Working solutions were freshly prepared three times a day from the same stock solution. The entire experiment was replicated for three consecutive days. All the solutions were protected from light throughout the study.

3.4. Construction of calibration curves

Five working standard solutions of NF and SDZ were used to construct the calibration curves. Linear regression analysis was made by using the least-squares method. The intensity of the first-derivative spectra of the solutions was measured at 272 nm for NF and 256 nm for SDZ, using a buffer of pH 7.4 as a blank. The first-order derived spectra dA/dλ were mathematically obtained from the absorbance spectrum using the OriginPro® 8.5 software. Spectra with a higher order of derivation had lower sensitivity and linearity. Thus, only first-order derivative spectra were selected for quantitative analysis.

3.5. Preparation of combined standard solutions of NF and SDZ

Aliquots from the stock solution of the two drugs were transferred into a series of 10-ml volumetric flasks to prepare six samples of an equimolar solution of NF and SDZ in a phosphate buffer of pH 7.4, to evaluate the specificity of the method for samples containing NF and SDZ.

3.6. Validation of the method

The method proposed was validated for linearity, limits of detection and quantification, range, precision (repeatability and intermediate precision), specificity, and accuracy according to the ICH guidelines. [19]

3.6.1. Linearity

The linearity of an analytical procedure is its ability to obtain test results that are directly proportional to the concentration of analyte in the sample. In the present study, a slope close to 1 and an intercept close to zero were taken as indicative of the linearity of the method.

Six different concentrations of NF and SDZ in the range of 10.0 to 0.5 μg/ml were analyzed three times on the same day, with a total of 18 determinations. The least-squares method evaluated the relationship between absorbance and concentration. The slope and the intercept were evaluated by using the student's t-test,

with a degree of significance of α=0.05.

3.6.2. Detection limit (DL) and quantitation limit (QL)

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantified as an exact value. The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, used particularly for the determination of impurities and/or degradation products.

DL and QL were calculated according to the following equations:

$$DL=3.3\ \sigma/s \qquad \text{[Eq. 1]}$$

$$QL=10\ \sigma/s \qquad \text{[Eq. 2]}$$

where σ is the standard deviation of the analytical signal and s is the slope of the corresponding calibration curve.

3.6.3. Range

The range of an analytical procedure is the interval between the upper and lower concentration of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy, and linearity.

3.6.4. Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered as repeatability and intermediate precision.

The repeatability of an analytical procedure, also termed intra-assay precision, expresses the precision under the same operating conditions over a short interval of time. To determine the repeatability of the equipment, a solution of NF and SDZ with a nominal absorbance (5 μg/ml) was analyzed and its absorbance was determined 12 times. The variation coefficient should be less than 1.0 %. The repeatability of the method was analyzed by repeating the assay of one different concentration three times on the same day and with the same analyst.

The intermediate precision, also termed inter-assay precision, expresses within-laboratories variations, i.e. those observed when considering different days, different analysts, different equipment, etc. In the present study, the previous procedures were repeated inter-daily on three successive days, on two different equipment, and with two different analysts for the analysis of the six selected concentrations. The samples were prepared in duplicate.

3.6.5. Specificity

The specificity of an analytical procedure is the ability of the method to accurately measure a compound in the presence of other components. The method specificity was evaluated by analyzing a mixture of NF+SDZ (fixed) and SDZ+NF (fixed) and subsequently constructing the calibration curve of the mixture.

Two sets of solutions were examined to evaluate the method specificity in samples containing both NF and SDZ. The first set contained the standard solution of $3.2\ 10^{-6}$ – $3.2\ 10^{-5}$ M of NF in the presence of $1.5\ 10^{-5}$ M of SZD. The second set contained the standard solutions of $3.2\ 10^{-6}$ – $3.2\ 10^{-5}$ M of SDZ in the presence of $1.5\ 10^{-5}$ M of NF. The similarity of the regression equations in the solutions of the first set and the second set to those of the pure drug solutions, as shown in Table 1, indicates the noninterference of one drug in the absorption measurements of the other at the chosen wavelengths. The student's t-test was performed for a level of significance α= 0.05 and the selected degrees of freedom t= (n1 + n2) – 2, where the null hypothesis indicates that the regression equations do not have significant differences. The value of t_{exp} was compared with the value of t_{tab}.

3.6.6. Accuracy (Recovery)

Finally, the accuracy of the method was evaluated by applying the standard addition technique, which consists in adding known amounts of the reference substance to the standard solution in the dissolution medium at 80, 100, and 120 % of the nominal assay value of NF and SDZ. The accuracy was calculated as the percentage of drugs recovered.

4. Conclusion

The present study is the first contribution to the simultaneous determination of NF and SDZ by a derivative spectrophotometric method. This technique allows the determination and quantitation of either drug in the presence of the other by applying the first-derivative spectrophotometric zero-crossing method in the spectra, without prior separation steps. The method demonstrated good linearity, precision, selectivity, and accuracy. The method proposed, described, and validated is simple, low-cost, and independent of expensive instruments or critical reagents. The first-order derivative spectrophotometric method proved its suitability to be applied for routine analysis of mixtures of NF and SDZ in quality control laboratories.

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