Pharmaceutica Analytica Acta

Kogawa and Salgadoa, Pharm Anal Acta 2016, 7:11
DOI: 10.4172/2153-2435.1000518

Research Article OMICS International

Optimization of Microbiological Method by Turbidimetry for Quantification of Rifaximin Tablets: Validation, Application and Evaluation of Degraded Compounds

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Received date: October 27, 2016; **Accepted date:** November 29, 2016; **Published date:** November 30, 2016

Abstract

Rifaximin, an oral antibiotic, is used for the treatment of hepatic encephalopathy, ulcerative colitis, irritable bowel syndrome, Clostridium difficile, travelers' diarrhea and acute diarrhea. The development of methods which evaluate the potency of antimicrobials is fundamental. So, this work presents the validation of a microbiological method by turbidimetry to evaluate the power of rifaximin tablets, as well as the evaluation of degraded drug in acidic, basic, neutral and photolytic conditions. For this, *Escherichia coli* ATCC 10536 were used. The method was linear over the concentration range of 50-98 μg mL^{-1} with correlation coefficients 0.9976 (standard) and 0.9999 (sample), precise (repeatability RSD=4.96 and interday RSD=3.92), exact (recovery 100.70%); robust against small and deliberate variations in method and indicative of stability because it allowed evaluating rifaximin degraded under stress conditions. The results were compared with data obtained by high-performance liquid chromatography. The validated turbidimetric method is very useful to the routine quality control for microbiological evaluation of rifaximin tablets.

Keywords: Rifaximin; Tablets; Antibiotic; Microbiological method; Turbidimetry; Indicative of stability; HPLC

Introduction

Rifaximin (Figure 1) is an oral antibiotic safe for presenting adverse effects compared to placebo [1-3].

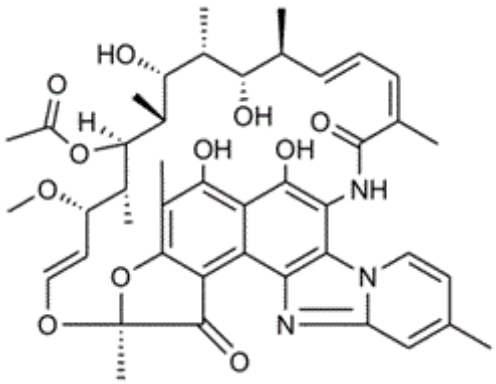

Figure 1: Chemical structure of rifaximin (CAS 80621-81-4).

Rifaximin is used for the treatment of various diseases such as hepatic encephalopathy, ulcerative colitis, irritable bowel syndrome, Clostridium difficile, travelers' diarrhea and acute diarrhea [1-9]. This makes rifaximin close to many people and therefore the proper quality control of this type of medicine is extremely important and required.

The question of the use of antimicrobials currently requires caution and, above all, information. The increasing human resistance to antimicrobials can make these drugs ineffective in the near future, leading the world into a period "post-antibiotic" in which, for example, a simple infection can be fatal [10].

The main cause of bacterial resistance is the abusive and indiscriminate use of antimicrobials, or by bad medical indication or self-medication, which creates an environment extremely favorable to the emergence of resistant bacteria [10]. Another relevant factor, but that is often overlooked is the lack of microbiological quality control of antimicrobials simultaneously with the physico-chemical tests.

Rifaximin does not present microbiological method described neither in official compendia [11-16] nor in the literature and they are extremely important and necessary in the evaluation of power of antimicrobials [17-24]. The physical-chemical methods are unable to provide this type of information. Therefore, the union of these two types of methods is essential in the release of result of antimicrobial product.

The lack of microbiological method to evaluate the power of rifaximin tablets was a gap that propelled us. Another motivation was to make the microbiological method more practical, dynamic, low-cost optimizing equipment and analysts. For this, we chose the microbiological test by turbidimetry.

So, this work presents the validation of a microbiological method by turbidimetry to evaluate the power of rifaximin tablets, as well as the evaluation of degraded drug in acidic, basic, neutral and photolytic conditions. The results were compared with data obtained by high-performance liquid chromatography.

Experimental

Equipment's

Apparatus Shaker MarconiTM model MA 420, oven OdontobrásTM model ECB2 at 35°C, autoclave PhoenixTM model AV 50 and automatic pipettors with capacity of 200 and 1000 µL were used. A QuimisTM spectrophotometer model Q-798 DRM was used to measure the turbidity of the tubes in absorbance.

LC analysis was performed on a Waters™ LC system equipped with Waters 1525 binary gradient chromatography pump, Rheodyne Breeze 7725i manual injector and Waters 2487 UV-Vis detector, Eclipse Plus™ C18 5 um column (150 mm × 4.6 mm, 5.0 µm particle sizes). Analytical balance model DV215CD Toledo™, ultrasonic bath Ultrasonic Cleaner Unique™, water purification system Millipore™ and membranes of polytetrafluoroethylene (PTFE) hydrophilic with pore 0.45 im and diameter 47.0 mm were used.

Chemicals and reagents

Rifaximin standard, content of 99.0%, acquired of the company NutraTech Development Limited (China). The pharmaceutical form used was rifaximin 200 mg tablets (labeled content), lot 12927, under the trade name Flonorm® of Laboratory Gonher Pharmaceuticals Ltd.

The turbidimetric method used broth Brain Heart Infusion (BHI) (Acumedia™), distilled water and formaldehyde 12% (Dinâmica™).

The chemical used were HPLC grade ethyl alcohol (J.T.Baker™), HPLC grade glacial acetic acid (Synth™), analytical reagent grade ethyl alcohol (Synth™), and deionised Milli Q water (Millipore™). Mobile phase was prepared by mixing water + 0.1% glacial acetic acid and ethyl alcohol in the ratio 52:48 (v/v) filtered through 0.45 µm membrane filter. The diluent used was the mobile phase.

Microorganism and inoculums

The strain of *Escherichia coli* ATCC 10536 were cultivated and maintained on BHI agar medium in the freezer and pealed to BHI broth (24 h before the assay) that was kept at 35 ± 2°C. On the test day, the bacteria, previously incubated in BHI broth, was also diluted with BHI broth to achieve a suspension turbidity of 25 ± 2% (transmittance) using a spectrophotometer and wavelength at 580 nm [11,14].

Turbidimetric conditions

The microbiological method describe followed the 3×3 assay design (3 doses of standard and 3 doses of sample).

The turbidimetric method consists of 8 tubes:

3 tubes for each standard concentration.

3 tubes for each sample concentration.

1 tube for the positive control.

1 tube for the negative control.

In 10 mL of BHI broth, contained in the tubes, were added 200 µL of each reference solution at concentrations of 50, 70 and 98 µg mL^{-1} and 200 µL of each sample solution at the same concentrations of 50, 70 and 98 µg mL^{-1}. The negative control contained only 10 mL of BHI and the positive control contained 10 mL of BHI with standardized inoculum to 25 ± 2% of transmittance.

The tubes were incubated at 35 ± 2°C for 4 h. The turbidity of the culture media, after this time, was measured by spectrophotometry.

Chromatographic conditions

Chromatographic analysis was carried out at ambient temperature on an Eclipse PlusTM C18 5 um column. The mobile phase was a mixture of water + 0.1% glacial acetic acid and ethyl alcohol in the ratio 52:48 (v/v). Flow rate was 0.9 mL min^{-1}. The detector wavelength was set at 290 nm with injection volume at 20 µL.

Pharmaceutical preparations

Stock and standard: 5 mg of rifaximin standard was weighed into a 25 mL volumetric flask, added 5 mL of ethyl alcohol and 20 mL of distilled water. It was sonicated for 10 min to complete dissolution of the drug. The mixture was diluted to the mark with distilled water. This stock solution (200 µg mL^{-1}) was filtered through filter paper and aliquots were removed and diluted in distilled water immediately before analysis.

Stock and sample: From a pool of 20 tablets of rifaximin pulverized, 9.44 mg (equivalent to 5 mg of standard) was weighed. This amount was transferred to a 25 mL volumetric flask, added 5 mL of ethyl alcohol and 20 mL of distilled water. It was sonicated for 10 min to complete dissolution of the drug. Then the mixture was diluted to the mark with distilled water, obtaining a concentration of 200 µg mL^{-1}. This stock solution was filtered through filter paper and aliquots were removed and diluted in distilled water.

Stock and placebo: The placebo was analyzed along with the standard and the sample to evaluate its influence on the turbidimetric analysis.

Methods

Development

In preliminary tests were tested microorganism, culture medium, diluent, inoculum concentration and drug concentration, as can be seen in Table 1.

Parameters	Description
Microorganism	*Bacillus subtilis* ATCC 9372 IAL 1027 *Micrococcus luteus* ATCC 9341 IAL 636 *Staphylococcus epidermidis* ATCC 12228 IAL 2150 *Escherichia coli* ATCC 10536 IAL 2393
Culture medium	Broth BHI, Broth Casoy, Broth Müller-Hinton
Inoculum concentration	0.8; 1.0; 1.5; 1.85; 1.9; 2.0; 3.5; 4.0; 4.5; 6.5; 8.0; 8.5; 10.0%
Diluent solution	Distilled water Phosphate buffer pH 6.8
Drug concentration	40, 60, 90 µg mL^{-1}; 50, 70, 98 µg mL^{-1}; 50, 100, 200 µg mL^{-1}; 25, 50, 100 µg mL^{-1}; 30, 60, 120 µg mL^{-1}

Table 1: Parameters tested during the development of microbiological assay by turbidimetry for analysis of rifaximin tablets.

Validation

The analytical parameters of linearity, selectivity, precision, accuracy and robustness were evaluated for validation of the turbidimetric method, according to AOAC [25], Brazil [26], ICH [27] and INMETRO [28].

Linearity

The linearity was evaluated by construction of three calibration curves developed on three different days and in triplicate. The concentrations used were 50, 70 and 98 μg mL^{-1}.

The data obtained in the construction of the calibration curve were analyzed to obtain the equation of the line by the least squares method and the check of linearity and parallelism was confirmed by analysis of variance (ANOVA).

Selectivity

The selectivity of the method was confirmed by the forced degradation of rifaximin tablets in HCl 0.01 M subject to 1 h in bath at 80°C, NaOH 0.001 M and distilled water (neutral degradation) subject to 6 h in bath at 80°C and in UV light at 25°C after 1 week of exposure. Degraded samples were analyzed by HPLC method to compare the results.

Precision

For evaluation of the intraday precision were performed six stock solutions of rifaximin standard, at concentration of 70 μg mL^{-1}, on the same day, same experimental conditions and same analyst. The evaluation of the results was done by calculating the Relative Standard Deviation (RSD).

For evaluation of interday precision were performed three stock solutions of rifaximin standard, at concentration of 70 μg mL^{-1}, on three different day, but the same experimental conditions and same analyst. The evaluation of the results was done by calculating the Test F (Snedecor) and Test t (Student).

Accuracy

The accuracy of the method was determined by the test of standard addition.

Stock solutions of rifaximin standard and sample were prepared at a concentration of 200 μg mL^{-1}. Of these solutions, aliquots of 1.25 mL of each one were taken and transferred to 5 mL volumetric flasks, obtaining a standard solution and another sample. They were completed with distilled water and obtained solutions with theoretical concentrations of 50 μg mL^{-1}.

From the sample stock solution, aliquots of 1.25 mL were removed and transferred to 5 mL volumetric flask added 150 μL of standard stock solution, completed with purified water to obtain a solution containing theoretical concentration of 56 μg mL^{-1}. Changing only the volume of standard stock solution, was similarly carried out for more two solutions through aliquots of 500 μL (theoretical concentration of 70 μg mL^{-1}) and 850 μL (theoretical concentration of 84 μg mL^{-1}).

The percent of recovered rifaximin was calculated by Equation I [25].

$$\%R = \left\{ \frac{(cr - ca)}{cp} \right\} \times 100 \qquad \text{(I)}$$

Where:

Cr=Concentration of sample solution added of standard (μg mL^{-1})

Ca=Concentration of sample solution (μg mL^{-1})

Cp=Theoretical concentration of standard solution added (μg mL^{-1})

Robustness

In parameter robustness the following conditions were changed and tested:

Incubation time of tubes (4 h to 3:40).

Concentration of inoculum (850 μL to 830 μL).

Shaker rotation speed (32 rpm to 30 rpm).

Mark of BHI broth (Acumedia™ to Himedia™).

Volume of culture medium in the tube (10 mL to 9.5 mL).

The evaluation of the results was done by calculating the Test F (Snedecor) and Test t (Student).

Results and Discussion

Development

The parameters in Table 2 were established in preliminary tests because they showed better performance in the analysis.

Validation

Linearity

Parameters	Description
Microorganism	*Escherichia coli* ATCC 10536 IAL 2393
Culture medium	Broth BHI
Inoculum concentration	8.5%
Diluent solution	distilled water
Drug concentration	50; 70 and 98 μg mL^{-1}

Table 2: Established parameters for the determination of rifaximin tablets by turbidimetry method.

Figure 2 shows the analytical curve for rifaximin standard and sample by turbidimetry.

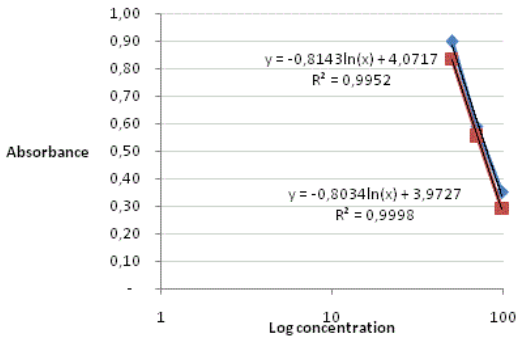

Figure 2: Analytical curve of rifaximin standard and sample solutions at concentrations of 50, 70 and 98 μg mL^{-1}, obtained by turbidimetric method.

The equation of the line, determined by the method of least squares, is: y=-0.8143 ln(x) + 4.0717, with coefficient of correlation (r) equal to 0.9994 for rifaximin standard and y=-0.8034 ln(x) + 3.9727, with coefficient of correlation (r) equal to 0.9999 for rifaximin sample. The analysis of variance (ANOVA) of data from the curves with rifaximin is shown in Table 3.

Specificity

The values of absorbances of the adjuvant solution and the diluent solution containing 20% ethyl alcohol were compared to the positive control, not interfering with the method.

The comparison of the results of turbidimetric and HPLC methods after forced degradation test of rifaximin tablets is shown in Table 4.

Accuracy

Table 5 shows the values obtained in the recovery test for rifaximin using turbidimetric method.

Precision

The intraday and interday precisions were made by repeatability and intermediate precision, respectively. The results are shown in Table 6.

Robustness

The results of the parameters evaluated in the robustness are in Table 7.

Potency of the antimicrobial agent

The potency of rifaximin tablets was checked by the method validated in this work and the result is shown in Table 8.

	Variation sources	Degrees of freedom	Sum of quadratic	Mean quadratic	Fcal	Ftab (0.05)
Linearity deviation	Preparation	1	0.0125	0.0125	24.24*	4.96
	Regression	1	0.8889	0.8889	1,726.57*	4.96
	Parallelism	1	0.0000	0.0000	0.08	4.96
	Quadratic	1	0.0016	0.0016	3.11	4.96
	Quadratic difference	1	0.0007	0.0007	1.31	4.96
	Between doses	5	0.90	0.18	351.06*	3.33
	Between tubes	2	0.02	0.01	3.67	4.10
	Within (error)	10	0.01	0.00	-	-
	Total	17	0.93	-	-	-

Table 3: Analysis of variance of the absorbances obtained from the analytical curve of rifaximin by turbidimetry. *Significant for p<5%.

Type of degradation	Method	
	HPLC	Turbidimetric
HCl 0.01 M	82.25%	86.77%
NaOH 0.001 M	77.01%	70.00%
Neutral	97.75%	99.59%
Photolytic	93.08%	43.18%

Table 4: Comparison of results of turbidimetric and HPLC methods for rifaximin tablets after stress test.

	Standard rifaximin added (μg mL^{-1})	Standard rifaximin recovered (μg mL^{-1})	Recovery (%)	Average recovery (%)	RSD (%)
R1	6	6.10	101.61	100.70	0.51
R2	20	19.93	99.67		1.84
R3	34	34.28	100.83		0.93

Table 5: Values obtained in the recovery test of rifaximin using the turbidimetric method.

ë	Level	Absorbance						RSD (%)
530 nm		1	2	3	4	5	6	
	Repeatability	0.563	0.523	0.582	0.538	0.515	0.634	7.93 (n=6)
	Intermediate precision	0.553	0.583	0.536	0.569	0.508	0.532	7.05 (n=12)
		0.543	0.554	0.562	0.627	0.627	0.627	

Table 6: Values determined for the parameter precision of rifaximin by turbidimetry.

Parameters	Normal condition	Modified condition	Test t	Robustness
Incubation time of tubes	4:00 hrs	3:40 hrs	0.56 (2.57)	Robust
Concentration of inoculum	850 μL	830 μL	2.38 (2.31)	No robust
Shaker rotation speed	32 rpm	30 rpm	12.91 (2.31)	No robust
Mark of BHI broth	AcumediaTM	HimediaTM	11.32 (2.31)	No robust
Volume of culture medium in the tube	10 mL	9.5 mL	0.30 (2.31)	Robust

Table 7: Robustness analysis of the turbidimetric method for rifaximin in tablets.

Day	Potency of rifaximin in tablets[a] (%)	Average	RSD (%)
1	107.79	106.78	1.15
2	107.14		
3	105.42		

Table 8: Values determined for the verification of potency of rifaximin in tablets by turbidimetric method. [a]Each value represents the mean of three determinations.

Microbiological methods have been replaced by physical-chemical tests, which are faster and more practical. However, the replacement of microbiological tests can create serious consequences on the safety and efficacy of antimicrobial [17,18,23].

In some cases, the content obtained by physical-chemical method does not correspond to the potency of the antimicrobial, generating false conclusions about quality of the product. This product, on the market, will not comply with its action and effectiveness and will maintain the individual's disease state, which suffocates the public health system, and additionally contribute to bacterial resistance.

The turbidimetric assay is a tool that combines microbiological test, required for the quality control of antimicrobial agents, and the optimization of time and analysts. It is a fast method, with the release of results in 4 h, and simple to run.

In the turbidimetric method validated for rifaximin in tablets were chosen concentrations 50-98 μg mL^{-1} and *Escherichia coli* as microorganism to 0.85%.

The results obtained in validation of the method demonstrated that rifaximin solutions showed linear correlation between the absorbances and concentrations in the range of 50 to 98 μg mL^{-1} and the data were validated by ANOVA.

The presence of adjuvants in the sample did not interfere in the analysis of rifaximin by turbidimetric method. This was proven by the absorbance values presented by adjuvants solution. They were

compared to the positive control, that is, they have no antimicrobial activity.

The purpose of forced degradation test is to validate an indicative method of stability. The turbidimetric method was sensitive to acid, basic, neutral and photolytic degradation. The results of HPLC for acidic, basic and neutral degradation are approximately similar to the microbiological results for power of rifaximin in tablets. However, the results of photolytic degradation by HPLC (93.08%) and turbidimetry (43.18%) showed results significantly different. This is the importance of conducting a microbiological method simultaneously with a physical-chemical method. The portion of the molecule essential for activity cannot be the same detected in the physical-chemical method, generating false conclusions about quality of the product.

The precision of the method was proven on two levels, intraday and interday. Both showed RSD lower than 8%.

In the study of accuracy, determined by the recovery test, the result was 100.70%, which proved the ability of the method to determine levels predefined accurately.

In robustness test was observed that there was no difference statistically significant when small changes are made in the shaker time and in the volume of medium in the tube. However, variations in mark of the culture medium, shaker speed and the concentration of the inoculum, in this case, were not robust. Therefore, care in these parameters is very important.

The potency of rifaximin in the pharmaceutical product analyzed was 106.78%.

This paper proposes a microbiological method for evaluation of rifaximin in tablets that values the quality and safety of pharmaceuticals and optimizes time and analysts.

Conclusion

A microbiological method should always accompany the analysis of an antimicrobial agent in order to present to the population a quality and safe product.

The turbidimetric method presented in this work showed linearity, selectivity, precision, accuracy, robustness and can be easily applied in routine analysis of rifaximin in tablets.

Acknowledgment

The authors acknowledge CNPq (Brasília, Brazil), FAPESP (São Paulo, Brasil), CAPES (São Paulo, Brasil) and PADC/FCF/UNESP (Araraquara, Brazil).

Declaration of Interest

The authors report no declarations of interest.

References

1. Pistiki A, Galani I, Pyleris E, Barbatzas C, Pimentel M, et al. (2014) *In vitro* activity of rifaximin against isolates from patients with small intestinal bacterial overgrowth. Int J Antimicrob Agents 43: 236-241.
2. Mantry PS, Munsaf S (2010) Rifaximin for the treatment of hepatic encephalopathy. Transplant Proc 42: 4543-4547.
3. Rivkin A, Gim S (2011) Rifaximin: New Therapeutic Indication and Future Directions. Clin Ther 33: 812-827.
4. Zhang X, Duan J, Li K, Zhou L, Zhai S (2007) Sensitive quantification of rifaximin in human plasma by liquid chromatography–tandem mass spectrometry. J Chromatogr B 850: 348-355.
5. Valentin T, Leitner E, Rohn A, Zollner-Schwetz I, Hoenigl M, et al. (2011) Rifaximin intake leads to emergence of rifampin-resistant staphylococci. J Infect 62: 34-38.
6. Mullen KD, Sanyal AJ, Bass NM, Poordad FF, Sheikh MY, et al. (2014) Rifaximin is safe and well tolerated for long-term maintenance of remission from overt hepatic encephalopathy. Clin Gastroenterol Hepatol 12: 1390-1397.
7. Ruiz J, Mensa L, O´Callaghan C, Pons MJ, González A, et al. (2007) In vitro antimicrobial activity of rifaximin against enteropathogens causing traveler's diarrhea. Diagn Micr Infec Dis 59: 473-475.
8. Jiang Z, Ke S, Dupont HL (2010) Rifaximin - induced alteration of virulence of diarrhoea producing *Escherichia coli* and Shigella sonnei. Int J Antimicrob Agents 35: 278-281.
9. Castro R, Domenichelli V, Lorenzo FPL, Prestipino M, Perrotta ML (1998) Rifaximin treatment for acute recurrent diarrhea in children with genitourinary disorders. Curr Therap Res 59: 746-752.
10. World Health Organization (2014) Antimicrobial Resistance: global report on surveillance, WHO Press: Geneva.
11. Brazilian Pharmacopoeia (2010) Brazilian Health Surveillance Agency, Atheneu: São Paulo.
12. World Health Organization (2005) Portuguese Pharmacopoeia, Infarmed: Lisboa.
13. Japonese Pharmacopeia (2011) Society of Japonese Pharmacopoeia: Tokyo.
14. The United States Pharmacopeia (2012) United States Convention Inc: Rockville MD.
15. European Pharmacopoeia (2011) Council of Europe: Strasbourg.
16. British Pharmacopoeia (2012) Her Majesty's Stationary Office: London.
17. Kogawa AC, Tomita LK, Salgado HRN (2012) Development and validation of a stability-indicative turbidimetric assay to determine the potency of doxycycline hyclate in tablets. Int J Microbiol Res 4: 316-321.
18. Tótoli EG, Salgado HRN (2013) Development and validation of a rapid turbidimetric assay to determine the potency of ampicillin sodium in powder for solution for injection. Anal Methods 5: 5923-5928.
19. Cazedey ECL, Salgado HRN (2013) A novel and rapid microbiological assay for ciprofloxacin hydrochloride. JPA 3: 382-386.
20. Vieira D, Fiuza T, Salgado HRN (2014) Development and validation of a rapid turbidimetric assay to determine the potency of cefuroxime sodium in powder for dissolution for injection. Pathogens 3: 656-666.
21. Pedroso TM, Salgado HRN (2014) Development and validation of a microbiological assay by turbidimetry to determine the potency of cefazolin sodium in lyophilized powder. Anal Methods 6: 1391-1396.
22. Silva LM, Salgado HRN (2015) Rapid turbidimetric assay to potency evaluation of tigecycline in lyophilized powder. J Microbiol Methods 110: 49-53.
23. Tótoli EG, Salgado HRN (2015) Rapid turbidimetric assay to determine the potency of daptomycin in lyophilized powder. Pharmaceutics 7: 106-121.
24. Curbete MM, Salgado HRN (2016) Rapid turbidimetric assay for quantification of fusidic acid in a dermatological cream. Talanta 153: 51-56.
25. AOAC (2002) Association of Official Analytical Chemists, Official Methods of Analysis Gaithersburg: AOAC.
26. ANVISA (2003) Agencia Nacional de Vigilancia Sanitaria. RE 899, de 29 de maio de 2003, Guia para Validação de Métodos Analiticos e Bioanaliticos, Brasília.
27. ICH Guidelines (2005) International Conference on Harmonization. Validation of Analytical Procedures, Text and Methodology Q2(R1), Geneva Switzerland.
28. INMETRO (2007) Instituto Nacional de Metrologia. Normalização e Qualidade Industrial, Orientação sobre Validação de Métodos de Ensaios Químicos DOQ-CGCRE-008, Brazil.