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ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 42 (2006) 192–199
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Stability evaluation of amoxicillin in a solid premix veterinary formulation by monitoring the degradation products through a new HPLC analytical method

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Received 4 January 2006; accepted 5 April 2006
Available online 9 June 2006

Abstract

A methodology (by VICH guidelines) for the stability evaluation of amoxicillin in granular premixes is described. This method is based on the monitoring of the degradation products formed during the stability study by a new HPLC-RP method, which has been developed and validated for the simultaneous determination of amoxicillin and its degradation products. The method uses a Nucleosil 120 C18 column and gradient elution. The mobile phase consisted of a mixture of methanol and buffer solution pH 3 ± 0.05 at different proportion according to a time-schedule programme, pumped at a flow rate of 1.750 ml min^{-1}. The DAD detector was set at 230 nm. The validation study was carried out fulfilling the VICH guidelines in order to prove that the new analytical method, meets the reliability characteristics, and these characteristics showed the capacity of analytical method to keep, throughout the time, the fundamental criteria for validation: selectivity, linearity, precision, accuracy, sensitivity (LOD, LOQ) and robustness. The method was applied during the stability study of an amoxicillin premix in order to quantify the drug (amoxicillin) and all its degradation products to evaluate the shelf life of the new veterinary dosage form. The method also proved to be suitable as a rapid and reliable quality control method.

Keywords: Amoxicillin; Degradation products; Premix; Liquid chromatography; Pharmaceutical dosage forms; Veterinary drug; Validation; Stability studies

1. Introduction

Antibiotics have for decades continued to play an important role in veterinary medicine as feed additives because of the broad spectrum and their economic advantages [1]. Amoxicillin is a β-lactam antibiotic that belongs to the group of penicillins. It is extremely active against both gram-positive and gram-negative microorganisms, including several pathogenic enteric microorganisms. Amoxicillin is widely used in veterinary practice for the treatment of gastrointestinal and systemic infection diseases. It is added to medical feeds at a level of 250–400 mg kg^{-1}, because of the resistance to gastric juice [2].

Amoxicillin is an antibiotic which degrades easily [3–11], and as a consequence, any manipulation of this active may lead to the formation of different degradation products.

Amoxicillin determination by means of microbiological assays, as well as with most of the antibiotics, is widely known. In the course of time, this determination has evolved to a more selective and sensitive techniques such as: thin layer chromatography [12–18], capillary electrophoresis [1] and HPLC, which is at the present time the most employed technique. In the revised bibliography there are methods described for the determination of the active as raw material [19–23], amoxicillin determination in different biological fluids [24–30], permeation studies [31] as well as different methods for amoxicillin determination in several dosage forms [32–38]. Moreover, in the literature there are methods described for the determination of impurities, related substances or degradation products in the raw material [6,7,18,19,33], finding few articles that quantify degradation products in the finished product [39,40].

As commented previously, amoxicillin is widely used in veterinary and that is why there are several methods described by HPLC (alone or together with mass spectroscopy) for

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doi:10.1016/j.jpba.2006.04.024

amoxicillin determination in different veterinary dosage forms [1,2,41].

Premixes are mixtures of one or more active ingredients usually in a suitable base of excipients that are prepared to facilitate feeding the active ingredients to animals. They are used exclusively in the preparation of medicated feeding stuffs by simple admixture with other ingredients [42,43]. Premixes occur in granulated or powdered form [42,43] or in pelleted form [44]. For the elaboration of these premixes, different kinds of vehicles are used as a base to incorporate the active [45]. The method implies that any active will suffer a strong manipulation throughout the whole manufacturing process of the premix, as well as afterwards, during its incorporation to the animal feed, as described in the consulted literature [46] where different premix formulations are studied with stabilized amoxicillin to find out the most stable formulation throughout the time.

A new premix of amoxicillin has been developed with a base of wheat husk to which different types of fats and oils are incorporated to achieve an stabilization of amoxicillin, due to its susceptibility to degrade as commented previously, by means of its coating with the previously mentioned fats and oils; moreover, a technologically acceptable granule must be obtained, as well as a good amoxicillin homogeneity. Even though, these requirements imply that during the manufacturing and later storage, degradation products of amoxicillin may appear.

The objective of this article is to study the stability of amoxicillin in premixes, taking into account the manufacturing method as well as the later storage (according to the corresponding VICH) [47–53], by means of the evaluation of the degradation products that may appear. For that, a new HPLC method is developed previously for the quantitative determination of amoxicillin and its degradation products. This new method is validated to prove that it has acceptable characteristics of suitability, reliability and feasibility [54–58].

The proposed method is also applicable as well for routine analysis: quantification of amoxicillin and quantification of degradation products of amoxicillin premix and complies with the validation requirements in the pharmaceutical and veterinary industry.

2. Experimental

2.1. Equipment

A high-performance liquid chromatographic system consisted of a Hewlett Packard 1100 featuring a column oven (79856A), a quaternary pump (G1311A), an automatic injector (G1313A) and a DAD detector (G1315A) which was set at 230 nm. Data acquisition was performed using a chromatography software package (Chemstation version A.07).

2.2. Materials and reagents

Methanol was of HPLC grade and was purchased from Panreac-Quimica S.A., Barcelona, Spain. Potassium phosphate monobasic and potassium phosphate dibasic were purchased from Roig Farma, Barcelona, Spain. The water used was deionized and purified by means of a purelab Plus system by Vivendi. Reactives used for the degradation study of the active (HCl 0.1N, NaOH 0.1N, H_2O_2 (33%, v/v), potassium permanganate) were analysis grade and supplied by Roig Farma, S.A. and Panreac-Quimica S.A.

Amoxicillin (A3OH-408) was purchased from Polichem S.A. and amoxicillin reference standard was purchased from European Pharmacopoeia (lot 2f). 4-Hydroxy-D-phenylglycine (4HDPG), penilloics acid (PA), penicilloics acid (PCA), amoxicillin diketopiperazine (ADP), degradation products of amoxicillin were provided also by European Pharmacopoeia. 6-Aminopenicillamic acid (impurity) was also purchased from European Pharmacopoeia to calculate the unknown degradation products amoxicillin was used. Amoxicillin premix was provided by laboratories Polichem S.A. This pharmaceutical preparation contains 10% of amoxicillin. The rest of excipients of the premix are: light liquid paraffin, glyceril monostearate and wheat husk. A placebo for the validation study is prepared with these excipients.

2.3. Chromatographic conditions

Chromatographic separation of the active and degradation products was performed using a Nucleosil 120 C18 column 250 mm × 46 mm i.d., 10 μm, made of stainless steel. The mobile phase consisted of methanol and buffer solution pH 3 ± 0.05 that were carried as a gradient program (Table 1). Both, methanol and buffer solution were degassed by filtering through a 0.45 μm GH-membrane filter. The flow rate was 1.75 ml min^{-1}. The DAD detector was operated at 230 nm. The injection volume was at 50 μl. The HPLC analysis was conducted at 40 °C. Each determination required 30 min.

2.4. Stock and working standard solutions

Working standard solution of amoxicillin was prepared at a concentration of 500 μg ml^{-1} dissolving the appropriated amount of the compound in buffer solution pH 6 ± 0.05. This standard solution is used to quantify the active on the final product. Based on this solution and by means of an adequate dilution, a 10 μg ml^{-1} was prepared to quantify unknown degradation products. These solutions could be stored at 25 °C (room temperature) for 3 h.

Due to the fact that there were not enough reference standards for the degradation products to carry on the whole stability study, by means of the validation a correction factor was established for

Table 1
Gradient program to carry out the chromatographic method

Minutes	Buffer solution (%)	Methanol (%)
0	98	2
15	98	2
20	70	30
25	70	30
28	98	2
30	98	2

Table 2
Correction factors to calculate degradation products in the finished product

Degradation product	Correction factor
4-Hydroxy-D-phenylglycine	0.413
Penilloics acid	2.095
Penicilloics acid	1.413
Amoxicillin diketopiperazine	0.765

each of these products. In this way, the degradation product can be calculated in relation to the amoxicillin reference standard of 10 μg ml^{-1}. The resulting factors for each degradation product in the study are listed in Table 2.

Out of the solutions obtained, a proportion was taken and filtered through a PVDF membrane filter (0.45 μm). The resulting filtered solution was placed in a HPLC vial. Each of the solutions prepared were injected by duplicate into the chromatograph, recording later the results obtained.

2.5. Assay of pharmaceutical preparations

555.54 mg of amoxicillin premix were weighed and placed in a 100 ml Erlenmeyer flask with 50 ml of petroleum ether. The solution was magnetically stirred for 20 min. Immediately after, 40 ml of buffer solution pH 6 ± 0.05 were added and the solution was magnetically stirred for 20 min more. The inside content of the erlenmeyer flask was transferred to a decantation funnel and the inorganic phase was collected in a volumetric flask. This last step was repeated twice more, adding to the decantation funnel 20 ml of buffer solution pH 6 ± 0.05: finally, level up the volumetric flask to 100 ml with more buffer solution. This solution could be stored at 25 °C (room temperature) for 3 h.

Out of the solution obtained, a proportion was taken and filtered through a PVDF membrane filter (0.45 μm). The resulting filtered solution was placed in a HPLC vial. Each of the solutions prepared were injected by duplicate into the chromatograph, recording later the results obtained.

2.6. Validation study

2.6.1. Specificity

In order to measure the specificity of the method, identification of the active was studied, comparing the raw material with a reference standard. Another study carried out was to check the absence of interference by the excipients which take part in the pharmaceutical preparation (placebo solution), as well as the absence of interference of the impurities or degradation products from amoxicillin.

In order to assure the selectivity and provide preliminary data of the stability-indicating properties of the proposed method, forced degradation studies were performed under various stress conditions. Thus, appropriate amounts of premix and placebo were stressed with HCl 0.1N and NaOH 0.1N (keeping the solutions to both, environmental temperature and 105 °C). Premix was also subjected to the effect of temperature (105 °C), UV light, IR light, high relative humidity (79% HR) for 24 h and exposed to daylight for a period of 15 days. Moreover, samples of premix and placebo were exposed to an oxidation treatment with H_2O_2 and $KMnO_4$ for 24 h. After the degradation treatments were completed, samples were analyzed according to assay sample preparation.

2.6.2. Linearity, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ)

A modified Eurachem method was employed [58,59] being the R.S.D. for the precision of LOQ 3.7% and accuracy (90–107%) in accordance with the AOAC [60].

To carry out all these studies, a total of three calibration curves were prepared for the known degradation products and seven calibration curves for the unknown degradation products. From these curves the above-mentioned parameters were calculated, starting with the calculation of the LOD and LOQ. The procedure was the following: a series of 13 different concentrations were prepared, from which the lineal points were detected (response factor ±10% (relationship between the area obtained and the studied concentration)). The next step was the preparation of three calibration curves for the known degradation products or seven for the unknown degradation products, from which linearity parameters, precision for the LOQ, 100% and 160% of the working concentration can be calculated, studying the relative standard deviation (R.S.D.) obtained for the response factor and accuracy of the same mentioned levels as for the precision, where the amount of degradation products recovered in relation to the added amount was calculated. This study was carried out on basis of the method described above.

Moreover, within the precision, the intermediate precision was also checked, where the variability between analysts and days were studied.

2.6.3. Robustness

In order to evaluate the robustness of the proposed method, the influence of small deliberate variations of the method parameters in the determination of degradation products (percentage of degradation products) was examined thoroughly. To carry out this study, only unknown degradation products (amoxicillin) were used because of the lack of degradation products. The factors selected to examine were the wavelength (nm), temperature (°C), flow rate (ml min^{-1}) and volume of injection (μl). Each factor was tested at three levels (−1, 0 and 1). One factor at a time was changed to estimate the effect. For each assay seven samples were studied together with a working standard solution.

2.7. Stability study for the amoxicillin premix

This study was fulfilled according to VICH guidelines for veterinary use [47–53], where three pilot batches of the premix were stored at different temperatures and relative humidity conditions during a period of time of 36 months. Within this period, at each established sampling time (see Table 3) samples were taken and evaluated with the previously developed method.

Table 3
Matrix for the stability study according to the VICH guidelines

Time (months)	Temperature/relative humidity		
	25 °C/60% HR	30 °C/60% HR	40 °C/75% HR
0	X	X	X
3	X	X	X
6	X	X	X
9	X	X	
12	X	X	
18	X		
24	X		
36	X		

3. Results and discussion

3.1. System suitability

Different liquid chromatography methods were investigated for their suitability to analyze amoxicillin and its degradation products, including the stability study of the solution in different buffer solutions, being the pH 6.0 buffer the one in where amoxicillin was more stable, as was mentioned in the literatures [61,62]. Finally, the best chromatographic separation, as explain above, was carried out with a C18 column (Nucleosil 120, 250 mm × 4.6 mm i.d., 10 μm particle size). The gradient used to elute all the compounds of the pharmaceutical preparation allows quantifying these compounds in the same chromatogram and only in 30 min. In Fig. 1, a representative chromatogram with all possible compounds (active, impurities and degradation products) is shown, where it was possible to observe a good separation between the different compounds.

It was concluded that the developed method is the optimum according to the studied parameters and complies with the accepted values for USP or ICH guidelines. Therefore, this method can be applied to routine, stability studies with no problems, its suitability being proved.

3.2. Validation study

3.2.1. Specificity

It was checked that amoxicillin raw material as well as amoxicillin reference standard elute at the same retention time, indicating thus a positive identification of the drug. In Fig. 1 it is observed that all the compounds of the injected sample elute at different retention times and do not interfere between them. The study of the purity of the peaks shows that the three spectrums obtained at different times are within the established threshold for these peaks. The placebo chromatogram shows there were not interference between the excipients of the pharmaceutical preparation, the active and degradation products.

The degradation studies carried out as stated in Section 2.6.1 was the last study to demonstrate the specificity of the method.

During the stress-testing study in acid media, at 25 °C (Fig. 2a) as well as 105 °C (Fig. 2b) various degradation products appeared, which eluted at 1.75 min, 20.05 min, 6.17 and 6.50 min and 22.61 and 23.61 min (105 °C), and they corresponded to 4HDP, PA, PCA, ADP, respectively. Unknown degradation products of amoxicillin were also formed which eluted at 3.5 and 5.0 min (25 °C). In basic media at 105 °C (Fig. 2c) the degradation product PA was observed [61].

With the high temperature treatment (105 °C) (Fig. 2d), degradation products 4HDP, ADP appeared and one unknown degradation product which eluted at 3.7 min. In the literature the degradation of amoxicillin by temperature is described [63]. When the premix was submitted to an oxidizing treatment the presence of several unknown degradation products, already seen in the other conditions were observed, and degradation products as PCA, were also formed (treatment with $KMnO_4$), in the treatment with H_2O_2 PA was formed.

When treating the sample with IR light and UV light PCA and PA were formed. 4HDP also appeared in the treatment with UV light.

To conclude, it can be stated that none of the peaks that could be generated by the stress treatment interfere with the peak corresponding to the active, therefore showing it was a selective method and suitable for routine work.

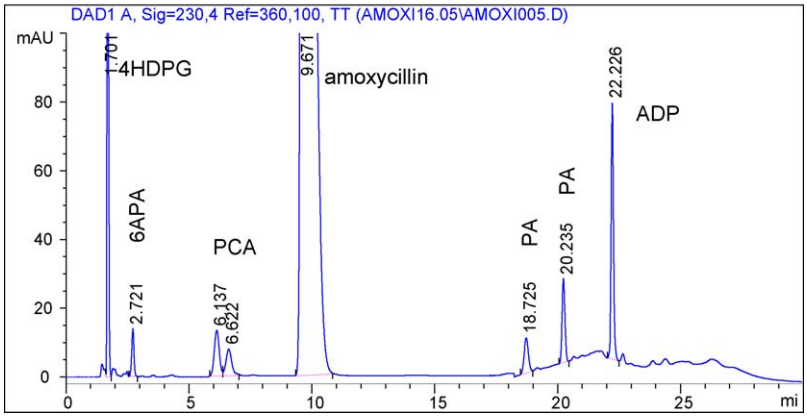

Fig. 1. Representative chromatogram obtained from a system suitability study, where impurities and degradation products appear together with amoxicillin. Amoxicillin elute at 9.67 min, 4HDPG elute at 1.70 min, 6APA (impurity), elute at 2.72 min, PCA elute at 6.14 and 6.62 min, PA elute at 18.73 and 20.24 min, ADP elute at 22.22 min.

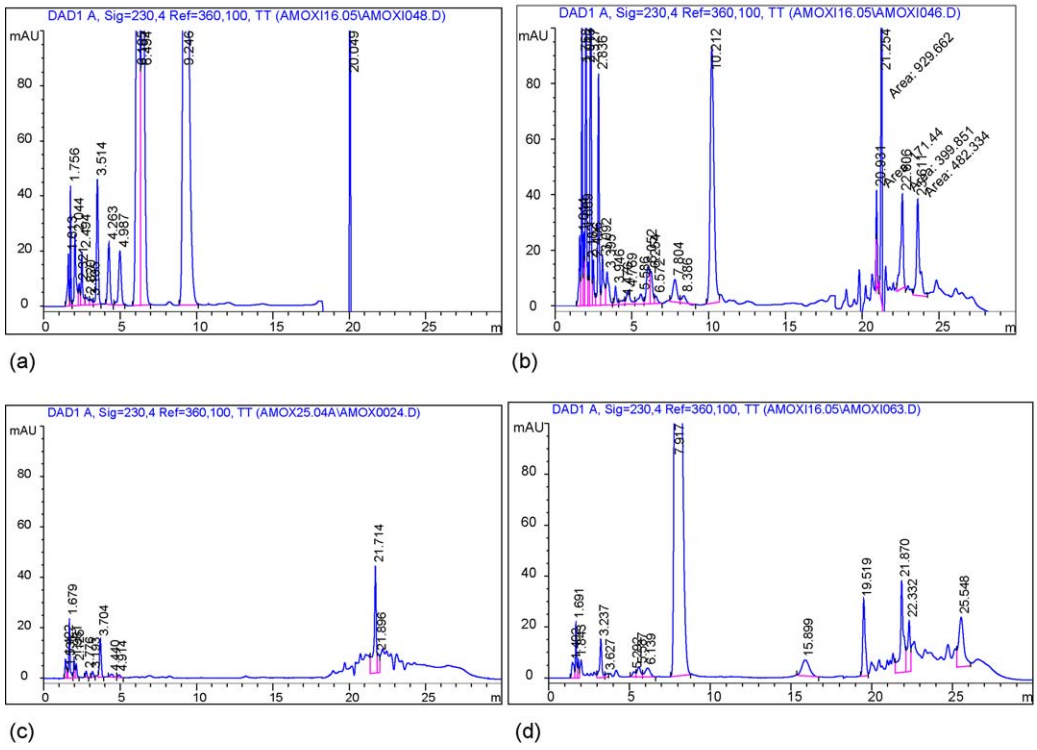

Fig. 2. Representative chromatograms obtained from the stress-testing study.

Table 4
Calibration equations for the determination of degradation products of amoxicillin by HPLC

Compound	Equations	r	r^2	$S_{x,y}$	S.D. intercept	S.D. slope
4HDPG	$Y = 86.2359X + 22.7238$	0.9999	0.9999	3.7446	2.2418	0.2458
PA	$Y = 8.8684X + 3.0449$	0.9985	0.9971	2.4399	1.9882	0.1951
PCA	$Y = 26.8596X + 8.1409$	0.9986	0.9972	6.7012	6.8177	0.6276
ADP	$Y = 24.8695X - 6.0592$	0.9997	0.9995	2.5114	2.5554	0.2286
UDP	$Y = 35.2127X + 3.0261$	0.9983	0.9966	8.6500	11.9243	1.0341

3.2.2. Linearity, precision, accuracy, LOD and LOQ

The method above mentioned (Section 2.6.2) was used to determine the linearity, precision, accuracy, LOD and LOQ.

The LOD for 4HDP was 1.39 μg ml^{-1}, for PA was 2.50 μg ml^{-1}, for PCA was 3.04 μg ml^{-1}, for ADP was 3.00 μg ml^{-1} and for unknown degradation products (UDP) was 4.00 μg ml^{-1}. The LOQ was established at 1.99 μg ml^{-1} for 4HDP, 3.00 μg ml^{-1} for PA, 4.06 μg ml^{-1} for PCA, 4.00 μg ml^{-1} for ADP and 6.00 μg ml^{-1} for unknown degradation products.

Linear relationship was obtained between the peak area of degradation products and the corresponding concentration, as shown by the equations presented in Table 4. The correlation coefficient, determination coefficient, standard error ($S_{x,y}$) of calibration line are also given, along with the standard deviations of the slope and intercept.

For the study of the precision of the method ($n = 9$ for known degradation products and $n = 21$ for unknown degradation products) the value of R.S.D. obtained for 4-hydroxy-D-phenylglycine was 3.23%, for penilloics acids was 2.43%, for penicilloics acids was 3.43%, for amoxicillin diketopiperazine was 2.96% and for unknown degradation products was 2.70%. These values were far below the value established (3.7%) at the beginning of the study (AOAC: Association of Official Analytical Chemists) [60].

For the intermediate precision, a study carried out by the same analyst working on different days ($n = 7$ number of analyses per day). The results were given both individually and as a whole observing that the inter-day R.S.D. corresponded to 1.87% for

Table 5
Accuracy for the determination of degradation products of amoxicillin by HPLC

Compound	Recovery (%)		
	Mean ± S.D.	R.S.D. (%)[a]	E_r[b]
4HDPG	101.78 ± 3.29	3.23	1.589
PA	100.11 ± 3.19	3.19	0.024
PCA	98.60 ± 3.38	3.43	−1.522
ADP	97.64 ± 2.89	2.96	−2.496
UDP	95.00 ± 3.36	3.54	−6.527

[a] Percentage relative standard deviation.
[b] Relative percentage error.

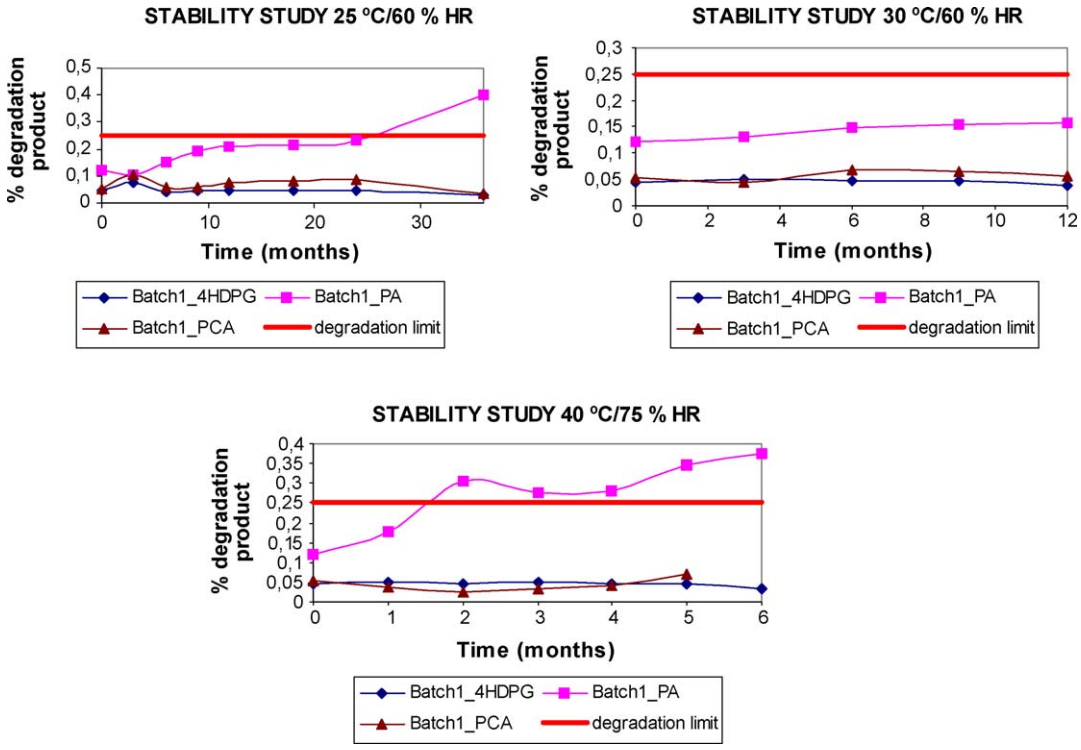

Fig. 3. Graphic representation of the stability study for batch #1 with the three different ICH conditions: 25 °C/60% HR, 30 °C/60% HR and 40 °C/75% HR.

4HDP, 2.82% for PA, 2.83% for PCA, 3.25% for ADP and 3.08% for unknown degradation products. The same study was carried out for different analysts ($n = 7$ number of samples per analyst) obtaining a R.S.D. of 3.37% for 4HDP, 2.11% for PA, 2.99% for PCA, 2.42% for ADP and 2.82% for unknown degradation products. Both results together with the individual results are below the established limit according to the AOAC (3.7%) [60], thus showing that the proposed analytical technique has a good intermediate precision.

The results obtained for the accuracy study (recovery method) from 9 samples studied ($n = 3$ for LOQ, $n = 3$ for 100% and $n = 3$ for 160%) for known degradation products and 21 samples studied ($n = 7$ for LOQ, $n = 7$ for 100% and $n = 7$ for 160%) for unknown degradation products are presented in Table 5 for all the studied compounds. The standard deviation (S.D.), relative standard deviation (R.S.D.) and relative percentage error are also given. The recovery obtained, individually and the mean for every compound studied are between the 90–107% established according to the AOAC [60].

Therefore, it can be concluded that the recovery study of the degradation products in the matrix for the developed method was correct, and therefore, the proposed analytical method was sufficiently accurate.

3.2.3. Robustness

Results obtained in this study are presented in Table 6. In this table the mean obtained ($n = 7$) for each level and factor studied is indicated, showing that the selected factors remained unaffected by small variations of these parameters because the recovery obtained, individually and the mean were between 90 and 107% for all degradation products studied, according to the AOAC [60]. Therefore, it can be concluded that the method is consistent in front of the wavelength, the temperature, the flow rate and the injection volume.

3.3. Stability study of amoxicillin premix

The proposed method is used to evaluate the stability of amoxicillin in the final dosage form (premix) during a period of time, according to what is indicated in Section 2.3 and Table 3, by monitoring the degradation products formed during the tem-

Table 6
Robustness evaluation of the developed HPLC method

Chromatographic change		Recovery % (mean ± S.D.)
Factor	Level	Unknown degradation products
(A) Wavelength (nm)		
227	−1	95.09 ± 2.53
230	0	97.21 ± 3.52
233	1	96.65 ± 2.71
(B) Temperature (°C)		
37	−1	95.71 ± 2.99
40	0	97.21 ± 3.52
43	1	101.53 ± 2.64
(C) Flow rate (ml min^{-1})		
1.650	−1	96.19 ± 1.47
1.750	0	97.21 ± 3.52
1.850	1	99.54 ± 4.97
(D) Injection volume (μl)		
45	−1	98.92 ± 2.09
50	0	97.21 ± 3.52
55	1	93.56 ± 2.61

perature and relative humidity conditions of the study. There are different methods described in the literature for the final evaluation of the stability of amoxicillin in several dosage forms [46,64,65], however, this study, unlike the others, bases the evaluation of the experimental results in what is described in the corresponding VICH [47,53]. Results obtained for batch 1 are represented graphically (Fig. 3), as an example, the studied temperatures and all the generated degradation products, together with the established specification of 0.25% for the degradation products in relation with the percentage of amoxicillin in the final product (10%). As observed in these graphical representations, degradation product PA increases progressively in all time points, temperatures and studied batches, and consequently, this degradation product will be the stability indicator for the premix under study. For the estimation of the shelf life data obtained from three batches at 25 °C/60% RH is used. Once the homogeneity of the variance is checked and no statistically significant differences of the slopes and intercepts of three batches under study are observed, a combined linear regression of all the raw data is performed, being the one that fits best: %DP = 0.098749 + 0.007303time, with a correlation coefficient of 0.95976 for a confidence level of 99%. From this equation the maximum stability time for the established limit of 0.25% can be estimated, giving a result of 20.712 months. Therefore, to assure the quality of the premix the estimated shelf life is 21 months.

4. Conclusion

A new simple analytical method has been developed to be applied for the evaluation of the stability of amoxicillin to quantify amoxicillin and its degradation products in a solid premix dosage form. The proposed high-performance liquid chromatographic method has been evaluated over the linearity, precision, accuracy, specificity, LOD and LOQ and proved to be convenient and effective for the quality control, stability studies of amoxicillin in the pharmaceutical dosage form studied (solid premix).

It has been proved that it was selective, linear, precise and accurate over the concentration range tested (70–160% of the working concentration).

The stability study of premix, by monitoring the degradation products by the HPLC method proposed, indicates that the estimated shelf life during which it keeps its initial quality attributes is of 21 months.

Acknowledgements

This study is part of the project number 302158 from “The Bosch i Gimpera” Foundation of the University of Barcelona, supported by Devinphar SL and Laboratories Polichem S.A.

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