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DEVELOPMENT AND VALIDATION OF A STABILITY-INDICATIVE TURBIDIMETRIC ASSAY TO DETERMINE THE POTENCY OF DOXYCYCLINE HYCLATE IN TABLETS

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Abstract- The doxycycline (DOX) is a broad-spectrum antibiotic used in several countries. This drug is part of the list of medicines to the SUS (Unified Health System), a model of health care in Brazil. This study describes the development and validation of a microbiological assay, applying the turbidimetric method for the determination of DOX, as well as the evaluation of the ability of the method in determining the stability of DOX in tablets against acidic and basic hydrolysis, photolytic and oxidative degradations, using *Escherichia coli* ATCC 10536 as micro-organism test and 3x3 parallel line assay design, with nine tubes for each assay, as recommended by the Brazilian Pharmacopoeia. The developed and validated method showed excellent results of linearity, selectivity, precision, accuracy and robustness. The assay is based on the inhibitory effect of DOX using *Escherichia coli* ATCC 10536. The results of the assay were treated by analysis of variance and were found to be linear (r= 0.9986) in the range from 4.0 to 9.0µg/mL, precise (repeatability R.S.D.= 0.99 and intermediate precision was confirmed by statistical analysis the mean values obtained from analysis by different analysts) and exact (97.73%). DOX solution exposed to direct UV light, alkaline and acid hydrolysis and hydrogen peroxide causing oxidation were used to evaluate the specificity of the bioassay. Comparison of bioassay and liquid chromatography showed differences in results between methodologies. The results showed that the bioassay is valid, simple and useful alternative methodology for DOX determination in routine quality control.

Keywords- Doxycycline hyclate, Tablets, Turbidimetric, Methods of analysis, Quality control, Validation

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Introduction

The synthetic pathway of doxycicline (DOX) involve metacycline (MTC) as an intermediate, during this process 6-epidoxycycline (EDOX) can be formed as a side product. DOX is a semisynthetic broad spectrum tetracycline antibiotic, widely used in veterinary medicine and as an animal feed supplement to prevent diseases [1].

Doxycycline presents itself in three forms: hyclate, monohydrate and hydrochloride. From the doxycycline hyclate is possible to obtain other forms.

The doxycycline hyclate [Fig-1] is the form hemihydrate and hemietanolada [2]. His description is hygroscopic yellow crystalline powder, should be stored in airtight containers and protected from light [3].

This drug presents the molecular formula $(C_{22}H_{24}N_2O_8.HCl)_2.C_2H_6O.H_2O$, CAS 24390-14-5 and molecular weight 1025.89g.mol^{-1} [3]. The doxycycline hyclate without burning fusion occurs in 201°C [4]. Tetracycline pKas values are approximately 3-4, 7-8 e 9-10. Shariati and collaborators (2009) [5] have the following pKa values for doxycycline hyclate: pK_{a1} 3.02±0.3; pK_{a2} 7.97±0.15; pK_{a3} 9.15±0.3. Doxycycline hyclate is sold in tablet dosage forms and lyophilized powder.

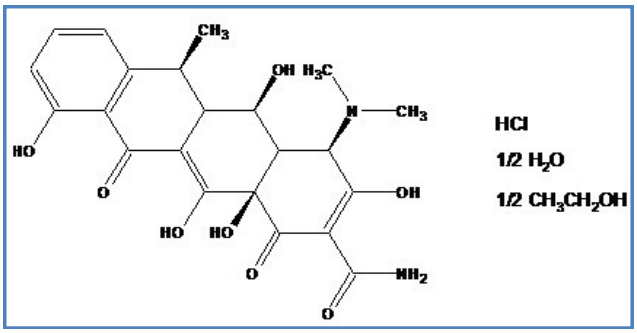

Fig. 1- Chemical structure of doxycycline hyclate (CAS 24390-14-5).

The doxycycline hyclate is a wide spectrum antibiotic used in several countries to treat infectious diseases and as an additive in animal nutrition to facilitate growth.

This drug is part of the list of medicines to the SUS (Unified Health System), a model of health care in Brazil, and it is free delivery in the public with a medical prescription. Thus, it is extremely important quality control of this medicine to be able to ensure their effectiveness and safety.

In Brazil this drug is marketed by pharmaceutical companies Apotex, EMS, Germed, Gross, Hexal, Legrand, Neo química, Neovita, Pfizer, Ranbaxy, Sanval, Teuto and União Química.

Tetracyclines inhibit bacterial protein synthesis through their link to the bacterial 30S ribosome, impeding access of aminoacyl-tRNA acceptor site in the mRNA-ribosome complex [6]. However, DOX has been studied as an inhibitor of matrix metalloproteinases (intercellular substance), an action unrelated to its effects on bacterial protein synthesis [6-7].

Doxycycline is more active than tetracycline against many species of bacteria including *Streptococcus pyogenes*, enterococci, anaerobic, and various *Nocardia* spp. Cross-resistance is common, although some *Staphylococcus aureus* resistant to tetracycline respond to doxycycline. Doxycycline is active against protozoa, particularly *Plasmodium* spp [8].

It is often used to treat chronic prostatitis, sinusitis, syphilis, Chlamydia and pelvic inflammatory disease [9]. Doxycycline has action against protozoa and must be administered in combination with quinine in the management of chloroquine resistant *Plasmodium falciparum*. Solutions of doxycycline are also used for malignant effusions [10] which occurring when there is an increase in the amount of fluids, usually associated with malignancies and lymphomas of the lung, breast and ovary [8].

Often the choice is doxycycline over than other tetracyclines in the treatment of infections by their better absorption and long half-life, which allows fewer daily doses [8].

The method of high performance liquid chromatography for determination of DOX is the choice of some Pharmacopoeias [11-13].

Kazemifard A.G. and Moore D.E. (1997) [14] showed that the official methods of microbial analysis are time consuming and poor in terms of sensitivity and specificity, and another problem is the fact that the degradation products, such as 6-epidoxiciclina, which are present in varying amounts in raw materials and finished products may also have antimicrobial properties, 2-5% of the activity of tetracycline. Thus, the results of the bioassay would not necessarily be an accurate representation of antimicrobial potency.

Materials and Methods

Chemical and Reagents

Doxycycline hyclate reference substance (assigned purity 97.10%) was supplied by União Química® (Brasil). Doxycycline hyclate tablets (Doxitrat®) claimed to contain 80mg of the active component was kindly provided by União Química. The placebo was prepared in the laboratory using amounts of pharmaceutical grade excipient: hydroxypropylmethylcellulose, polyethylene glycol 4000, mannitol, talc, starch, cellulose microcrystalline, magnesium stearate and croscamelose. The qualitative composition of placebo was the same as claimed in Doxitrat® 80mg. Ultrapure water was obtained from a MilliQ® Plus apparatus (Millipore®, USA) and it was used to prepare all solutions for the bioassay and HPLC methods. All chemical used were of analytical grade and all solvents were of HPLC grade. Acetonitrile was purchased from J.T. Baker® (USA), trifluoroacetic acid was obtained from Sigma Aldrich® (Germany), hydrochloric acid was obtained from Synth® (Brazil) and formaldehyde 12%. Brain Hearth Infusion (BHI) broth was purchase from Acumedia® (USA).

Apparatus

Photodegradation studies were carried out in a photostability UV chamber equipped with a UV lamp. A spectrophotometer (Quimis®, Brazil) was used to measure the turbidity of the tubes in absorbance. An LC system consisting of Waters®, model 1525 (Waters Chromatography systems, CA, USA) was connected to a UV/Visible Waters 2487 and an injector fitted Rheodyne Breeze 7725i with a 20µL loop. The chromatographic separation was carried out under isocratic reversed-phase conditions on an Luna CN column particle of 5µm (250mmx4.6mm) and pore 10 A^0 (Phenomenex®, USA). The mobile-phase consisted of water +0.1% trifluoroacetic acid (TFA): ACN+0.1% TFA (60:40, v / v), flow rate 1ml/min, injection volume 20µL, using a UV-VIS detector at 360nm and at room temperature.

Preparation of Samples Solutions

Twenty tablets were weighed and the powder was mixed. An amount of powder equivalent to 5mg of doxycycline hyclate was weighed the equivalent to average weight of one tablet and transferred to 50mL volumetric flask and the volume was completed with ultrapure water to obtain a solution with a concentration of 100.0mg/mL. Aliquots of this solution were diluted in ultrapure water to obtain the concentrations of 4.0; 6.0 and 9.0µg mL^{-1} (T1, T2 and T3 respectively), which were tested against S1, S2 and S3.

Microorganism and Inoculums

The strain of *Escherichia coli* ATCC 10536 were cultivated and maintained on tryptic soy agar medium in the freezer and pealed to BHI broth (24h before the assay) that was kept at 35±2°C. The microorganism standardization was made according to the procedure described in the Brazilian and The United States Pharmacopoeias [15, 13]. 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 Quimis®, model Q-798 DRM, and wavelength at 530nm.

Turbidimetric Bioassay

The bioassay describe followed the 3x3 assay design (three doses of the standard and three doses of the sample). The test consists of 8 tubes, 3 tubes for each standard concentration, 3 tubes for each sample concentration, 1 tube for the positive control and 1 tube for the negative control, according to the Brazilian Pharmacopoeia [15]. The broth was composed of Brain Hearth Infusion (BHI). They were added to the tubes 10ml of BHI broth and 200µL of each reference solution (4, 6 and 9µg/mL) in the nine tubes and 200µL of each sample solution in the other nine tubes (4, 6 and 9µg/mL). Eighteen and one negative control (containing only 10ml

of BHI broth) and one positive control (containing 10ml of BHI broth and standardized inoculum to 25±2%). The tubes were incubated at 35±2°C for 4 hrs. The turbidity of the culture media were measured by the apparatus spectrophotometric. All experiments were performed in a biological safety cabinet and the infected material was decontaminated before being discarded and all safety procedures (wearing masks, gloves and cap) were adopted.

Method Validation

The development of effective and reliable analytical methods for quality control of marketed drugs is extremely important and aims to provide reliable information about the nature and composition of the materials under analysis [16]. Validation is an important part of quality assurance program and aims to demonstrate that the analytical method is suitable for the intended proposal and it is safe to run [17], and the procedures included in the standards of Good Manufacturing Practices (GMP) required by the U.S. FDA, and applied in pharmaceutical industries and should also occur according to good laboratory practice (GLP) [18].

The method was validated according to the parameters of linearity, selectivity, precision, accuracy and robustness [13,17].

Linearity- in order to assess the validity of the assay, three concentrations of the standard substance were used. The linearity was evaluated by linear regression analysis. Correlation coefficient and analysis of variance (ANOVA) were calculated and presented.

Precision- in the precision was performed dosage tests of DOX tablets. Six stock solutions of DOX were performed, from which were withdrawn aliquots of each one to obtain solutions with theoretical concentration of 6µg mL^{-1}, in the same day and with the same experimental conditions for the evaluation of precision intra-day and calculate the coefficient of variation. And for three consecutive days, for evaluation of inter-analyst precision, the test was performed by different analysts and the results were statistically analyzed.

Accuracy- Stock solutions of doxycycline hyclate SQR and tablets were prepared at a concentration of 100µg mL^{-1}. These solutions were taken aliquots of 400µL of each and transferred to a 10mL volumetric flask, obtaining two balloons of 400µL each, one with SQR and the other with finished product. They were supplemented with purified water and obtained theoretical solutions with concentrations of 4µg mL^{-1}. From the stock solution of DOX tablets, were taken aliquots of 400µL and transferred to a 10mL volumetric flask and added 80µL of stock solution of DOX SQR, supplemented with purified water to obtain a solution with theoretical concentration of 4.8µg mL^{-1} (R1). Changing only the volume of stock solution of SQR, was done the same way to obtain more two solutions through aliquots of 200µL (theoretical concentration of 6µg mL^{-1}-R2) and 320µL (theoretical concentration of 7.2µg mL^{-1}-R3). The preparations of samples are exemplified in [Table-1].

Specificity- the ability of the proposed method to determine DOX in the presence of degradation products was assessed by comparing the results obtained from the degraded samples analyzed by bioassay and by HPLC method (developed and validated in the previous studies). Under all conditions, the sample solutions were assayed and compared to freshly prepared DOX reference substance solutions at the same theoretical concentrations. Specificity was also checked using the excipient of the formulation to determine whether this substance could interfere with the assay. The preparations of the degraded DOX and excipient solutions were the following.

Table 1- Preparation of solutions for testing the accuracy of the turbidimetric method for the DOX

	Volume added of DOX sample in the concentration of 100µg mL^{-1} (µL)	Volume added of DOX SQR in the concentration of 100µg mL^{-1} (µL)	Final theoretical concentration (µg mL^{-1})[a,b]
Sample	400	-	4,0
R1	400	80	4,8
R2	400	200	6,0
R3	400	320	7,2
Standard	-	400	4,0

[a]*diluted in a 10mL volumetric flask*

[b]*each concentration level was prepared in triplicate*

Acid Hydrolysis- the sample solutions were prepared and maintained in 0.1M HCl for 4 hrs. in bath a 70°C. After that, aliquots of these solutions (100µg mL^{-1}) were removed, neutralized with 0.1M NaOH and diluted in ultrapure water to achieve theoretical concentrations of 4.0, 6.0 and 9.0µg mL^{-1}.

Basic Hydrolysis- the sample solutions were prepared and maintained in 0.1M NaOH for 4 hrs. in bath a 70°C. After that, aliquots of these solutions (100µg mL^{-1}) were removed, neutralized with 0.1M HCl and diluted in ultrapure water to achieve theoretical concentrations of 4.0, 6.0 and 9.0µg mL^{-1}.

Photodegradation- aqueous sample solution (100µg mL^{-1}) was exposed to UV lamp for 4 hrs. The stress degradation study was performed exposing the solution in quartz cell in the photodegradation chamber. After that, this solution were removed and diluted in ultrapure water to achieve theoretical concentrations of 4.0, 6.0 and 9.0µg mL^{-1}.

Excipients- the amount of formulation excipient containded in equivalent to 5mg DOX tablets was accurately weighed and dissolved in a 50mL volumetric flask with ultrapure water. The same aliquots used to prepare the sample solutions were withdrawn and diluted in ultrapure water.

Robustness- The robustness of the method was determined by comparing the results obtained by varying the incubation time of the micro-organism from 20 to 24 hrs. The results obtained by varying this parameter were analyzed by F-test (Snedecor) and t-test (Student).

Calculation

To calculate the activity of DOX, the Hewitt equation was used [19]. The assays were calculated statistically by the linear parallel model and regression analysis and verified using analysis of variance (ANOVA).

HPLC Method

An LC system consisting of Waters®, model 1525 (Waters® Chromatography system, CA, USA) was connected to a UV/Visible Waters® 2487 and an injector fitted Rheodyne Breeze® 7725i with a 20µL loop. The chromatographic separation was carried out under isocratic reversed-phase conditions on a Phenomenex Luna CN® C18 column, 5µm, 250mmx4.6mm (Phenomenex®). The

mobile phase consisted of water +0.1% trifluoroacetic acid (TFA): ACN+0.1% TFA (60:40, v/v), flow rate 1ml min^{-1}, using a UV-VIS detector at 360nm and at room temperature.

Comparison of Methods

The results obtained in this study were compared with those by a high-performance liquid chromatography (HPLC) method described previously. The methods were statistically analyzed using the analysis of variance (ANOVA), which indicates whether there is a significant difference between these methods at a 5% significance level.

Results

Method validation

Linearity- the results of microbial growth of DOX reference substance are shown in [Table-2]. The calibration curve for DOX [Fig-2] was constructed by plotting absorbance versus log of concentration (µg mL^{-1}) and showed good linearity between 4.0 and 9.0µg mL^{-1} range. The representative linear equation for DOX was y= -0.536Ln(x)+1.5978, where x is log dose and y is absorbance. The correlation coefficient (r= 0.9989) was highly significant for the method and there were no deviations from parallelism and linearity with the obtained results (P < 0.05) [Table-3].

Table 2- Absorbances of the solutions reference of DOX obtained for standard curve

Concentration (µg mL^{-1})	Absorbances*	Average of absorbance	RSD (%)
	0.865		
4	0.856	0.861	0.532
	0.862		
	0.64		
6	0.619	0.627	1.849
	0.621		
	0.416		
9	0.439	0.427	2.716
	0.425		

**Each value represents the mean of three determinations.*

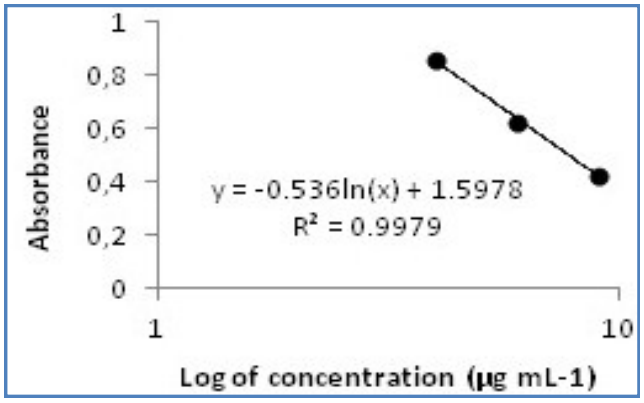

Fig. 2- Analytical curves of solutions of DOX SQR at concentrations of 4.0, 6.0 and 9.0µg/mL, obtained by turbidimetric method.

Precision- the method precision in terms of repeatability (intra-assay) was evaluated by the determination of sample in the same day, at the same concentration and under the same experimental conditions. The results obtained showed RSD values lower than 0.99% [Table-4]. The inter-analyst precision was confirmed by statistical analysis, the mean values obtained by different analysts in the analysis and the result is shown in [Table-5].

Table 3- Parameters of linearity for determination of DOX[a] and summary of ANOVA

Parameter	530 nm
Linearity range (mg)	4.0 - 9.0
Slope	-0.536
Intercept	1.5978
Correlation coefficient (r)	0.9989
Detection limit (µg mL^{-1})	0.23
Quantification limit (µg mL^{-1})	0.71
Regression	2408.23 (4.96)
Lack of fit	0.74 (4.96)

[a]Values are reported as mean±S.D. of three calibration curves generated on three consecutive days (n= 3)

Table 4- Intra-assay precision data for the bioassay of DOX in pharmaceutical formulation

Day	Content of DOX tablets* µg mL^{-1}	Content of DOX tablets* %	Average content ± s.e.m.	RSD (%)
1	6,06	101,01		
2	6,10	101,70	101,90 ± 1,09	0,99
3	6,18	103,00		

**Each value represents the mean of six determinations*

Table 5- Values calculated in the test-F and test-t of inter-analyst precision of turbidimetric method

Test-F: two samples for variance	Test-t: two samples assuming equal variances
Fcal 4,40 < Ftab 19	tcal 1,48 < ttab 2,78
Pvalue 0,18 > 0,05	Pvalue 0,21 > 0,05

Accuracy- [Table-6] shows the recovery values obtained for each concentration level tested by turbidimetric method.

Table 6- Values of the recovery test of turbidimetric method

	DOX SQR added (µg mL^{-1})	DOX SQR recovered (µg mL^{-1})	Recovery (%)	Average recovery (%)	RSD (%)
R1	0,8	0,79	98,60		
R2	2,0	1,95	97,44	97,73	0,78
R3	3,2	3,11	97,15		

Specificity- the same degradation conditions used to validate the stability-indicating HPLC method, previously validated in our laboratory, were employed in the microbiological assay.

Degradation under acidic conditions (0.1M HCl for 4 hrs. in bath a 70°C): Under HPLC conditions, the drug concentration decrease about 8.74%. Such instability was also detected during the microbiological assay, when the drug activity decreased about 26.01% in 0.1M HCl for 4 hrs.

Degradation under basic conditions (0.1M NaOH for 4 hrs. in bath a 70°C): Under HPLC conditions, the drug concentration decrease about 82.63% and by microbiological assay, the drug concentration decrease about 98.61%.

Under photolytic conditions (UV radiation): the drug concentration decreased about 22.95% by microbiological assay and the drug concentration decreased about 0.95% by HPLC method.

During the specificity analysis using the formulation excipient: there was no microbial growth in the tubes, and the absorbance values equivalent the values of the positive control, revealing the absence of interference from this substance.

Robustness- the average variation in time of incubation of the

microorganism to evaluate the robustness parameter were analyzed statistically and the results found in [Table-7].

Table 7- Tests F and t for variation in time of incubation of the microorganism in the turbidimetric method for the determination of DOX

Test	Incubation time (hours)
	20-24
Fcal	1.73
Ftab	19
Pvalue	0.36
tcal	1.63
ttab	2.78
Pvalue	0.18

Comparison of the Methods

The results obtained in this study were comparable with those obtained by HPLC [Table-8]. For the microbiological assay method, the mean potency found was 101.90% and for HPLC method, 110.07%. The methods developed and validated provided different results for DOX quantification in tablets. However, the power provided by the turbidimetric method is within the specification described in the Pharmacopoeia of 95-105% for DOX in tablets [12].

Table 8- Assay results of DOX by two different methods, HPLC and turbidimetry

Day	HPLC (%)[a]	Mean (%)	Turbidimetric (%)[a]	Mean (%)
1	110.39		101.01	
2	109.53	110.07	101.7	101.9
3	110.3		103	

[a]*Means of three replicates*

Discussion

Biological methods are advantageous because the measured parameter and the therapeutic properties of the drug are the same. Therefore, microbiological or biological tests are the best methods to eliminate any doubts about the potential loss of activity [20].The development and validation of analytical methods for the potency determination has received considerable attention recently, mainly from regulatory agencies, because of their importance in pharmaceutical analysis [20-23]. For this reason, a microbiological assay was proposed as a suitable method for the determination of DOX in tablets. The potency of an antibiotic may be demonstrated under suitable conditions by comparing the growth inhibition of sensitive microorganisms produced by known concentrations of the antibiotic to be studied and a reference standard [13,15,24].

The microbiological assay by turbidimetric method allows the determination of drug potency, by measuring the turbidity (absorbance), caused by inhibition of growth of micro-organism by antimicrobial.

In developing the turbidimetric method for DOX was tested concentrations of the chemical reference ranging from 4.0 to 24.0µg mL^{-1}. Finally, the concentrations were chosen 4.0, 6.0 and 9.0µg mL^{-1}, with a ratio of 1.5, since it showed the best response against the micro-organism and maintained correlation between dose and response of analyte, as recommended by the Brazilian Pharmacopoeia [15]. The use of micro-organism *Escherichia coli* ATCC 10536 IAL 2393 is based on their sensitivity to the front of doxycycline hyclate and ease of maintenance and growth of this microorganism.

Some other parameters were tested earlier to stablish the conditions described as different culture media, inoculum concentration and diluent solutions. At the end of the preliminary tests were chosen to work with the microorganism *Escherichia coli* ATCC 10536 IAL 2393, 10% inoculum and ultrapure water as diluent solution at the concentrations mentioned above.

Antibiotic assays must be designed in such a way that they allow the mathematical model's validity to be examined based on a potency equation. According to the European, Brazilian and The United States Pharmacopoeias [13,15,24], if a parallel-line model is chosen, the two log dose-response lines of the preparations to be examined as well as the reference preparation must be parallel and they must be linear over the range of doses used in the calculation. These conditions must be verified by validity tests for a probability, generally P= 0.05. The method was validated by means of the analysis of variance, there was no deviation from parallelism and linearity with the obtained results ($P < 0.05$).

The linearity of the method was proven by the correlation coefficient and analysis of variance of the analytical curve. Statistical analysis demonstrated that there is no deviation from linearity of the analytical curve originating from the chemical reference. The correlation coefficient is 0.9989, a value close to unity, which is excellent in the case of a biological assay.

Through all the stress conditions used to validate both methods, it is possible to conclude that the drug is unstable under photolytic, acid and alkaline conditions.

The intraday precision was confirmed with the value obtained was 0.99% and inter-analyst precision is confirmed by not statistically significant for a significance level of 5% between the mean values obtained during testing, thus validating the precision turbidimetric method for the analysis of DOX.

The lower values for the calculated limit of detection and quantitation of DOX (DL= 0.23µg mL^{-1} and QL= 0.71µg mL^{-1}) indicate a good sensitivity of the method for determination of drug.

The accuracy was confirmed by the recovery test and determined the average of 97.73%.

The robustness of the method was confirmed by varying the incubation time of the micro-organism. The results show no statistically significant difference. Thus, the method can be considered robust.

The potency of DOX tablets showed an average content of 101.9% in the turbidimetric analysis. This value shows the real microbial activity of the antibiotic and approve the lot analyzed. However, the potency provided by HPLC analysis was 110.07%, this value does not show the actual activity and disapprove the lot (95-105%) [12].

The turbidimetric method is not time consuming (4 hrs.), it showed be sensitive and specific for the analysis of DOX tablets.

Conclusion

The quantification of antibiotic components by chemical methods such as HPLC and UV spectrophotometry, although widely used, cannot provide a true indication of biological activity. Despite HPLC be the method of choice for the analysis of DOX tablets, microbiological testing is essential. The result of content 100% of an antibiotic in HPLC is not always the value of its biological activity, as

shown in this work. The microbiological test evaluates the potency of the drug and HPLC not always detects changes in the structure of the molecule, so the importance of microbiological test be held in conjunction with a physical-chemical method.

The turbidimetric method validated in this work for quantification of DOX showed that the procedure might be successfully implemented into routine quality control testing as a good alternative methodology for pharmaceutical analysis of DOX in tablets.

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