An application of Doehlert matrix optimization on developing of flow injection analysis of neomycin in pharmaceutical samples

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Abstract

This work proposes a Flow Injection Analysis (FIA) system to determine neomycin in pharmaceutical formulation, utilizing ninhydrin as chromogenic reagent, with absorbance signal measured at 570 nm. To check the significant flow parameters, a screening with 2^4 full factorial designs was optimized, and a Doehlert matrix used to obtain the optimum variable values. The method presents two linear responses ranging from 29.8 up to 300 mg L^{-1} (r^2=0.9933) and 800.0 up to 2500 mg L^{-1} (r^2=0.9926). The Limits of Detection and Quantifications were 8.94 mg L^{-1} and 29.8 mg L^{-1}, respectively. The readings frequency was of 33 reading h^{-1}. The method accuracy was checked through comparison with HPLC method and the results found were similar (t-test, 95% confidence level). The method was applied in pharmaceutical samples with very good performance and the precision (RSD) was always below 8.0%. The reagent and sample consumption was very low, what is in agreement with the green chemistry concept

Keywords neomycin, factorial designs, Doehlert, FIA, green chemistry

1. Introduction

Infectious diseases have been over the years a great threat to human health, as well as important morbidity and mortality causes. It is estimated that more than fifty percent of the antimicrobial items produced in the world have been used for animal treatment [1].

Neomycin sulfate is a veterinary antimicrobial used in treatment of: external otitis, dermatitis, furunculosis and gastrointestinal tract infections [2]. This drug consist of a mixture of compounds produced by *Streptomyces fradiae,* the main component being sulfate 2-deoxy-4-O(2,6-diamino-2,6-dihydroxy-α-D-glucopyranosyl)-5-O-[3-O-(2,6-diamino-2,6-dihydroxy-β-L-idopyranosyl)-β-D-ribofuranosyl]-streptamine sulfate, which was discovered by Waksman and Lechevalier in 1949 [3]. It has acid resistance (pH below 2), and also supports water boiling temperature. When it was discovered it showed a wide action range, including the Gram-positive and Gram-negative ones, mainly the *Proteus vulgaris* [4].

The veterinary drugs have been widely employed and applied to cure or prevent diseases [5]. Due to the great importance of the drug to animal health, and consequently for human health, its quality control becomes essential to verify mainly properties such as concentration, activity, efficiency, purity and safety [6].

For the drug quality control, the recommended method described in the official literature consists of microbiological assay to quantify the analyte by diffusion in agar [7,8]. To ensure the method's reliability, parameters such as incubation, temperature and time must be rigorously controlled [9]. Besides, the method is slow (72 h), complex and of hard reproducibility [7].

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Other methods have been developed for neomycin sulfate determination, such as HPLC with several detectors, e.g. UV-Vis, Mass spectrometry, Fluorimetry and Capillary electrophoresis [10,11]. The HPLC with UV-Vis detector has a drawback related to the non existence of chromogenic groups in the neomycin, thus a derivatization is needed, besides a rigorous control of reaction conditions and a time-consuming internal calibration, which is strongly recommended [10,11].

The HPLC with fluorimetric detection has also been described, nevertheless this method has no good selectivity, thus it also requires derivatization both before and after the column, besides a rigorous sample treatment.

The HPLC with Mass Spectrometry detectors presents good sensitivity, but have high cost, and the calibration is done mainly by internal standards. The determination by capillary electrophoresis do not quantify neomicyn directly [11].

The present work proposes a FIA system employing ninhydrin to form a colored compound with neomycin, which is monitored by spectrophotometry on 570 nm [7]. The reaction was performed in flow (FIA), thus securing a better accuracy of the results, less contamination risk, higher reading frequency and less consumption of reagents and sample, in agreement with green chemistry.

It is important mentioning that some works was done using FIA system to determine neomycin with different detection techniques, *e.g.*, electrocatalytic [12] and chemiluminescence [13], nevertheless, they used monovariate tools for optimization.

In the present work multivariate tools were used for optimization, which presents some advantages as lesser number of experiments, being also faster and cheaper because of less reagent and sample use, and for being ecologically correct (green chemistry). A common multivariate optimization procedure consists in an initial screening through factorial design plan, followed by a Doehlert matrix, which permits a surface response chart construction and a mathematical equation to calculate optimum values of each variable. Additionally, multivariate tools yield information about interaction among variables and also

more reliable results [14].

2. Experimental

2.1. Reagents

All reagents and solutions were prepared with deionized water in Milli-Q system (Millipore, USA). To prevent contamination, all glasses were decontaminated with nitric acid (Merck, Germany) 10% (v/v) at least for 24 hour.

The stock solution of neomycin standard (1000 mg L^{-1}) was prepared by dissolution of the salt in phosphate buffer pH 8 (0.1 mol L^{-1}), and the working standard solutions were prepared by dilution of the stock solution with the same buffer. The ninhydrin solution, 2,2-dihydroxyindane-1,3-dione (Merck, Darmstadt), 2% (w/v) was prepared by dissolution of the salt in a mixture containing 50 mL of phosphate buffer pH 8 (0.1 mol L^{-1}) and 50 mL of ethylic alcohol.

2.2 Apparatus

To measure the absorbances, used was a single beam UV-Vis spectrophotometer (BIOSPECTRO SP-220) with a flow cell of 1 cm optical path. The wavelength was set at 570 nm and the data acquisition was done with software written with Visual Basic language.

A peristaltic pump (Ismatec IPC-08, Switzerland), with Tygon® tubes, was used for fluid propulsion on the FIA system. The manifold of the system was built with 0.8 mm i.d. polyethylene tubes. A lab-made autosampler and commutator to flow injection analysis (FIA) system were also used [15]. A pHmeter (Handylab 1 Schott, UK), with combined glass electrode, was used to measure the pH of solutions.

For the comparison method, it was used HPLC (Shimadzu) model LC 20A with spectrophotometric detector. The samples were solubilized in 0.02 mol L^{-1} borate solution and placed in an ultrasonic bath, over 10 minutes, for the analyte extraction. After that, a derivatization of neomycin with dinitrofluorobenzene was carried out. The mobile phase was a sodium phosphate buffer (30%) and acetonitrile (70%) mixture, with flow rate of the 0.5 ml min^{-1}, temperature 35°C and injected volume of 25 µL [16].

2.3 Sample preparation

To prepare the pharmaceutical samples [7], 10 g of the semi-solid (ointments) samples were placed into a separator flask with 50 mL of ethyl ether, then four aliquots (25 mL) of phosphate buffer pH 8 (0.1 mol L^{-1}) were added. The contents were joined and brought to determination. The solid samples were weighed 0.1 g for feedstock and 1.5 g for pharmaceutical formulation, and transferred to volumetric flasks of 50 mL, which were filled with phosphate buffer pH 8.0.

The liquid sample was determined without pre treatment, i.e. by direct introduction in the FIA system.

2.4 FIA system

Figure 1 shows the FIA manifold for neomycin determination. The sample aliquot was inserted into the system, and at confluence C receives the ninhydrin reagent that causes the neomycin hydrolysis in the reactor R1, resulting in the colored compound. In the rector R2 the sample is cooled to avoid temperature gradient that could cause problems in the base line. Finally, the sample was conducted to the detector to perform absorbance measurement at 570 nm. All process is controlled by personal computer through the software written in Visual Basic® Language.

2.5 FIA system optimization

Table 1 presents the full factorial design to verify what the significant parameters were. The Doehlert matrix, employed to obtain the optimum values of each parameter, was presented on Table 2. All statistical multivariate calculus and plots are done employing a Statistica® software package (StatSoft, Tulsa, USA).

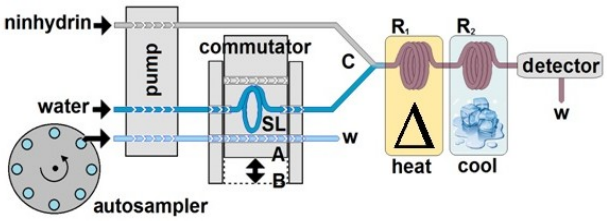

Fig.1. Flow system manifold for neomycin determination. SL: sample loop; C: confluence; R1: reactor 1; R2: reactor 2; W: waste.

Table 1. Factors, levels and analytical responses obtained from the 2^4 full factorial designs.

Factors		Levels			
		Low (-)		High (+)	
CR (% w/v)		2.0		4.0	
SL (cm)		30		60	
R1 (cm)		350		700	
R2 (cm)		50		100	
Runs	CR[a]	SL[b]	R1[c]	R2[d]	Absorbance[e]
1	-	-	-	-	0.193/0.213
2	+	-	-	-	0.640/0.669
3	-	+	-	-	0.864/0.856
4	+	+	-	-	0.501/0.506
5	-	-	+	-	0.575/0.597
6	+	-	+	-	0.442/0.450
7	-	+	+	-	1.35/1.44
8	+	+	+	-	0.253/0.242
9	-	-	-	+	0.515/0.484
10	+	-	-	+	0.196/0.206
11	-	+	-	+	0.751/0.780
12	+	+	-	+	0.940/0.945
13	-	-	+	+	0.391/0.388
14	+	-	+	+	0.682/0.651
15	-	+	+	+	0.936/0.938
16	+	+	+	+	1.88/1.86

[a]CR: concentration of the ninhydrin reagent
[b]SL: sample loop
[c]R1: reactor 1
[d]R2: reactor 2
[e]The experiments were done with 2.5 g L^{-1} neomycin

3. Results and discussion

3.1 Factorial design and Doehlert matrix

Figure 2 shows the Pareto chart obtained from full factorial design performed on the first step of the optimization. As can be noted from the Pareto chart, the sample loop, R1 and the interaction CRxR2 presented significant effects on the system at a 95% confidence level.

The positive effect for SL indicates a signal increase directly proportional to the sample volume in the studied range.

The correlation between dispersion and sample volume is well known in FIA systems, i.e, when a higher volume is introduced, a lower dispersion is observed, resulting in higher signal.

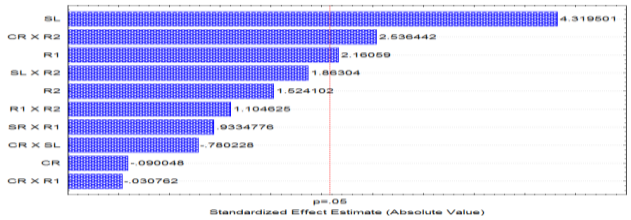

Fig 2. Pareto chart showing the factors effects. Confidence level of 95%.

The reactor R1 also shows a positive effect (2.1606), indicating that longer reactor contributes to signals improvement. The increasing in signal proportional to reactors can be related to the low reaction rate between neomycin and ninhydrin. Thus, a longer reactor promotes much more time to the reaction, therefore increasing the signal.

Regarding the factors CR and R2, these reveal no significant effects, thus their values were set at 2.0% (w/v) and 50 cm, respectively. The other SL and R1 factors were optimized through Doehlert matrix for two factors [17]. The central point was performed in triplicate to estimate the experimental error. The Doehlert matrix level and responses are summarized in Table 2 and the surface response chart is shown in Figure 3.

Table 2. Doehlert matrix used in the sample pH optimization and concentration factors buffer.

Runs	[a]R1 (cm)	[b]SL (cm)	[c]Absorbance
1	700	130	0.501
2	700	130	0.489
3	700	130	0.450
4	700	110	0.225
5	650	120	0.139
6	650	140	0.171
7	700	150	0.222
8	750	140	0.227
9	750	120	0.302

[a]R1=reactor 1
[b]SL: sample loop
[c]The experiments realized with 1.0 g L^{-1} neomycin.
Figure 3, shown maximum point at 130 and 700 cm for SL and R1, respectively.

3.2 Interference studies

In order to evaluate the interference of coexisting substances, binary solutions were prepared from the pharmaceutical samples containing the analyte at concentration of 2.0 g L^{-1} and the interferent at proportion in weight of 1:1, 1:5 and 1:10. The studied interferents were: kaolin, bacitracin, lidocaine and dexamethasone, lactose, starch, cellulose and $CaCO_3$. It was adopted as interferent those which present recovery values lesser than 90% and or higher than 110% [18]. As can be noted in Table 3, none of the studied concomitant presented significant interference, thus, despite of spectrophotometric detection, the method has very good selectivity.

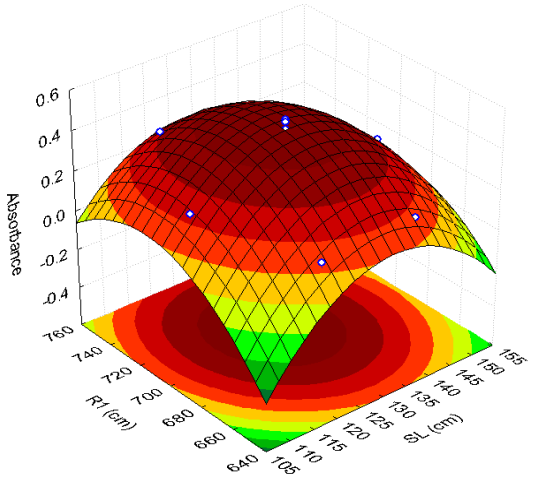

Fig. 3 Response surface relating the sample loop (SL), reactor 1 (R1), and absorbance. The ninhydrin concentration and reactor 2 (R2) values were fixed as 2% (w/v) and 50 cm, respectively.

Table 3. Influence of coexisting substances in the neomycin determination.

Interferents	1/1	1/5	1/10
Kaolin	101.93	107.74	107.1
Bacitracin	106.97	108.39	91.46
Lidocaine	98.99	97.48	99.50
Dexamethasone	98.49	97.34	95.98
Lactose	98.00	96.08	92.65
Starch	92.00	96.5	94.5
Cellulose	102.00	100.5	95.00
$CaCO_3$	104.5	96.00	91.00

3.3 Analytical figures of merit

At optimized conditions the method presents two linear ranges: 29.8 up to 300 mg L^{-1}, (Abs=1.1x10^{-4}(neomycin mg L^{-1}) + 1.9x10^{-3}; R^2 =0.9933) and 800 up to 2500 mg L^{-1} (Abs=1.1x10^{-3} (neomycin mg L^{-1}) – 8.90x10^{-1}; R^2 = 0.9926). The detection limit [19] was 8.94 mg L^{-1} and the quantification limit was 29.8 mg L^{-1}. The reading precision presented RSD always lower than 8.0 % (n=3) The analytical frequency was about 33 readings h^{-1}, and as above mentioned, there are no significant interferences.

3.4 Method application and accuracy studies

Table 4. Neomycin determination in pharmaceutical samples using proposed and reference methods.

Sample	[c]Proposed method	[c]HPLC method	Labeled value
Pharmaceutical A	[a]5.05±0.06	5.20±0.03	5.0
Pharmaceutical B	[b]1.05±0.08	1.03±0.01	1.0
Pharmaceutical C	[b]5.30±0.02	5.14±0.02	5.0
Pharmaceutical D	[b]5.60±0.02	5.05±0.02	5.0

[a]Results expressed in mg L^{-1}
[b]Results expressed in mg g^{-1}.

The results are expressed as mean±RSD, (*n=3*). *Test t* 95% confidence.

The developed method was applied to the neomycin determination in four pharmaceutical formulations. The accuracy was checked through comparison with HPLC method [16]. In Table 4, it can be noted the agreement among the results (*t test*, p=0.05). The results were also similar to the labeled values.

4. Conclusion

The proposed method showed satisfactory results to determine neomycin in pharmaceutical samples. The main features are rapidity expressed by sampling rate of 33 readings per hour; low reagent consumption: 62 mg of neomycin, 136 mg of ninhydrin per readings, and exploration of kinetic neomycin/ninhydrin reaction. There is no interference by concomitants of the samples studied. The method was compared with HPLC and the results were in agreement at a 95% confidence level (*t-test*). The readings, presenting RSD always lesser than 8.0%, show the method's good precision. Thus, the proposed method is one feasible alternative to neomycin determination in veterinary samples

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