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HPLC

Clean, safe and fast method by HPLC for quantification of rifaximin-based samples

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Resumo

Rifaximina, é um medicamento antimicrobiano oral, pertence à Classe IV, de acordo com o Sistema de Classificação Biofarmacêutica e apresenta baixa solubilidade e permeabilidade. Assim, três amostras baseadas em rifaximina foram desenvolvidas por complexação à β-ciclodextrina, usando diagrama de solubilidade de fase, malaxagem e diminuição do tamanho de partícula usando moagem úmida. A efetividade do preparo de novas amostras à base de rifaximina foi previamente confirmada por técnicas de análise térmica e espectroscópicas, mas a quantificação de rifaximina ainda não foi realizada por não haver método disponível para este fim. Um método ecologicamente correto por CLAE foi desenvolvido e validado para avaliar o conteúdo de rifaximina nestas amostras. A análise foi realizada usando-se uma coluna C18, água purificada com ácido acético a 0,1% e etanol (52:48, v/v) como fase móvel, 0,9 mL min^{-1}, 290 nm e 25°C. O método foi linear na faixa de 5 a 50 μg mL^{-1} e preciso, com desvios padrão relativos de 1,15% para o nível intra-dia e 0,47% para o nível inter-dia. A exatidão foi confirmada pela recuperação de padrão com resultados entre 100,99 e 101,32%. O método foi robusto a pequenas alterações no comprimento de onda e temperatura utilizadas, fonte de água purificada, proporção de etanol e ácido acético na fase móvel e vazão. A seletividade foi confirmada pela avaliação de possíveis interferências e pelos testes de degradação forçada em meios ácido, básico, oxidativo, neutro e fotolítico e mostrou-se indicativo de estabilidade. O método também envolve características da Química Analítica verde em análise farmacêutica: não utiliza solventes orgânicos tóxicos, é rápido, com tempo de aproximadamente 5 minutos, e geração de baixo volume de resíduos.

Palavras chaves: amostras baseadas em rifaximina, CLAE, validação de método analítico, controle de qualidade, análise farmacêutica.

Abstract

Rifaximin, is an oral antimicrobial drug, belongs to Class IV, according to the Biopharmaceutic Classification System, and has low solubility and permeability. Three rifaximin-based samples were developed by complexation to β-cyclodextrin using phase solubility diagram and malaxation, and decreasing particle size using wet milling. The effectiveness of preparation of new rifaximin-based samples has been previously confirmed and characterized by thermal and spectroscopic techniques, but the quantification of rifaximin has not yet been performed because there is no method available. So an ecologically correct method by HPLC was developed and validated to evaluate the rifaximin content in them. The analysis was performed using a C18 column, water with 0.1% glacial acetic acid and ethanol (52:48, v/v) as mobile phase, 0.9 mL min^{-1}, 290 nm, and 25°C. The method proved to be linear in the range of 5-50 μg mL^{-1} and precise with relative standard deviations of 1.15% for intra-day and 0.47% for inter-day precision. Accuracy was confirmed by standard recovery with mean recoveries between 100.99 and 101.32%. The method was robust to small changes in wavelength and temperature used, purified water source, the proportion of ethanol and acetic acid in the mobile phase and flow. Selectivity was confirmed by the evaluation of possible

interferences and forced degradation tests in acidic, basic, oxidative, neutral, and photolytic media and it proved to be indicative of stability. The method also involves characteristics of green Analytical Chemistry for a pharmaceutical analysis: it does not use toxic organic solvents, it is fast (approximately 5 minutes), and waste generation is low.

Keywords: rifaximin-based samples, HPLC, analytical method validation, quality control, pharmaceutical analysis.

1. Introduction

Rifaximin is an antimicrobial, marketed as tablets, used for the treatment of hepatic encephalopathy, ulcerative colitis, irritable bowel syndrome, *Clostridium difficile*, travelers' diarrhea, and acute diarrhea (1-10). Administration of high doses, 600 to 800 mg per day, is required for successful therapeutic effect. Rifaximin belongs to class IV according to the Biopharmaceutic Classification System (BCS). It presents low solubility in water and low permeability (11).

Whitin this context, people who have trouble swallowing, such as children and the elderly, may have to do that two to three times per day. In order to facilitate the pharmacotherapy, three rifaximin-based samples were developed by complexation to β-cyclodextrin using phase solubility diagram, malaxation, and decreasing particle size using poloxamer and wet milling. The identity of rifaximin in these new samples is usually confirmed by spectrophotometry in the infrared region, differential scanning calorimetry and X-ray diffraction (XRD), since their characteristics were different from the ones of pure drug and also from physical mixtures between drug and adjuvants. However the content of rifaximin has not yet been investigated (11).

Different research groups have already reported methods for quantitative evaluation of rifaximin in tablets, such as spectrophotometry in the ultraviolet (12-13), visible (14) and infrared regions (15), capillary electrophoresis (16), thin layer chromatography (17), high performance thin layer chromatographic (18) and high performance liquid chromatography (HPLC), but not for the developed rifaximin-based samples (19-21). HPLC is the most commonly used method for the analysis of rifaximin in tablets, so it was chosen to evaluate rifaximin-based samples. Therefore, an HPLC method, covering the principles of green Analytical Chemistry, was developed specifically for these rifaximin-based samples, counting on the possible impacts of β-cyclodextrin and poloxamer in the quantification of the antimicrobial agent.

2. EXPERIMENTAL

2.1. Equipment

HPLC system (Waters, Santa Clara CA, USA) equipped with binary gradient chromatography pump (Model 1525), manual injector (Model Breeze 7725i Rheodyne Bensheim, Germany) and UV-Vis detector (Model 2487), Eclipse Plus C18 column (Agilent, Santa Clara CA, USA), 5.0 µm particle size, 150 mm x 4.6 mm, analytical balance (Model 410, Kern, Germany), ultrasonic bath (Ultrasonic Cleaner Unique, Indaiatuba, Brazil) and water purification system (Millipore, Darmstadt, Germany).

2.2. Material and reagents

Rifaximin standard (4-deoxy-4′-methylpyrido [1′,2′-1,2] imidazo [5,4-c] rifamycin, 99.0% purity, CAS No. 80621-81-4, NutraTech Development Limited, China), rifaximin-based samples in powder form named X, Y, and Z, β-cyclodextrin (Roquette, Lestrem, France) and poloxamer 188 (O-Basf, Helsinki, Finland) were used.

Rifaximin samples have been obtained according to a former study of this group. Sample X refers to the rifaximin: β-cyclodextrin complex, obtained through a phase solubility diagram, sample Y refers to the rifaximin: β-cyclodextrin complex obtained by malaxation, while sample Z refers to the rifaximin: poloxamer 188 complex obtained by wet milling (11).

Reagents used were ethyl alcohol HPLC grade (J.T.Baker, Aparecida de Goiânia, Brazil), glacial acetic acid HPLC grade (Synth, Diadema, Brazil), ethyl alcohol analytical reagent grade (Synth).

2.3. Chromatographic conditions and pharmaceutical preparations

Chromatographic analyses was carried out using a mobile phase consisting of water with 0.1% glacial acetic acid and ethyl alcohol (52:48, v/v), the flow rate at 0.9 mL min^{-1}, injection volume 20 µL, wavelength set at 290 nm and temperature was 25°C. Standard and sample stock solutions equivalent to 100 µg mL^{-1} of rifaximin were prepared by dissolving an accurately weighed amount of standard drug in mobile phase.

2.4. Methods

2.4.1. Validation

Linearity, selectivity, precision, accuracy, robustness, and limit of detection and quantification were the parameters evaluated for validation of the method according to national and international guidelines (22-25).

2.4.2. Linearity

Three calibration curves were constructed in three days. The concentrations used ranged from 5 to 50 µg mL^{-1} (6 points = 5, 10, 20, 30, 40, 50 µg mL^{-1}), and they were analyzed in triplicate. Linearity evaluation was performed by linear regression analysis and analysis of variance (ANOVA).

2.4.3. Limit of detection (LOD) and Limit of quantification (LOQ)

LOD and LOQ were determined using data from the mean analytical curve obtained in the linearity and the Equations 1 and 2, respectively.

$$LOD = (3.3 \text{ standard deviation of the average intercept})/\text{ slope} \quad (1)$$

$$LOQ = (10 \text{ standard deviation of the average intercept})/\text{ slope} \quad (2)$$

2.4.4. Selectivity

Selectivity was confirmed by the forced degradation of rifaximin-based samples in a bath at 80°C in addition to acidic (HCl 0.01 *M*), alkaline (NaOH 0.001 *M*), oxidative (H_2O_2 10%), photolytic (UV light) and neutral (H_2O) conditions (26). Aliquots were taken and analyzed immediately by the proposed method.

2.4.5. Precision

Precision was evaluated by intraday (analysis of 6 solutions on the same day, under the same experimental conditions and by the same analyst) and intermediate (analysis of six solutions on different days, under the same experimental conditions and by two different analysts) levels using the concentration of 30 µg mL^{-1}. The relative standard deviation (RSD) was calculated.

2.4.6. Accuracy

Accuracy was determined through standard recovery tests. Different amounts of rifaximin standard solutions (19, 25 and 31 µg mL^{-1}) were added to a 5 µg mL^{-1} sample solution to obtain solutions with final concentrations of 24 µg mL^{-1} (80%), 30 µg mL^{-1} (100%) and 36 µg mL^{-1} (120%). Table 1 summarizes the planning of accuracy assays.

2.4.7. Robustness

Parameters such as wavelength (normal = 290 nm and modified = 295 nm), temperature (normal = 20°C and modified = 25°C), purified water source (normal = Lab 1 and modified = Lab 2), the proportion of ethanol in the mobile phase (normal = 48% and modified = 46%), the proportion of acetic acid in the mobile phase (normal = 0.1% and modified = 0.08%), flow (normal = 0.9 mL min^{-1} and modified = 0.88 mL min^{-1}), injection volume (normal = 20 µL and modified = 16 µL) were evaluated by Youden Test (27).

Table 1. Information about the preparation of sample solutions for the analyses of accuracy

Level (%)	Concentration of standard solutions of rifaximin used to reach final solutions (µg mL^{-1})	Total solution concentration (standard and sample) (µg mL^{-1})
80	19	24
100	25	30
120	31	36

2.4.8. Evaluation of rifaximin content in rifaximin-based samples

After method validation, standards and samples were prepared at a concentration of 30 µg mL^{-1} and immediately analyzed under the conditions of the proposed method.

3. RESULTS AND DISCUSSION

3.1. Linearity

The method can be considered linear. It showed a correlation between rifaximin concentration and peak area in the range of 5 to 50 µg mL^{-1}, with significant regression and no linearity deviation (Table 2).

3.2. Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were 0.11 µg mL^{-1} and 0.34 µg mL^{-1}, respectively.

3.3. Selectivity

The method can be considered selective, because it was able to detect rifaximin in the presence of adjuvants such as β-cyclodextrin, poloxamer, as well as degradation products formed in the forced degradation tests, which also indicates stability of the process (Figure 1).

3.4. Precision

The method showed proximity among results, with low RSD values for both intraday and intermediate precision (Table 3). Thus, it can be considered precise.

3.5. Accuracy

The proximity of the results to the true value and the average recovery showed values within the specification

Table 2. Data related to method linearity

	Parameters	Results
Linearity	Range (µg mL-1)	5-50
	Slope	34089
	Intercept	16325
	Correlation coefficient (r)	0.9999
	Among concentrations	428.43* (3.11)
	Regression	2141.81* (4.75)
	Lack of fit	0.09 (3.26)
	Retention time (min)	~ 4.7

* significant for p <5%

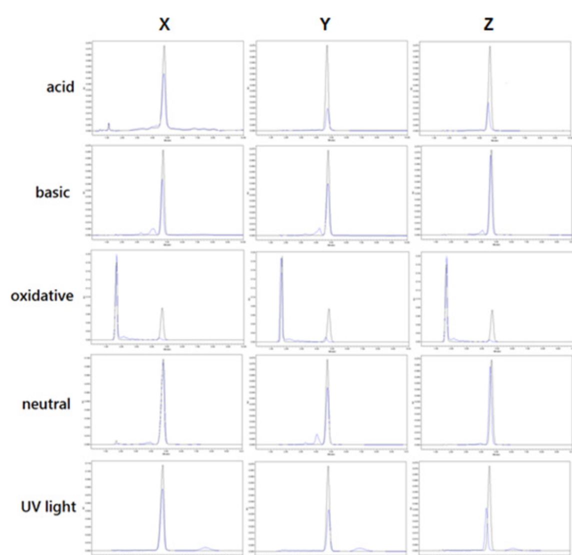

Figure 1. Chromatograms of the degradation of samples X, Y and Z, respectively, under acid, basic, oxidative, neutral conditions and in UV light. Black = zero condition, no degradation. Blue = degraded condition.

of 98 to 102% (Table 3) (28-29), proving the accuracy of the method.

3.6. Robustness

The method can be considered robust for variations in wavelength (nm), temperature (°C), purified water source, proportion of ethanol in the mobile phase (%), proportion of acetic acid in the mobile phase (%) and flow (mL min^{-1}), according to information given in the section 2.4.7. It was not robust for the change in injection volume (µL). Therefore, this parameter must be carefully controlled, and the exact 20 µL volume must be used (Table 4).

Table 3. Results of method precision and accuracy experiments

Precision

λ	Level	Peak area 1	2	3	4	5	6	RSD (%)
290 nm	Intraday	989145	1015057	985196	985990	987184	994910	1.15 (n=6)
	Intermediate	1000339	1004508	1006675	1007168	1009267	1005688	0.47(n=12)
	precision	993853	1000037	1007563	998869	1006240	999039	

Accuracy

Sample	Level (%)	Standard rifaximin added (µg mL-1)	Standard rifaximin recovered (µg mL-1)	Recovery (%)	Average recovery (%)	RSD (%)
X	**80**	19	19.13	100.68	101.10	1.18
	100	25	25.38	101.50		0.05
	120	31	31.35	101.12		0.41
Y	**80**	19	19.10	100.55	101.32	0.82
	100	25	25.43	101.73		0.28
	120	31	31.52	101.69		0.10
Z	**80**	19	19.10	100.54	100.99	1.10
	100	25	25.39	101.56		0.29
	120	31	31.27	100.87		1.60

Table 4. Results of method robustness

Condition	Experiments								Effect	Limit*
	1	2	3	4	5	6	7	8		
Wavelength (nm) A: 290; a: 295	A	A	A	A	a	a	a	a	-1.18	
Temperature (°C) B: 20; b: 25	B	B	b	b	B	B	b	b	0.74	
Purified water source C: lab 1; c:lab 2	C	c	C	c	C	c	C	c	-0.84	
Ethanol (%) D: 48; d: 46	D	D	d	d	d	d	D	D	-0.78	5.95
Acetic acid (%) E: 0.1; e: 0.08	E	e	E	e	e	E	e	E	0.61	
Flow (mL min^{-1}) F: 0.9; f: 0.88	F	f	f	F	F	f	f	F	-3.26	
Injection volume (µL) G: 20; g: 16 µL	G	g	g	G	g	G	G	g	6.90	
Results (%)	96.95	93.53	93.34	97.21	91.40	103.01	100.04	91.33		

*standard deviation multiplied by the square root of 2. Capital letters (A, B, C, D, E, F, G) = normal conditions. Small letters (a, b, c, d, e, f, g) = modified conditions.

3.7. Evaluation of rifaximin content in rifaximin-based samples

The method was able to quantify rifaximin content in the samples and can be applied to evaluate the quality of these new formulations (Table 5). It also has advantages such as the analysis speed (retention time of rifaximin less than 5 minutes), which consequently streamlines analyses and improves work performance. Also, ecological awareness is highlighted due to experimental choices such as solvents (ethanol and acidified water), column (15 cm), low number of steps involved, solvent (mobile phase), temperature (ambient), and flow rate (less than 1 mL min^{-1} decreases waste generation) (30-33).

4. CONCLUSIONS

The analytical method proved to be linear, selective, precise, exact, robust, and able to quantify

Table 5. Results of the evaluation of rifaximin content in rifaximin-based samples

Sample	Rifaximin content* %			Average	RSD (%)
X	83.91	92.61	88.41	88.31	4.93
Y	95.96	94.58	98.74	96.43	2.20
Z	100.92	98.76	99.41	99.69	1.11

* Each value represents the mean of three determinations. X, Y and Z designate the three rifaximin-based samples mentioned in section 2.2.

rifaximin content in the three different types of samples of this pharmaceutical drug. It is reliable, fast, and presents ecologically correct characteristics, as it generates low amounts of waste and do not involve toxic organic solvents.

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CONFLICT OF INTEREST

The authors have no financial or other potential conflicts of interest.

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