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Validation of a chromatographic method for amoxicillin determination in wastewaters after its degradation by advanced oxidation process

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ABSTRACT

A rapid analytical procedure based on high performance liquid chromatography for identification and quantification of amoxicillin in wastewaters after its degradation by advanced oxidation process (AOP) was developed and validated. The proposed methodology was validated concerning to linearity, precision, limit of quantification, and limit of detection according to the required INMETRO and ANVISA (regulatory agencies in Brazil) standards. The method was applied to the determination of amoxicillin in wastewaters from wastewater treatment plant of a Brazilian pharmaceutical industry spiked with amoxicillin after AOP treatment process on a pilot scale reactor. The method allows the determination of the referred drug in a concentration range of 10–100 mg L^{-1} with a detection limit of 2.32 mg L^{-1}, a quantification limit of 7.04 mg L^{-1}, and enabling control of contaminant removal.

Keywords: Amoxicillin; Development and validation; HPLC; Wastewater; AOP

1. Introduction

Amoxicillin is a β-lactam antibiotic usually used for the treatment of gram-negative bacterial infections. Like other antibiotics from the same group, this drug inhibits cell wall biosynthesis causing their osmotic break [1]. Among other drugs, antibiotics are those with higher consumption worldwide [2], and they have been widely used in human and veterinary medicine not only to treat or to prevent diseases in humans and animals, but also as animal growth promotion. Both their daily excretion as well as the parent compounds into the environment (soils, wastewaters, groundwaters, surface waters) has contributed to significant environmental disturbances such as the development of bacterial resistances. According to Jones [3], antibiotics are extremely toxic to microorganisms (EC50 below 0.1 μg mL^{-1}), leading to the development of acquired resistance mechanisms. However, reports on the amoxicillin effects differ greatly. Recent studies conducted on *Synechocystis* sp. [4] showed that exposure of microorganisms to amoxicillin at the concentration of 150 μg mL^{-1} turned the color of these live organisms to yellow after 12 h

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and inhibited the production of O_2 by about 80% after 24 h. The EC50 value for inhibitory growth of the algae *Microcystis aeruginosa* was of 0.0037 μg mL^{-1}, while for *Selenastrum capricornutun* was of 0.1 μg mL^{-1}. Tests performed on zebrafish (*Danio rerio*) showed an EC50 value of 132.4 μg mL^{-1} premature hatching of embryos, inhibited catalase and induced glutathione-S-transferase in adults [5,6]. Their presence is considered dangerous in both low and high concentrations [2]. It also persists in the environment for long periods of time since biological process can induce limited degree of transformation and cause bio persistence of its transformation products [7]. Waste treatment systems, in particular for wastewater uses, resorts in most cases physical removal or biological processes whose efficiency is low. Treatment with UASB reactor, Parshall channel, sandbox, and neutralization tank are not able to remove all the organic persistent pollutants such as drugs [8–12]. Biological treatments such as pond aeration, disinfection with chlorine, and decantation are also common, but with low efficiency [13]. These aspects have attracted the attention of health authorities and researchers. Not only the analytical aspects, but also the removal of these contaminants from environment have been the subject of intensive research [14,15].

Regarding the removal of pharmaceuticals from aqueous systems, advanced oxidation process (AOP) has proved quite effective [16–18] and was adopted in several countries is an alternative way to promote the degradation of recalcitrant compounds [19]. This technique consists of generating high reactive hydroxyl radicals (which are oxidants and are able to react with different organic compounds) leading to complete degradation of the target pollutant. Besides presenting a high efficiency (yields values greater than 80%), the final products generated such as CO_2 and H_2O are nontoxic and are environmentally biodegradable [11,20]. To provide control of its efficiency, expeditious analysis must be performed to assure accuracy of results. Several analytical techniques have been proposed for the determination of amoxicillin and its derivatives in waters based on GC/MS [21], LC/MS [22,23], and HPLC with a mixture of organic eluents [24]. In some cases [21–23], the equipment used is very sophisticated and expensive and not suitable for routine analysis. In turn, HPLC procedure [24] is based on the mixture of high volumes of organic solvents with buffers being themselves concerning contaminants. To overcome this problem, an HPLC method using only phosphate buffer as eluent was developed and validated according to INMETRO and ANVISA standards [25,26]. The proposed methodology was evaluated in the control of amoxicillin degradation efficiency after the AOP process.

2. Materials and methods

2.1. Reagents and solutions

Analytical grade chemicals without further purification and double deionized water were used to prepare all solutions. The following chemicals were acquired from F. Maia: potassium dihydrogen phosphate, potassium hydroxide (KOH), and hydrogen peroxide. Amoxicillin trihydrate was purchased from Sigma–Aldrich. The chromatographic grade acetonitrile was from Merck.

A volume of 500 mL of phosphate buffer solution was prepared by adding KH_2PO_4 solution (0.2 mol L^{-1}) to a 1 mol L^{-1} KOH until the pH was settled to 5.0 ± 0.1. The final volume was fulfilled to 1.0 L by addition of deionized water.

A stock solution 1,000 μg mL^{-1} of amoxicillin was prepared by rigorous weighing of 100.0 mg of raw powder followed by complete dissolution with phosphate buffer solution (pH = 5) in a 100 mL volumetric flask. Whenever necessary, more diluted solutions with concentrations 10–100 μg mL^{-1} were prepared by simple dilution in the same buffer.

2.2. Apparatus

As chromatographic system, an HPLC Shimadzu (Tokyo, Japan), model Prominence, equipped with a UV detector of the same brand, model SPD-20A, set to the wavelength of 285 nm was used. A C18 reversed phase column ULTRA (5 μm, 4.6 × 250 mm) was used for chromatographic separations. The separations were performed in isocratic conditions using a solution of deionized water acidified with 10% (v/v) of acetic acid and acetonitrile at the volumetric ratio of 65:35 as mobile phase. The temperature of the oven was maintained at 313.15 K, the flow rate kept constant at 0.700 mL min^{-1}, and at the pressure of 53 kgf.

The water remediation process was implemented in a benchtop homemade photochemical reactor comprising an annular-shaped quartz reactor placed around an UV radiation. The sample flows through the inner compartment of the reactor (inner volume of about 0.7 L) while thermostated water at 298.15 K flows through the external compartment. A peristaltic pump, a stainless steel recirculation tank, a pH meter coupled to a combined pH electrode for the automatic control of this parameter, and a thermostatic bath completed the photochemical reactor. A medium

pressure HPLN 80 W Phillips UV radiation lamp was used in the experiments to promote the degradation process.

2.3. HPLC validation parameters

Standard solutions of amoxicillin with different concentrations were daily prepared by using phosphate buffer as solvent. After HPLC separation, amoxicillin was detected at a wavelength equal to 285 nm. Following the above experimental conditions, chromatographic peaks were obtained with retention times between 2.95 and 3.02 min (Fig. 1). In that condition, an analytical curve ranging between 10 and 100 mg L^{-1} was obtained and the validation parameters were studied.

The average of peak areas from the seven curves were calculated, as well as the corresponding standard deviation for five degrees of freedom, and the scattering of the results, were obtained by using Grubb's test (Eq. (1)):

$$G_{<} = \frac{\bar{X} - X_{i<}}{s} \text{ and } G_{>} = \frac{X_{i>} - \bar{X}}{s} \quad (1)$$

where $G<$ and $G>$ refer, respectively, the calculated Grubbs values for the lowest and highest measured value $\bar{X}$ is the average values for the peak areas obtained after replicate injection of the same sample; $X_{i<}$ and $X_{i>}$ the lowest and highest values of peak area and s the estimated standard deviation for $n = 7$.

Linear regression was used to calculate the determination coefficient (R^2) for the curve. The precision of the method was obtained by calculating the variation coefficient CV, also called relative standard deviation. In this case, the calculation was based on Eq. (2).

$$\mathrm{CV}(\%) = \frac{s}{\bar{X}} x100 \quad (2)$$

The limit of quantification (LQ) was determined by the relationship between the estimated standard deviation of the response and the slope of the calibration curve (Eq. (3)).

$$\mathrm{LQ} = 10x\frac{s}{S} \quad (3)$$

where s is the estimated standard deviation and S is the slope of the calibration curve.

Likewise, the limit of detection (LD) was determined by the method based on parameters of the calibration curve, using the Eq. (4).

$$\mathrm{LD} = 3,3x\frac{s}{S} \quad (4)$$

2.4. Degradation procedures

The degradation studies by AOP process were performed on amoxicillin spiked synthetic samples collected from a Brazilian pharmaceutical industry treatment plant effluent. The degradation studies were conducted with the above-described homemade reactor having an inner volume of 0.7 L and further coupled to an external tank to allow treatment of 2 L of the effluent which circulated at a flow rate of 1.95 L min^{-1}. Volumes of 250 μL of hydrogen peroxide were added at regular time intervals of 0.30 and 60 min, totalizing a substance volume corresponding to 750 μL. The total reaction time of two hours was set for the complete AOP process.

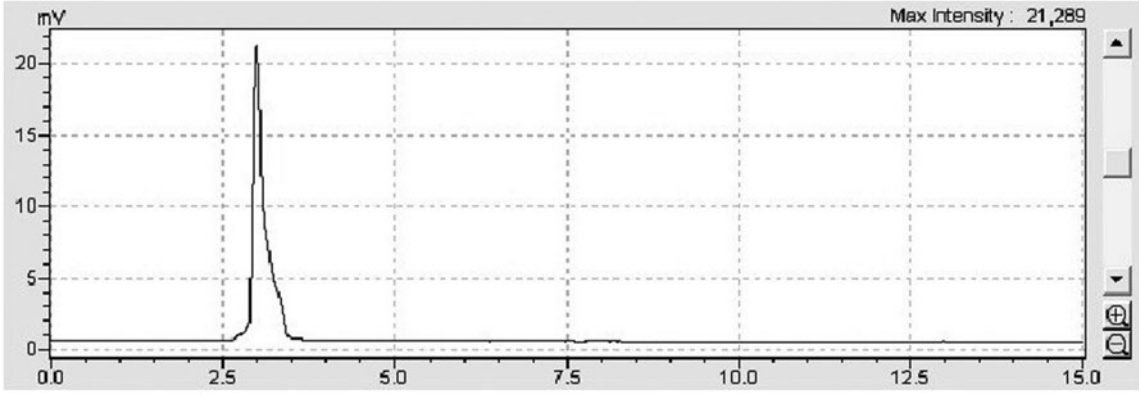

Fig. 1. Chromatogram of amoxicillin standard solution with concentration of 100 mg L^{-1}.

In order to ensure a reactor flow rate meeting the standards specified by CONAMA 430, as well as the time of exposure of the effluent to UV radiation, preliminary optimization studies were performed. Previous studies carried out with lamps of different powers (80, 125, and 250 W) showed to yield similar final degradation percentages [27]; so the lowest power lamp was selected for further work considering the operating costs.

3. Results and discussion

3.1. Validation of the HPLC method

Two procedures were used to validate the technique following the guidelines provided by the Brazilian National Agency for Sanitary Surveillance-ANVISA and the National Institute of Metrology, Standardization and Industrial Quality-INMETRO. According to ANVISA, the validation "must ensure, through experimental studies, that the method meets the requirements of analytical applications, ensuring reliable results". To ensure the quality of the study, some validation parameters including Linearity, Precision, LQ, and LD were determined [28].

For method validation, injection of amoxicillin solutions with concentrations ranging from 10 up to 100 μg mL^{-1} were performed. The obtained peak areas are presented in Table 1.

The average of the results corresponding to the peak areas, the standard deviation and dispersion analysis of the results, using the Grubb´s test, are shown in Table 2.

The values obtained are in accordance with the requirements of regulatory agencies. Based on the results presented in Table 2, it was possible to set the calibration curve and to determine its analytical linear range.

One of the validation parameters required is the test of linearity, indicating in which range the peak area is proportional to the concentration. Some authors claim that linearity can be tested in two ways: a priori ("not taking into account any non-linear model") and a posteriori ("relates a given non-linear" model). Not often, the correlation of the measured signal (or area) and the concentration occurs a priori, because the mathematical relationship is given empirically, using an equation of a straight line, known as the calibration curve [28]. Linear regression by the least squares method is widely used to estimate the regression coefficients (R^2) of an analytic curve. Fig. 2 represents the calibration curve obtained for amoxicillin standards in the concentration range between 10 and 100 μg mL^{-1}. The regression line obtained by the least squares method enabled the equation: peak area = 2,633(±94)x | $amoxicillin_{\mu g/mL}$ | + 8,000(±5,560), with a $R^2 = 0.9990$. ANVISA recommends a determination coefficient better than 0.99, while INMETRO considers $R^2 > 0.90$ as an adequate curve value. The value obtained in this work matches with the specification of ANVISA and the INMETRO.

The second parameter analyzed was the reproducibility based on the quantification of coefficient of variance (CV).

Eq. (1) was used to perform the corresponding calculations and the values obtained are also shown in Table 2. In order to consider a result as accurate, supplied CV values must be lower than 20% [29] and as described above were obtained for all the concentrations examined. Based on these assumptions, a LD of 2.32 mg L^{-1} and a LQ of 7.04 mg L^{-1} were established.

3.2. Analysis of real samples prior to degradation process by AOP

After validation of the chromatographic methodology, synthetic samples were analyzed before and after being subjected to the degradation process through AOP. Fig. 3 shows the chromatogram corresponding to the analysis of synthetic samples before the degradation process. By a chromatographic analysis, a high content of amoxicillin was obtained (3.22 mg.L^{-1}). In addition to amoxicillin peak, another one with a

Table 1
The peak areas values obtained for the seven calibration curves using seven different concentrations

Amoxicillin (mg L^{-1})	Peak area						
10	34,716	33,228	30,451	30,254	30,032	29,961	31,068
20	59,360	64,094	60,440	72,252	64,260	75,248	59,991
40	105,184	119,067	105,717	107,936	119,074	116,514	112,408
50	136,888	145,779	136,937	130,194	142,264	139,360	143,709
60	172,816	168,826	173,122	148,102	166,854	174,211	167,606
80	225,083	222,985	225,008	194,296	216,518	222,683	205,515
100	271,367	277,389	271,178	255,225	275,637	284,568	273,970

Table 2
Calculated values for the means, standard deviations, Grubbs test, and variation coefficient CV%*

Amoxicillin (mg L^{-1})	Average area	Standard deviation	Grubb's test		CV%
			$G_<$	$G_>$	
10	31,387.14	1,853.04	0.770	1.796	5.90
20	65,902.14	6,279.80	0.913	1.617	9.65
40	112,271.43	6,089.07	1.164	1.117	5.42
50	139,304.43	5,236.51	1.740	1.236	3.76
60	167,362.42	8,974.42	0.057	0.763	5.36
80	216,012.57	11,820.63	1.837	0.767	5.47
100	272,762.00	8,967.69	1.956	1.317	3.29

Note: *Test the tabulated value for 95% confidence level is 2.020 ($t^{0.05}6$).

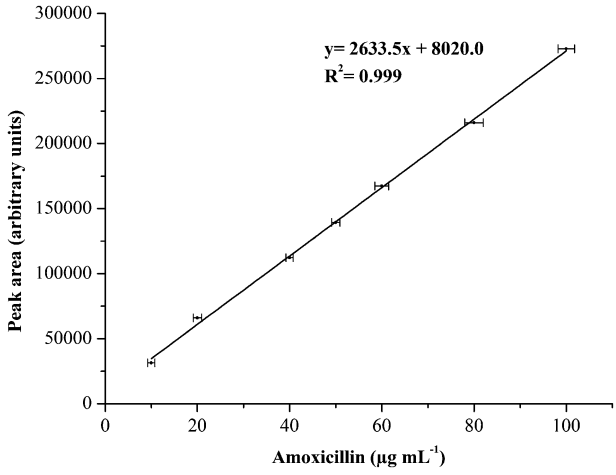

Fig. 2. Calibration curve for amoxicillin standards ranging from 10 to 100 µg m L^{-1}.

retention time of about 9.25 min has been revealed when compared with chromatogram of standard solution (Fig. 3). According to [30], this peak will be related with its hydrolyzed product. The transformation product occurs in synthetic samples once they were prepared from the original aqueous effluent with a pH different from the standards.

3.3. Assessment of the degradation of amoxicillin

The efficiency of AOP process of synthetic samples after degradation was evaluated by chromatographic analysis. Fig. 4 shows the corresponding chromatogram. As can be seen, a substantial decrease for amoxicillin peak was registered while the hydrolysis product remains constant.

Based on the validation process described, the initial quantification was carried out by using the analytical curve equation and concentration of 3.22 mg L^{-1} (amount of drug before degradation process). After 2 h of treatment, the presence of amoxicillin is detected, although in a concentration below the LD (2.32 mg L^{-1}) leading to the conclusion that the AOP procedure has an efficiency superior to 90%. However, this efficiency is only related to amoxicillin

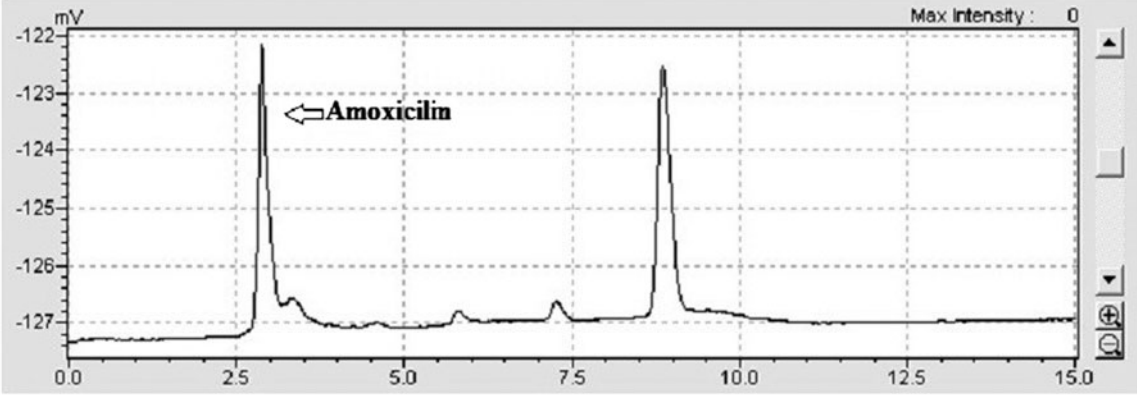

Fig. 3. Chromatogram of a synthetic sample before the degradation process.

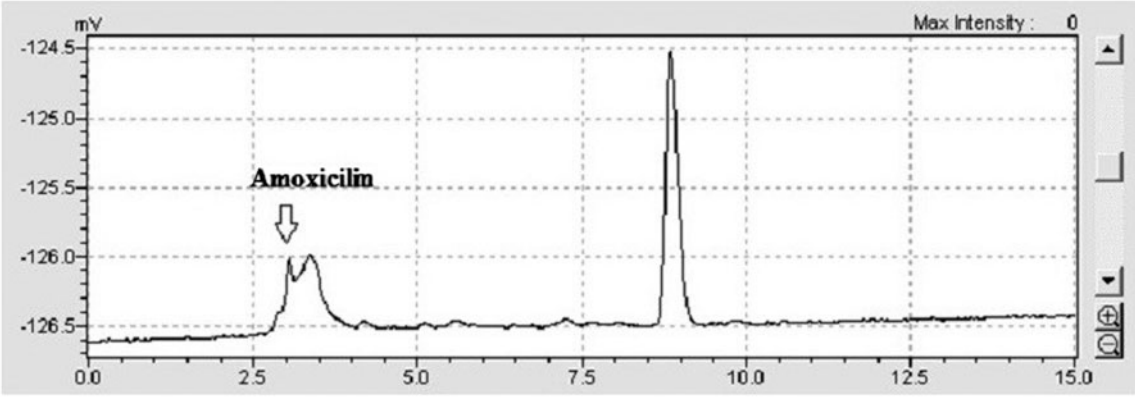

Fig. 4. Chromatogram of a synthetic sample after the degradation process.

and not to the transformation products. With the validated analytical method, it is possible to ensure on one hand the efficiency of contaminant removal process, but also evidence that its efficiency is not complete when this type of drug is released into the aquatic environment by the pharmaceutical industry.

4. Conclusions

Validation of an analytical method based on HPLC with UV/Vis detection, according to the standards regulated by regulatory agencies in Brazil (INMETRO and ANVISA) allowed more accurate analysis of water samples subjected to conventional treatment processes in water treatment plants. Also, the same method applied to the control of the remediation process by AOP leads to the conclusion that, although the process is effective for the parent drug, it does not have the same efficiency to the substrates hydrolyzed. Compared to other more sophisticated techniques, the proposed method is more economical and robust enough to control the AOP process, nowadays considered as highly efficient.

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