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Development and validation of a new HPLC analytical method for the determination of alprazolam in tablets

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Abstract

A new analytical method is developed together with its latter validation study, by means of a high resolution liquid chromatography (HPLC) of reverse phase to quantify alprazolam (8-chloro-1-methyl-6-phenyl-4H-[1,2,4] triazole [4,3,-α]-[1,4] benzodiazepine) in tablets. Determination is carried out by means of an ODS C18 column (200 mm × 4.6 mm i.d., 5 μm particle size); the mobile phase consisted of a mixture of 0.02 M buffer solution of phosphates (pH 6.0) and acetonitrile (45:55, v/v). It is pumped through the chromatographic system at a flow rate of 0.50 ml min^{-1}. The UV detector is operated at 254 nm. The validation study is carried out fulfilling the ICH guidelines in order to prove that the new analytical method, meets the reliability characteristics, and these characteristics show the capacity of an analytical method to keep, throughout the time, the fundamental criteria for validation: selectivity, linearity, precision, accuracy and sensitivity. The method is applied during the working day for the quality control of commercial alprazolam tablets in order to quantify the drug and its degradation products and to check the content uniformity test.

Keywords: Alprazolam; Liquid chromatography; Pharmaceutical dosage forms; Tablets; Validation; Quality control

1. Introduction

Alprazolam (8-chloro-1-methyl-6-phenyl-4H-[1,2,4] triazole [4,3,-α]-[1,4] benzodiazepine) is a benzodiazepine derived from 1.4-benzodiazepines of new generation [1,2]. It is a benzodiazepine mainly used to treat anxiety disorders. On a short time basis it is used to palliate symptoms of anxiety or anxiety associated to symptoms of depression. It should be borne in mind that neither alprazolam nor any other benzodiazepine is effective when it comes to treating anxiety and strain caused by daily stress. Besides this, alprazolam is also used to treat panic disturbances with or without agarophobia [1–5].

Most of the analytical methods carried out by high performance liquid chromatography (HPLC) to determine alprazolam found in the consulted literature, are aimed at quantifying alprazolan in biological fluids, for postmortem analysis [6–10], to determine the raw material [9] and its related substances [11,12]. Few

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of the methods described by HPLC that are found, are aimed at the study of alprazolam in final product [13–16].

The target of this study is to develop a new, simple and fast analytical method by HPLC to quantify alprazolam and its degradation products in a final product, together with its latter validation study. This validation study is defined as the process by which it is established, by laboratory studies, that the performance characteristics of the method meet requirements for the intended analytical application [17].

This work describes the validation parameters stated either by USP 26 [18] or by the ICH guidelines [19,20] to achieve an analytical method with acceptable characteristics of suitability, reliability and feasibility. Ensuring, in this way that the findings achieved, when this method is applied, are correct, and so the drug fulfils are the required specifications showing its quality is the right one.

2. Experimental

2.1. Equipment

The chromatographic system used to develop this technique is a Hewlett Packard 1100 featuring a column oven (79856 A), a quaternary pump (G1311 A), an automatic injector (G1313 A) and a DAD detector (G1315 A) which is set at 254 nm. Data acquisition is performed using a chromatography software package (Chemstation version A.07).

2.2. Materials and methods

The acetonitrile used is HPLC grade, supplied by JT Baker. Dihydrogen potassium phosphate and monohydrogen phosphate, all of analytical reagent grade, supplied by Roig Farma, Barcelona, Spain. *Ortho*-phosphoric acid 85%, for analysis is purchased from JT Baker. The water used is deionized and purified by means of a purelab Plus system by Vivendi.

The reactives used for the degradation study of the active (HCl 0.1N, NaOH 0.1N, H_2O_2 (33%, v/v), potassium permanganate) are analysis grade and supplied by Roig Farma, S.A. and Panreac S.A.

Alprazolam, batch 94E223 and its related substances, 7-chloro-1,3 dehydrophenyl-2H-1,4-benzodiazepine-2-thione, 7-chloro-1,3-dehydro-5-phenyl-2H-1, 4-benzodiazepine-2-one and 2-amino-5-chlorobenzophenone are supplied by the Orion Corporation Fermion Laboratories and also the standard reference of alprazolam (batch 94E174).

Tablets used correspond to the "Alprazolam Cinfa 1 mg" pharmaceutical preparation and its declared excipients are: lactose monohydrate, microcrystalline cellulose PH 101, corn-starch, sodium docusate, sodium benzoate, colloidal silicon dioxide, magnesium stearate, E-132 coloring agent and hydrated aluminium oxide. A placebo for the validation study is prepared with these excipients.

2.3. Chromatographic conditions

Chromatographic separation of the active, related substances (synthesis impurities) and its degraded products was performed using an Hypersil ODS column 5 μm 200 mm × 4.6 mm i.d., made of stainless steel. The mobile phase consisted of 55% of acetonitrile and 45% of a buffer solution pH 6.0 (0.02 M). To prepare the buffer solution, 4.8 g of dihydrogen potassium phosphate and 1.2 g of monohydrogen potassium phosphate were weighed and dissolved in about 900 ml of HPLC grade water. Once dissolved, the pH was adjusted to 6.0 ± 0.05 with *ortho*-phosphoric acid, reactive analysis. Finally, it was diluted up to 1000 ml with additional HPLC grade water. Both, the acetonitrile and the buffer solution were filtered through a 0.45 μm GH-membrane filter.

The mobile phase was pumped through the column at a flow rate of 0.5 ml min^{-1}, at the same time the temperature of the column was held at 40 °C. The injection volume to carry out the chromatography was set at 50 μl. Each test required 25 min.

2.4. Stock and working solutions

Standard solution of alprazolam was prepared at a concentration of 200 μg ml^{-1} dissolving the appropriated amount of raw material in mobile phase. This standard solution will be used to quantify the active on the final product. Based on this solution and by means of an adequate dilution, a 0.5 μg ml^{-1} was prepared to quantify unknown degraded products.

To carry out the sample solution (assay of pharmaceutical preparation), 10 tablets were taken and

weighed individually, obtaining afterwards the average weight of these tablets, finally they were ground. An appropriate portion of this powder, equivalent to 2 mg of alprazolam was weighed and placed in a 10 ml volumetric flask, dissolving it with 5 ml of mobile phase (55% acetonitrile, 45% of buffer solution, pH 6.0 ± 0.05). This solution was sonicated for 10 min to dissolve and remove all the active from the tablet. Once the time had elapsed and the volumetric flask reached the environmental temperature (25 °C), it was diluted up to 10 ml with additional mobile phase.

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 an HPLC vial.

Each of the solutions prepared were injected in triplicate into the chromatograph, recording later the results obtained.

2.5. Validation study

2.5.1. Specificity

For the specificity study, identification of the active was studied, comparing the raw material (mobile phase + alprazolam) with a standard of reference (mobile phase + alprazolam CRS). 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 study which was carried out to determine the absence of interference on behalf of the prospective impurities purchased from the supplier of the raw material (mobile phase solution with alprazolam and the three known related substances, 7-chloro-1,3 dihydro-5-phenyl-2H-1,4 benzodiazepine-2-thione, 7-chloro-1,3 dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one and 2-amino-5-chlorobenzophenone).

Within the study of specificity, a series of degradation studies were carried out where the samples were subjected to different degrees of stress. The samples corresponded to placebo and final product and were subjected to stress conditions such as NaOH 0.1N, HCl 0.1N (keeping the solution to both, environmental temperature and 105 °C). The powder was also subjected to the effect of temperature (105 °C), UV light, IR light during 24 h and 15 days for the daylight. The samples were also subjected to an oxidation treatment with H_2O_2 and $KMnO_4$ for 24 h.

After the stress assays, the samples were analyzed as shown in the chromatographic conditions.

2.5.2. Linearity

To carry out this study, seven levels of concentration within the range 70–130% of the work-concentration (200 μg ml^{-1}) were prepared. Each of the levels of concentration were prepared in triplicate, individually weighing the amount of active and the corresponding amount of placebo (three independent calibration equations were obtained). The experimental results were represented graphically, obtaining a calibration graph and carrying out the corresponding statistic study (ANOVA).

2.5.3. Precision

For the precision study four different tests were carried out. The first one consisted of checking instrumental system precision, where a sample corresponding to a concentration of 200 μg ml^{-1} was injected 10 times, consecutively into the chromatograph, repeating the operation on a second day. The second test consisted of testing the standard solution precision where three solutions were prepared at 70%, three solutions at 130% and seven solutions at 100% of the work concentration, studying the relative standard deviation (S.D.) obtained for the response factor (relationship between the area obtained and the studied concentration). The third test consisted of checking the precision of the method, operating as described in the standard solution previously mentioned: seven individual samples were prepared and the relative standard deviation (R.S.D.) was studied for the response factor obtained. Lastly, the intermediate precision was studied. Preparing the samples according to the precision of the method and studying the variability which takes place when the same analyst works on different days or when the analysts change.

2.5.4. Accuracy (recovery method)

The recovery method was studied at concentration levels of 70% (three samples), 100% (seven samples) and 130% (three samples) where a known amount of the active was added to a determined amount of placebo and it was calculated the amount of alprazolam recovered in relation to the added amount. This study was carried out on basis of the method described above.

Table 1
Experimental design matrix for the robustness study where the eight experiments with their particular conditions are shown

Experiment number of runs	Wavelength (nm)	Temperature (°C)	Flow (ml/min)	Mobile phase (%) ACN	Injection volume (μl)
Experimental design					
1	257.0	43.0	0.4	60.0	45.0
2	251.0	43.0	0.6	50.0	45.0
3	251.0	37.0	0.6	60.0	45.0
4	257.0	37.0	0.6	50.0	55.0
5	251.0	37.0	0.4	60.0	55.0
6	257.0	43.0	0.6	60.0	55.0
7	251.0	43.0	0.4	50.0	55.0
8	257.0	37.0	0.4	50.0	45.0

2.5.5. Robustness

The study of robustness was carried out to evaluate the influence of small but deliberate variations in the chromatographic conditions for the determination of alprazolam in tablets (percentage alprazolam). The factors chosen for this study were the wavelength (nm), temperature (°C), flow (ml min^{-1}), mobile phase (percentage acetonitrile) and volume of injection (μl).

To carry out this study, a fractional factorial design of eight experiments and five factors (2^{5-2}) was planned with the aid of the Statgraphics 3.1 Plus statistics program. The combination of the different factors to obtain the eight experimental runs are detailed in Table 1.

2.5.6. Detection limit and quantitation limit

To determine the detection and quantitation limits, the first point was to prepare a battery of different concentrations by means of dilutions starting from a parent solution, to establish a calibration curve, where the 100% of the work concentration corresponds to 0.5 μg ml^{-1}, a value which at the same time corresponds to the maximum percentage allowed for degraded products (0.25%). The range of prepared concentrations was from 0.05 to 0.65 μg ml^{-1}. Once the results of the first linearity were obtained, the response factor was calculated (relationship between the area and the concentration) for each of the points studied. Apart from this, a linear regression was also calculated to obtain the calibration curve ($Y = a+bX$). The value obtained for the slope (b) was used to calculate the ±5%, thus obtaining an interval of the slope. The response factors calculated previously, which did not fit within the calculated intervals for the value of the slope do not have a linear behavior. Once the concentrations that did not show a linear relationship with the response were rejected, seven linearities with the final concentrations were analyzed. From here on, the mean, S.D. and R.S.D. were calculated for both, concentrations and response factors. Afterwards, the concentrations in relation to the R.S.D. obtained of the response factors from each of the concentrations was represented graphically. The R.S.D. set as a limit for the study of degraded product in accordance with the AOAC [21] was 5.3%. The first point which does not fulfil this R.S.D. corresponds to the detection limit, and the first point which fits into this specified value corresponds to the quantitation limit.

3. Results and discussion

3.1. System suitability

The chromatographic separation, as explained above, was carried out with a C18 column (Hypersil ODS, 200 mm × 4.6 mm i.d., 5 μm particle size). To evaluate the chromatographic parameters (capacity factor-K', number of theoretical plates, asymmetry of the peaks, tailing factor and resolution between two consecutive peaks) the chromatogram obtained for the test of lack of interference for the impurities provided by the supplier of the raw material (7-chloro-1,3 dihydro-5-phenyl-2H-1,4 benzodiazepine-2-thione, 7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepine-2-one and 2-amino-5-chloro-benzophenone) was used.

In Fig. 1, a representative chromatogram is shown, which corresponds to the chromatographic separation of these substances. The capacity factor (K') of the first peak was 2.158 and 7.483 for the last peak. The

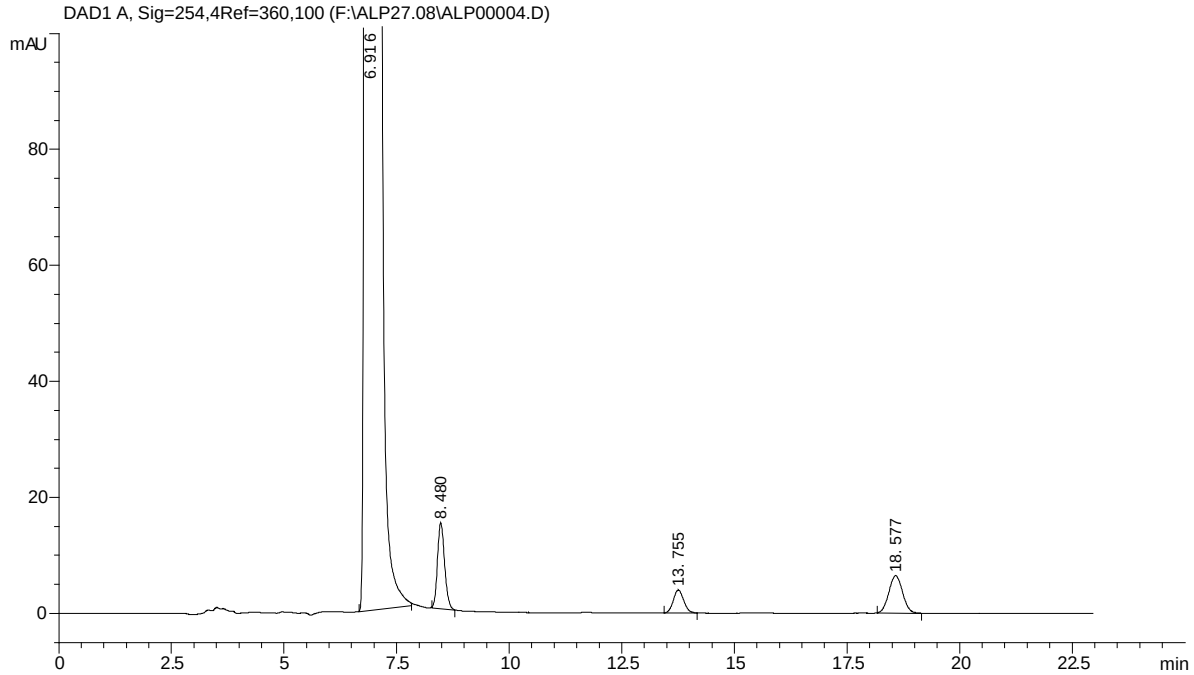

Fig. 1. Representative chromatogram obtained from a system suitability study, where the related substances appear together with alprazolam. Alprazolam elute at 6.9 min, 7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one elute at 8.5 min, 7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-thione elute at 13.8 min and 2-amino-5-chloro-benzophenone elute at 18.6 min.

number of theoretical plates (per meter of the column) in order of elution were 39,896, 71,707, 86,133 and 89,587. The results obtained for the asymmetry of the peak and the tailing factor parameter were the following: 0.667 and 0.977, respectively for alprazolam, 0.800 and 1.129 for the impurity 7-chloro-1,3-dihydro-5-phenyl-dH-1,4-benzodiazepin-2-one, 0.902 and 1.098 for impurity 7-clhoro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-thione and finally 0.930 and 1.034 for the impurity 2-amine-5-chloro-benzophenone. The resolution obtained between alprazolam and the impurity which appears at 8.5 min was 5.3.

It was concluded that the developed method is the optimum according to the studied parameters. The capacity factor obtained is within the accepted values, above 2 for the first peak and less than 10 for the last peak. The values of the number of theoretical plates were higher than the accepted value of 2000 (minimum value to consider, it is an acceptable method). The tailing factor, another parameter that ICH guidelines consider as a factor to be controlled, was within the limits established by these guidelines. Lastly, the resolution factor between two consecutive peaks, in the developed method, approximately represents twice the minimum request to consider there was a good separation between both peaks. Therefore, this method can be applied to routine with no problems, its suitability being proved.

3.2. Stability of the solution

Fig. 2 shows the results obtained in the study of the solution (both, reference and sample solution) where it can be noticed that solutions were stable for at least 48 h, as during this time the result does not decrease below the minimum percentage (95%).

3.3. Validation study

3.3.1. Specificity

Comparing both substances, raw material and standard reference (alprazolam CRS) in the same chromatogram, it was noticed that both eluted at the same

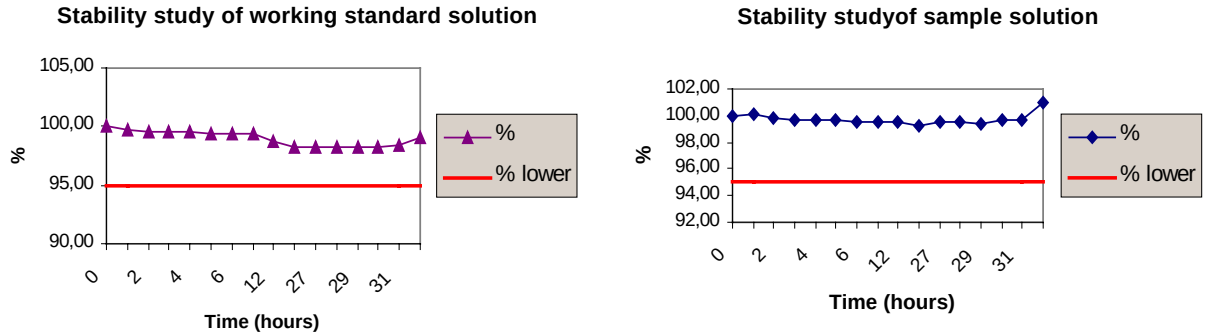

Fig. 2. Representative graphics of stability study, both, working standard solution and sample solution.

retention time and therefore, it was concluded that it was the same substance (Fig. 3). The study of the purity of the peak shows that the three spectrums obtained at different times are within the established threshold for this peak.

It was observed in both, the test to show the absence of excipients interference for pharmaceutical preparation (Fig. 4) and the test to show the absence of impurity interference provided by the supplier of the raw material, that none of the peaks appears at the same retention time than alprazolam peak, then, it was concluded that the developed method is selective in relation to the excipients of the final preparation and the synthesis impurities provided by the supplier of raw material.

Another study carried out to check the selectivity of the method was the degradation test carried out under different stress conditions, as stated in Section 2.5.1.

During the study of stress with HCl, the presence of an unknown degraded product was noticed, which eluted at 5.4 min approximately. When NaOH was used as a stress media, and the solution was kept at 105 °C of temperature, an unknown degraded peak appeared at 10.83 min.

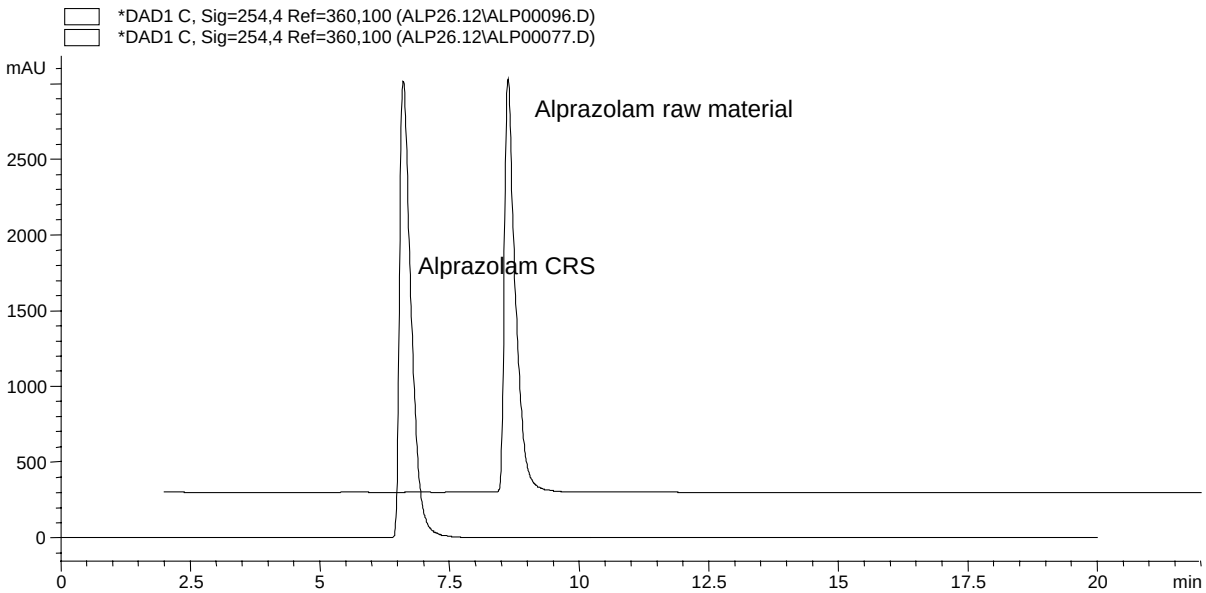

Fig. 3. Representative chromatogram where the peak obtained with raw material and the peak obtained with alprazolam CRS are compared.

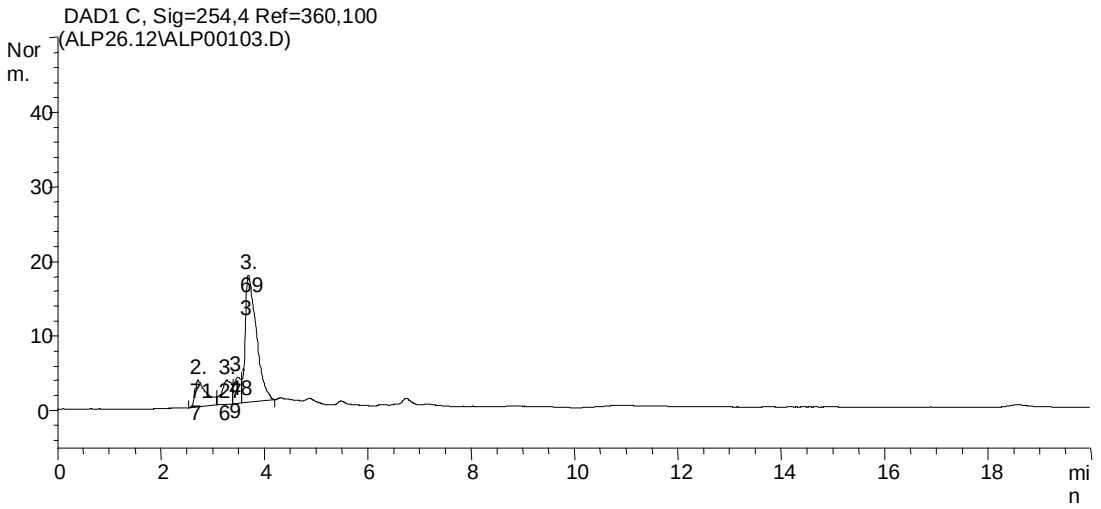

Fig. 4. Representative chromatogram of a placebo solution.

When the sample was subjected to 105 ° C of temperature, and after 24 h of study, no unknown peak appeared, whereas in the samples subjected to high RH (79%) a degraded peak appeared which eluted at 10.1 min. When the samples were subjected to an oxidation treatment, with H_2O_2 33%, peaks appeared which eluted at 5.78, 6.73 and 8.07 min corresponding to unknown degraded products. When $KMnO_4$ was used, no known or unknown degraded peaks appeared.

When treating the sample with IR light, no apparent change was noticed, whereas when the sample was subjected to UV light, an unknown peak appeared, which eluted at 5.4 min [22].

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.

3.3.2. Linearity

The equation of the regression curve obtained (with all the values) relating the tested concentrations and the response obtained correspond to $Y = 1041.5346 + 203{,}424X$ (Fig. 5), with a standard error ($S_{x,y}$) of 701,416 and a correlation coefficient of 0.997, which is higher than the value established at the beginning of the study, which corresponds to 0.995.

The *R*-squared showed there was a correlation of 99.3% between the tested concentration and the response obtained, within the 95% interval of confidence.

3.3.3. Precision

In the study of the instrumental system precision where, a R.S.D. of 0.557% was obtained for retention time, and of 0.215% for the area obtained corresponding to the first day, being 0.530 and 0.176% for the second day, respectively (with $n = 10$ number of analyses per day).

The inter-day study ($n = 20$ analyses) carried out showed a R.S.D. of 0.210% for the area obtained and 0.552% for retention time. In all these cases the R.S.D. obtained was far below 1%, the limit percentage set for the precision study of the instrumental system, thus showing that the equipment used for the study worked correctly for the developed analytical method, and being highly repetitive.

The precision study for standard solution ($n = 7$ analyses) showed a R.S.D. of 1.093% for the response factor. For the study of the precision of the method ($n = 7$) the value of R.S.D. corresponded to 0.946%. Both studies with values far below the value established (2.7%) at the beginning of the study.

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 2.045. The same

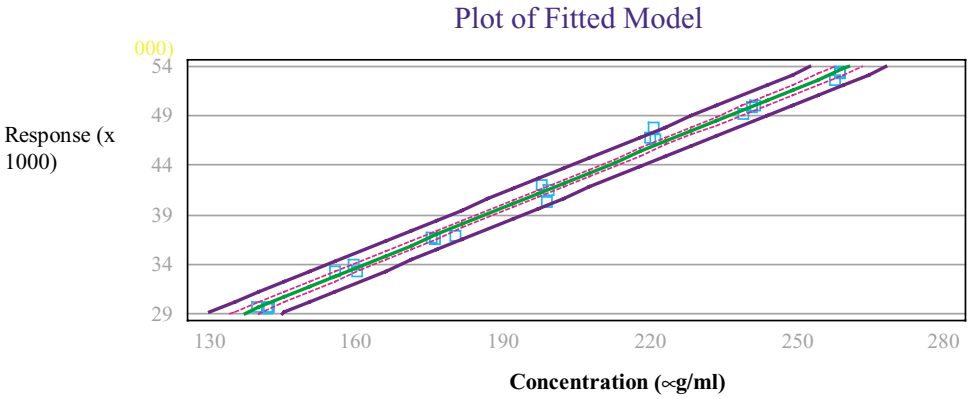

Fig. 5. Representative graphic where the linearity of the obtained results together with the confidence levels are shown.

study was carried out for different analysts ($n = 7$ number of samples per analyst) obtaining a R.S.D. of 2.322%. Both results together with the individual results are below the established limit according to the AOAC (2.7%), thus showing that the proposed analytical technique has a good intermediate precision.

3.3.4. Accuracy (recovery method)

The results obtained for the accuracy study (recovery method) from 13 samples studied ($n = 3$ for 70%, $n = 7$ for 100% and $n = 3$ for 130%) indicated that the mean of the recovery was 100.69%, S.D. was 2.41 and R.S.D. was 2.39%. It was also studied the experimental t of the recovery percentage, which value was 1.041 being it far below the 2.17 established in the tabulated t (95% level of probability, 12 d.f.).

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

3.3.5. Robustness

By means of the Statgraphics Plus 3.1 software, a 2^{5-2} factorial design was constructed as is reflected in Section 2.5.5 with the aim of calculating the effects of the main factors and its interactions for the response of alprazolam percentage. The ANOVA study shows the importance of the effects (statistic significance). Fig. 6 shows the influence of each of the variables studied in percentage alprazolam as a response, where none of them exceeds the limit set in the graph and therefore showing that the study of the main factors does not affect the result obtained for percentage alprazolam. Therefore, it can be concluded that the method is consistent in front of the wavelength, the temperature,

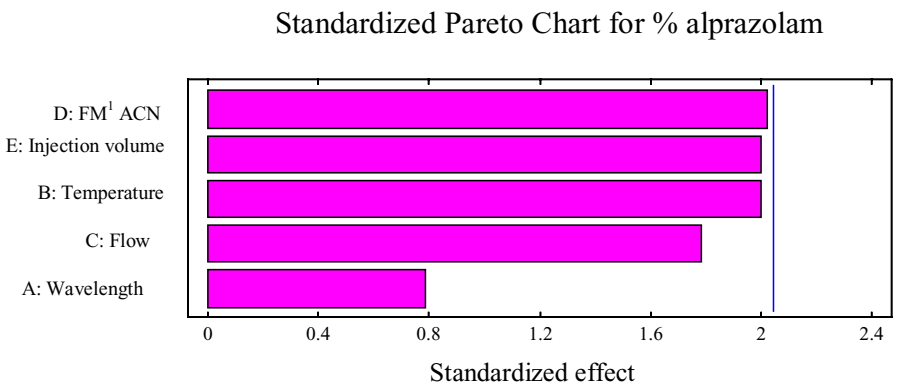

Fig. 6. Representative graphic of Pareto to show the influence of the different variables studied in the response of percentage of alprazolam.

the flow rate, the mobile phase and the injection volume.

3.3.6. Detection limit and quantitation limit

According to the study carried out with the seven linearities and graphically summing up the results of the relative standard deviation versus the concentration, the quantitation limit is set at 0.30 $\mu g\,ml^{-1}$ which corresponds to a 0.15% of the work concentration (200 $\mu g\,ml^{-1}$) showing that this point is linear, precise and exact, the detection limit is set at 0.20 $\mu g\,ml^{-1}$, a concentration equivalent to 0.1% of the work concentration and which could not demonstrate its linearity, precision and accuracy.

4. Conclusion

A simple and quick, new analytical method has been developed to be applied in routine to determine alprazolam and its degraded products in tablets. The method proposed by HPLC to determine alprazolam in tablets has been proved in a linear, precise, accurate and selective manner to be applied in routine and in quality control of alprazolam tablets.

It has been proved that it was selective, linear between 70 and 130% of the work concentration (200 $\mu g\,ml^{-1}$) for alprazolam and between the quantitation limit and the 130% for degraded products, with a correlation coefficient higher than 0.995, exact, precise, accurate and robust regarding the wavelength, flow rate, mobile phase, injection volume and temperature.

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