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Validation of Ninhydrin Quantitative Method for Cephalexin Generic Tablets

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Abstract: The Medicinal Plant of Corrientes, Argentina, elaborates, distributes and provides free 500 mg cephalexin tablets in hospitals and health care centers. HPLC-UV is the reference method to quantify this antibiotic. The spectrophotometric ninhydrin method was applied for cephalexin quantification and analytical parameters for its validation were determined. The linearity of this method was in the range 0.04-0.20 mg/mL, responding to $y = 2.2338x + 0.0007$, with $R^2 = 0.9995$. The RSD% found was 0.41, indicating a good repeatability of the analytical procedure. Exactitude in recovery experience was 98.50-101.33%. Comparison using t-tests and F-test indicates that there are no significant differences between results using both methods, with a confidence level of 95%. Specificity and intermediate precision assays were satisfactory. These parameters complete the validation of ninhydrin method, according to requirements of both United States and Argentinean Pharmacopoeia, for its application on cephalexin quality control in generic tablets.

Keywords: Antibiotic, β-lactamic, Cephalexin, Spectrophotometry, Validation.

INTRODUCTION

Bacterial infections are extremely prevalent and have high mortality. Antibiotics are the most frequently prescribed drugs, due to the safety profile of many of them [1]. The selective toxicity of this group of drugs is their main advantage, because they interfere with vital functions of bacteria, without affecting host cells. Among the wide spectrum of antibiotics, β-lactams are one of the most important. Its (β-lactam) chemical structure contains a four member ring, consisting of three carbon atoms and one nitrogen adjacent to a carbonyl group (C=O).

Cephalexin ($C_{16}H_{17}N_3O_4S$ – MM = 347.39), 7-(D-α-amino-α-phenylacetamido)-3-methyl-3-cephem-4-carboxylic acid, is a second-generation cephalosporin and one of the most commonly used cephalosporin antibiotic. Its structure has a β-lactam ring attached to a six-member ring containing sulfur as a heteroatom. Cephalexin is active against *Streptococci* and *Staphylococci*. It is useful in skin and soft tissue infections, and is commonly used as prophylaxis against postoperative infections. It is closely related to the penicillin family and is used for patients with penicillin allergy [2].

β-lactam antibiotics base their therapeutic action in inhibition of bacterial wall synthesis. The bacterial wall, of both gram-positive and gram-negative bacteria, is a rigid structure of peptidoglycan that protects the bacteria from osmotic rupture. These microorganisms produce proteins called penicillin-binding proteins (PBP) in their wall. Cephalexin inhibits PBP synthesis [3].

The Pharmaceutical Plant of Corrientes (PLAMECOR), under the Ministry of Public Health of the province, produces 500 mg of cephalexin tablets. These are distributed without cost in public hospitals and primary health centers.

According to the United States Pharmacopoeia (USP), European Pharmacopoeia and the Argentinean Pharmacopoeia of the National Administration of Medicaments, Food and Medical Technology (ANMAT), HPLC-UV [4-6] is the analytical method of reference to quantify Cephalexin, both active ingredient and its presence in tablets. Several cephalexin determination methods for pharmaceutical preparations have been reported, but most of them require long reaction time, expensive equipment, or the reagents used are heavy metals or toxic compounds [7]. These include densitometry [8], solid-phase extraction [9], electrochemical [10-12], chromatographic [13-17], chemiluminescent [18,19] spectrofluorimetric [20-21] and spectrophotometric [22-23-24-25] methods.

This spectrophotometric method uses ninhydrin as a chromogenic reagent. The amino group ($-NH_2$) electron donor acts as a ligand in complex formation with electron acceptor atoms or molecules. The reaction of α-amino group of cephalexin with ninhydrin ($C_9H_6O_4$ - MM = 178.14) generates an orange adduct with maximum absorbance at 480 nm.

However, the analytical attributes of this method, established by the pharmacopoeias, have not been yet reported.

In this paper we establish the parameters required by USP and Argentinean pharmacopoeia for cephalexin quantitative analysis in pharmaceutical generic tablets compared to HPLC, in the presence of common excipients.

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EXPERIMENTAL

Reagents and Samples

- Cephalexin active ingredient. Lot 0607110. Origin: China. 99.3% purity
- Cephalexin tablets of 500 mg, elaborated by PLAMECOR. Lot 032.
- Excipients: microcrystalline cellulose (13.20%), sodium starch glycolate (5.10%), polyvinylpyrrolidone (5.60%), magnesium stearate (1.00%), talc (2.00%).
- Ninhydrin, analytical reagent grade. Biopack (Argentina)
- Sulfuric acid 98%, analytical reagent grade. Cicarelli (Argentina)
- Acetonitrile, HPLC grade. Biopack (Argentina)
- Potassium phosphate monobasic, analytical reagent grade. Cicarelli (Argentina)
- Potassium hidroxide, analytical reagent grade. Anedra (Argentina)

Equipment

- UV-Visible Spectrophotometer Boeco S-26, range 190-900 nm.

- HPLC with variable UV wavelength detector Agilent 1120.

Spectrophotometric Procedure

Stock solution of Cephalexin was prepared dissolving 0.2500 g of pure Cephalexin in 500.0 mL of distilled water, obtaining a 0.5 mg/mL solution. Work solutions of sulfuric acid 20.0% v/v, and ninhydrin 0.2% w/v were prepared. Standards contained 1.0; 2.0; 3.0 and 5.0 mL of stock solution of cephalexin; 4.0 mL of 20% v/v sulfuric acid and 0.5 mL of 0.2% w/v ninhydrin. Blank used the same amount of reagents without cephalexin solution. These aliquots were diluted to 10 mL with distilled water in a volumetric flask, homogenized, and taken to 100 ºC water bath for 15 minutes for color development. Reaction was stopped using a cold bath. Absorbance was recorded as function of concentration at 480 nm.

From a pool of 20 cephalexin tablets, an average mass equivalent to one tablet (756.8 mg) was dissolved by stirring for 5 minutes in 500 mL of distilled water. A 15 mL aliquot was centrifuged. Procedure described above was applied on supernatant.

The concentration of active ingredient in the sample was determined by calibration curve.

HPLC-UV Procedure

A solution was prepared by dissolving 6.8 g of monobasic sodium phosphate in 1 L of distilled water. pH was adjusted to 5.0 ± 0.1 with 10% potassium hydroxide. This solution was used in the preparation of mobile phase and to dissolve the stock and sample solutions. The method used an RP-18C column of 125x4.5 mm; solution of monobasic potassium phosphate: acetonitrile (80:20) as mobile phase, flow rate of 1.5 mL/min and detection at 254 nm. The standard was a 1.0 mg/mL of active ingredient solution. For the sample, a solution of about 1.0 mg/mL was prepared weighing 153 mg of pool of tablets and dissolving in 100 mL of distilled water. Then it was stirred for 5 minutes. An aliquot was centrifuged for 15 minutes. Supernatant and standard were injected into the chromatograph (50 μL), recording the signal corresponding to the peak of cephalexin (t_R = 2.45'). The concentration of cephalexin in solution of pool of tablets is calculated from the ratio of peak areas according to Equation 1:

$$C_{sample} = C_{std} \cdot A_{sample}/A_{std} \quad (1)$$

Validation of Spectrophotometric Method

According to the last edition of the USP and Argentinean Pharmacopoeia, the validation schema of the proposed method for cephalexin corresponds to Category I. This includes analytical methods for the quantification of the major components of raw materials or active ingredients in pharmaceutical formulations. Data requirements for validation of an analytical method category I (ANMAT) are: accuracy, repeatability, intermediate precision, specificity, linearity and linear interval.

Specificity

Specificity is the ability of a method to assess unequivocally the analyte in the presence of many others components, such as impurities, degradation products, matrix compound, etc. [4, 5]. Absorption spectra of a) reagents, b) reagents and placebo and c) reagents and sample were determined. Spectrophotometric readings of placebo were assesed by excipients in the same proportion as found in 500 mg cephalexin tablets.

Linearity

Linearity represents the method's ability to produce results directly proportional to analyte concentration within a given interval. A calibration curve in 0.04 to 0.20 mg/mL interval was prepared using a standard solution of cephalexin 0.4 mg/mL. Each analysis was performed by triplicate. This parameter was set using linear regression.

Precision

Precision is the degree of agreement among individual test results when the method is applied repeatedly to multiple aliquots of a homogeneous sample. It is expressed as standard deviation, SD, or relative standard deviation, RSD (or coefficient of variation, CV) of a series of measurements. The precision shall be considered at two levels: repeatability and intermediate precision.

Repeatability expresses the precision under the same operating conditions in a short time interval. Twelve (12) determinations were conducted on the same sample, by a single operator, same equipment and in the same day.

Intermediate precision expresses the intra-laboratory variations. To evaluate this parameter a single homogeneous sample of one concentration was analyzed, and readings were made by two analysts on two different days and taking three different aliquots from the sample solution.

Accuracy

The proximity between the experimental results and the actual value is evaluated. To determine the accuracy two ways can be used. The first one is to carry out recovery assays of cephalexin when the analytical method is applied on placebo solution enriched with active ingredient. The other one compares the results of the proposed analytical method with another one whose accuracy has been established (reference or official method) [26].

RESULTS AND DISCUSSIONS

Specificity

The absorption spectra of reagents, and reagents plus placebo solution, were very similar between them (Fig. **1**). This shows that the excipients that accompany the active ingredient in the formulation are chemically indifferent to the reagents used. The Specificity of the method was demostrated.

Linearity and Interval

The method was linear over the studied range. It is not necessary to explore the linearity of the response in a lower concentration range as it is quality control of the active ingredient of a drug.

The confidence intervals for the intercept and slope were calculated using a Microsoft Excel ® spreadsheet, Table **1**. The interval of the intercept includes the point (0;0) with a confidence level of 95%.

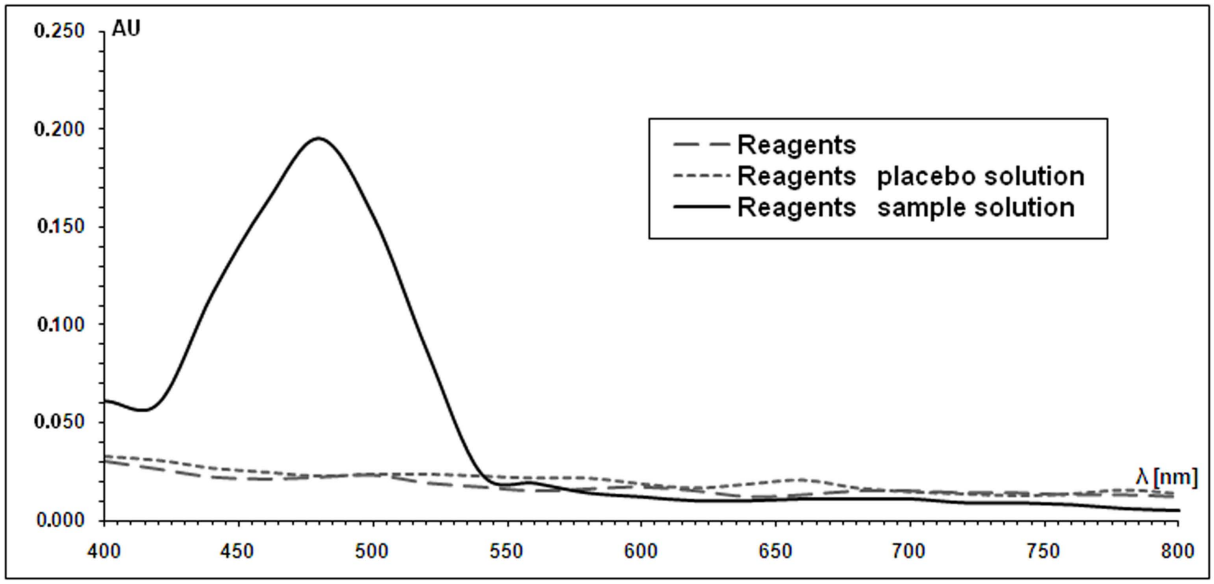

Fig. (1). – Specificity assay.

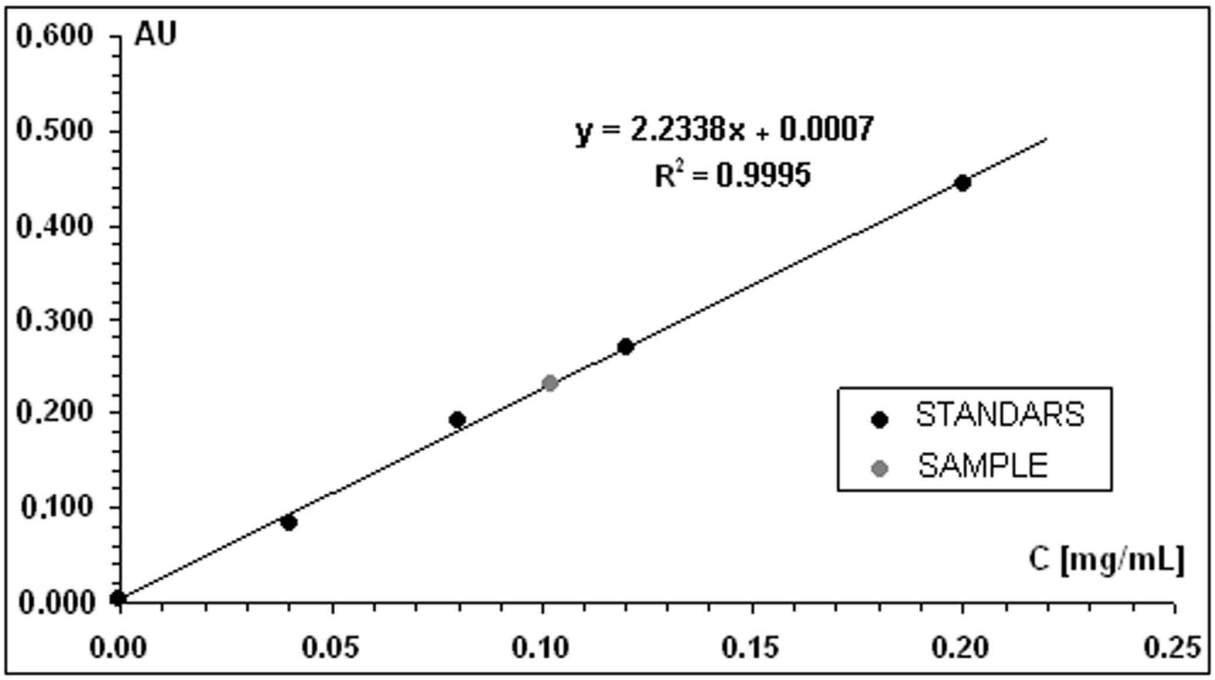

Fig. (2). – Linearity of spectrophotometric method cephalexin - ninhydrin.

Table 1. Analysis of Variance of Linear Regression with a Confidence Level of 95%

VARIANCE ANALYSIS	
Adjusted Correlation R^2	0.9995
Intercept	0.0007 ± 0.0031
Slope	2.2338 ± 0.0279

Repeatability

According to the procedure described above, twelve sample aliquots were used. Results are shown in Table **2**.

The method gives a RSD% of 0.412, this is less than 2%, which is required for this type of analytical determinations [4, 5].

Intermediate Precision

The study was conducted from three different aliquots of a solution 0.50 mg/mL of cephalexin, prepared from a pool of tablets, following the spectrophotometric method as described. Each analysis was performed in triplicate. The results, expressed as mass of cephalexin per tablet, are shown in Table **3**.

The RSD% for intermediate precision study was less than twice the RSD% of repeatability. The precision evaluated as repeatability and intermediate precision, shows satisfactory results [4, 5].

Recovery Assay

This assay was performed by triplicate and in three levels of concentration, Table **4**. Placebo solutions enriched with different volumes of 1.0 mg/mL of cephalexin solution were analyzed.

The recovery rate found reached 98.50 to 101.33%; values that fall within the requirements set by USP and ANMAT (98.0-102.0%) [4, 5].

Comparison with the Reference Method

The results obtained using the official method (HPLC-UV) are shown in Table **5**.

The proposed method is compared with the reference method using the F test for precisions and the t test for means, Table **6**. Calculations were performed using Microsoft Excel ® spreadsheet.

Statistically, it is found that there are no significant differences between the results obtained using the spectrophotometric method and the reference for the determination of cephalexin, with a confidence level of 95%.

Table 2. Repeatability of the Proposed Method

Nº	Mass Recovered [mg]	$X_{average}$ [mg]	SD [mg]	RSD%
1	501.65	500.55	2.062	0.412
2	503.15			
3	500.95			
4	501.96			
5	499.87			
6	498.60			
7	501.23			
8	502.36			
9	500.15			
10	496.95			
11	502.56			
12	497.16			

Table 3. Intermediate Precision for the Proposed Method

V_{CFX} 0.50 mg/mL	ANALYST 1		ANALYST 2		REPRODUCIBILITY	
	DAY 1	DAY 2	DAY 1	DAY 2		
1.0 mL	499.87	503.35	499.65	503.54		
	501.37	501.69	502.16	501.45		
	500.74	500.60	497.69	501.45	**n =**	**12**
M [mg]	500.66	501.88	499.83	502.15	**M [mg] =**	**501.13**
SD [mg]	0.753	1.385	2.241	1.207	**SD [mg] =**	**1.607**
RSD%	0.150	0.276	0.448	0.240	**RSD%**	**0.321**
2.0 mL	502.15	498.63	496.89	504.11		
	501.56	499.66	497.98	501.54		
	500.95	500.96	498.63	502.36	**n =**	**12**
M [mg]	501.55	499.75	497.83	502.67	**M [mg] =**	**500.45**
SD [mg]	0.600	1.168	0.879	1.313	**SD [mg] =**	**2.108**
RSD%	0.120	0.234	0.177	0.261	**RSD%**	**0.421**
3.0 mL	502.36	503.60	497.21	501.98		
	501.56	502.46	501.69	496.50		
	501.96	500.67	502.59	499.32	**n =**	**12**
M [mg]	501.96	502.24	498.65	499.27	**M [mg] =**	**500.99**
SD [mg]	0.400	1.477	2.882	2.740	**SD [mg] =**	**2.205**
RSD%	0.080	0.294	0.578	0.549	**RSD%**	**0.440**

M_{GLOBAL} [mg]	500.80
SD_{GLOBAL} [mg]	2.010
$RSD\%_{GLOBAL}$	0.401

Table 4. Results of the Recovery Assay

M_{CFX} added [mg]	M_{CFX} recovered [mg]	% Recov.	%Recov average	Precision	
				Parameter	Value [mg]
1.00	1.03	103.0	101.33	**Xm =**	1.013
	1.02	102.0		**SD =**	0.021
	0.99	99.0		**RSD% =**	2.054
2.00	1.98	99.0	98.50	**Xm =**	1.970
	1.96	98.0		**SD =**	0.010
	1.97	98.5		**RSD% =**	0.508
3.00	3.03	101.0	100.00	**Xm =**	3.000
	2.99	99.7		**SD =**	0.026
	2.98	99.3		**RSD% =**	0.882

Table 5. Determination of Cephalexin by HPLC-UV Method

RUN	SIGNAL	CONCENTRATION [mg/mL]	MASS RECOV [mg_{CFX}/tablet]	Xm	SD	RSD%
Std.	3051.6	1.00	-	-	-	-
1	3114.5	1.02	504.53	500.41	2.02	0.403
2	3074.9	1.01	499.59			
3	3178.3	1.01	499.59			
4	3082.1	1.01	499.59			
5	3089.7	1.01	499.59			
6	3068.5	1.01	499.59			

Table 6. Statistical comparison between proposed and reference methods.

Parameter	Spectrophotometric Method	HPLC-UV Method
Xm ± SD	500.55 ± 2.06	500.41 ± 2.02
RSD%	0.412	0.403
S^2	4.252	4.067
F test	1.045 (4.704)	
t test	0.896 (2.228)	

Values in parentheses correspond to those tabulated for p = 0.05.

CONCLUSION

The results obtained enable the application of this spectrophotometric methodology for the quantification of the active ingredient in pharmaceutical tablets containing cephalexin as a single drug, with accuracy and precision comparable to the reference method, without interference from common excipients and with de requirements established by the USP and ANMAT.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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