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Validation of a simple reversed Phase-HPLC method for determination of Blacofen in tablets

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Baclofen is a muscle relaxant used as a first option to treat spasticity and muscle spasms in patients with spinal cord injuries, which is available in Brazil as 10 mg tablets. The compendia methods employ HPLC by ion pairing that requires the use of specific reagents and column conditioning, increasing the waste generation and the cost of analysis. In this study, an isocratic, simple and stability-indicating HPLC method was validated to assay baclofen tablets. A C-18 column (Luna®, 150 x 4.6 mm, 5µm), mobile phase composed by triethylamine 10 mM pH 7.0, methanol and acetonitrile (80:15:5), flow rate 1 mL/min and detection at 220 nm was used. The baclofen retention time was 6.2 min and the method was linear in the range of 5 – 100 µg.mL^{-1} (r = 0.9999). Method selectivity was demonstrated by the forced degradation study and simultaneous analysis of baclofen impurity. The method showed accuracy (mean recovery 99.27%) and precision (RSD < 2%). The robustness was evaluated by factorial design, and the method was robust regarding the proposed variations. The developed method met the requirement of current guidelines, being indicative of stability and suitable for the determination of baclofen in tablets.

Keywords: Baclofen; baclofen tablets; validation; stability indicative

Introduction

Baclofen (Figure 1) is a muscle relaxant useful in the reduction of spasticity and muscle spasms resulting from multiple sclerosis, particularly for the relief of flexor spasms and concomitant pain, clonus, and muscular rigidity (1). Is a GABA agonist, derived from aminobutyric acid (GABA), which acts on the presynapse inhibiting calcium channels, hyperpolarizing the cell membrane and thus decreasing the release of excitatory neurotransmitters such as glutamate and aspartate, and also in postsynapse by activating potassium channels (2,3).

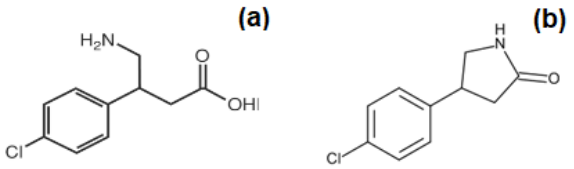

Figure 1. Chemical structure of baclofen (a) and baclofen impurity A, (4*RS*)-4-(4-chlorophenyl)pyrrolidin-2-one (b).

In Brazil, baclofen is available in 10 mg tablets, Lioresal® (Novartis) is the reference drug product and Baclofen® (Pfizer/Teuto) is the similar brand. In other countries, such as Germain, Canada, United States and Spain there is an injectable solution (0.5 mg/mL) for intrathecal injection.

The monographs of baclofen tablets on The United States Pharmacopoeia (USP) (4) and the British Pharmacopoeia (5) propose a HPLC method for the assay, by *in situ* ion pairing technique, using a C-18 column. This technique is also used to analyze carboxylic acids, sugars, analgesics, vitamins, inorganic anions and metallic cations (6). In the case of baclofen, sodium pentanesulfonate or sodium hexanesulfonate are added to the mobile phase and saturate the stationary phase, which is then able to retain the drug by electrostatic interaction. Elution occurs by a combination of the drug displacement from its pair by sodium ions and by migration of the ion pair itself in the mobile phase. Besides the high cost of the ion pairing reagents, this technique requires a column pre- and post-conditioning step, increasing the analysis time and the solvent waste (7).

Several methods for the determination of baclofen in different matrices by HPLC reverse phase are reported in the literature, but none of them is indicative of stability (8-16). Such studies are of great importance since no product is stable indefinitely, and they are performed to monitor the product throughout its shelf life, as well as the generation of toxic or inactive products (17-21).

Thus, in view of the limitations of the available HPLC methods for the baclofen tablets assay, this study aimed to develop a new HPLC analytical method by reverse phase technique. Stress testing was performed and a pharmacopeial baclofen impurity was used to evaluate the stability-indicating capacity of the method. The method was also applied to the dissolution test.

Experimental

Standards and chemicals

The baclofen reference substance (Pharmanostra, Anápolis, Brazil) was identified and assayed following the official monograph (5) after receiving (purity 98.93%). The baclofen impurity "A" was obtained from European Pharmacopoeia (Lot B0200050). Baclofen tablets (10 mg) were purchased in the local market (Lioresal®, lot ZC996, Novartis, Taboão da Serra, Brazil). To prepare the samples and the mobile phase, the following reagents were used: methanol and acetonitrile of HPLC grade (Merck, Darmstadt, Germany), hydrochloric acid (Dinâmica, São Paulo, Brazil), triethylamine (Tedia, Fairfield, USA) and

ultrapure water from a Milli-Q system (Millipore®, Bedford, MA, USA).

Chromatographic system and mobile phase

A Shimadzu LC-system (Kyoto, Japan) equipped with a CBM-20A system controller, LC-20AT pump, SPD-M20A PDA detector, DGU-20A5 degasser and SIL-20AHT autosampler was used. A C-18 column was used to perform the analysis (Phenomenex Luna® 150 x 4.6 mm; 5µm) and the mobile phase consisted of a mixture of triethylamine aqueous solution 10 mM adjusted to pH 7.0 with phosphoric acid 18% (v/v), methanol and acetonitrile in the proportion 80:15:5 (v/v/v). The mobile phase was filtered through a 0.45 µm membrane filter (Millipore, Bedford, USA) and run at 1.0 mL min^{-1}; the detection was obtained at 220 nm and the injection volume was 20 µL.

Reference substance and sample solutions preparation

A stock solution containing 500 µg mL^{-1} was prepared by weighing accurately 10 mg of baclofen reference substance and dissolving with 20 mL of hydrochloric acid 0.1 M. The final solution (50 µg mL^{-1}) was obtained by dilution with the mobile phase.

To assay baclofen tablets, at first the mean weight was determined from 20 units and then tablets were crushed. An amount equivalent to 10 mg of baclofen was weighed, transferred to a 20 mL-volumetric flask and hydrochloric acid 0.1 M was added. This mixture was stirred for 10 minutes and then the volume was made up, obtaining a 500 µg mL^{-1} solution. This solution was centrifuged at 4000 rpm for 10 minutes and then one milliliter of the supernatant was diluted in a 10 mL-volumetric flask with the mobile phase, giving a 50 µg mL^{-1} solution.

Selectivity

The method specificity was studied by forced degradation of sample solutions in alkaline, acidic and oxidative mediums, temperature and radiation exposure. In all degradation conditions, sample solutions at 500 µg mL^{-1} were used, prepared as described in the "*Reference substance and sample solutions preparation"* section.

For acidic and alkaline hydrolysis 2.5 mL of the sample solution were mixed with 2.5 mL of 5 M HCl or with 5 M NaOH and remained for 10 days (at room temperature) and for 8 hours (at 80ºC). At the end of the degradation treatment, the solutions were neutralized as necessary and then diluted with the mobile phase to the theoretical final concentration of 50 µg mL^{-1}.

For the oxidative degradation, 2 mL of the sample solution and 2 mL of 30% hydrogen peroxide were mixed and kept reacting for 8 hours and for 10 days. At the end, the solutions were diluted to 50 µg mL^{-1} with the mobile phase.

To study the effect of temperature, 2.0 mL of sample solution were kept under heating in an oven (50ºC for 8 hours and for 10 days) and after that, it was diluted to the final concentration.

To promote photolysis, 1 mL of sample solution was transferred to a transparent container and exposed to UV light (352 nm, Blacklight blue lamp, Orion, 30 W) for 8 h and for 10 days inside a chamber with mirrored internal surfaces. Samples submitted to the same procedure, but protected from light were also analyzed. Before the analysis, all the solutions were diluted to the theoretical concentration of 50 µg mL^{-1}.

In addition to the stress testing, specificity was also evaluated by adding baclofen impurity A (Figure 1), which is a known impurity from the baclofen synthesis. Aliquots of baclofen reference substance and impurity A solutions were dissolved in mobile phase to obtain a solution having known concentrations of 50 µg mL^{-1} and 250 µg mL^{-1} of baclofen and impurity A, respectively. The same procedure was performed using the baclofen sample solution. Chromatographic parameters such as the resolution between the peaks and the peak purity index were considered for the chromatograms analysis.

Linearity and limit of detection (LOD) and limit of quantitation (LOQ)

The linearity was evaluated by analysis of five solutions at 5, 10, 25, 50 and 100 µg mL^{-1}, in triplicate. The results obtained were plotted in a graph of analyte concentration (µg mL^{-1}) versus response (peak area). Both the regression equation and the correlation coefficient (r) were obtained by ordinary least squares method. The data obtained were further statistically analyzed to prove that they met the assumptions for a linear regression. Analysis of variance (ANOVA) was used to determine how well the model fit the data (α=0.05). Ryan-Joiner, Durbin-Watson and Cochran tests were performed to assess normality, independence, and homoscedasticity of residuals, respectively. The values for LOD and LOQ were calculated through the analytical curves following the USP equations: LOD= 3.3(σ/ α) and LOQ= 10(σ/ α), where σ is the standard deviation of the y-axis intercept and α corresponds to the mean slope, all obtained from three independent regression lines (22).

Accuracy

Accuracy was assessed by the recovery method, by adding known amounts of baclofen reference substance to samples at the beginning of the process in three levels. To prepare them, aliquots of 1 mL of sample solution (300 µg mL^{-1}) were placed in 10 mL volumetric flasks to which 0.1, 0.4 and 0.7 mL of reference sample (500 µg mL^{-1}) were added. Dilutions were performed in mobile phase to achieve final concentrations of 35, 50 and 65 µg mL^{-1}, respectively, named as R1, R2 and R3 which correspond to 70, 100 and 130% of the usual working concentration. This procedure was performed in triplicate. Solutions of sample (30 µg ml^{-1}) and reference substance (50 µg ml^{-1}) were also prepared and analyzed and the baclofen content was calculated using the equation below (22), where R% = recovery percentage, CR = concentration of baclofen in sample solution (µg mL^{-1}) spiked with baclofen reference substance; CA = concentration of baclofen (µg mL^{-1}) in unspiked sample solution; C_{SQR} = spiked baclofen concentration (µg mL^{-1}).

$$R\% = \left[\frac{(C_R - C_A)}{C_{SQR}} \right] X\,100$$

Precision

Precision was evaluated in the levels of repeatability and intermediate precision. The repeatability was determined by the assessment of six independent sample solutions in the same concentration and conditions (day and analyst). Intermediate precision was verified by the analysis of six other sample solutions prepared independently, with the same nominal concentration, but changing the analyst and day. The relative standard deviation (RSD) was calculated and values below 2% were considered acceptable (22).

Robustness

To assess the robustness of the proposed method, a full factorial design with two levels and three factors (2^3) with three central points was carried out, totaling 11 experiments. The variables were the aqueous phase pH (6.5, 7.0 and 7.5), methanol ratio (10, 15 and 20%) and mobile phase flow rate (0.8, 1.0 and 1.2 mL/min). A sample solution at 50 µg mL^{-1} was used to measure the effect of factors and the selected experiments were performed randomly as shown in Table 4. The experimental design and analysis were performed employing the statistical software Minitab 17® (Pennsylvania, USA).

Application of the developed method

The developed method was used in the dissolution test of baclofen tablets. The test followed the conditions described in USP (4), using as dissolution medium 500 mL of 0.01N hydrochloric acid, apparatus 2 at 50 rpm, performing collections after 30 minutes. The aliquots were filtered through a nylon filter (0.45 µm) and analyzed without additional dilution. The test was carried out in a Pharma Test PTW II (Hainburg, Germany) dissolution equipment. Not less than 75% (Q) of the labeled amount of baclofen should be dissolved in 30 minutes.

Results and discussion

Several preliminary tests were performed aiming to develop a method that would meet the recommended analytical parameters with a reasonable retention time. Baclofen has two ionizable groups, acidic (pKa 3.89) and basic (pKa 9.79) (23) and in the usual pH range of chromatographic assays both groups will be ionized, which could decrease the retention time. By using a C-18 column and a mobile phase with a high proportion of aqueous phase (triethylamine aqueous solution 10 mM pH 7.0, methanol and acetonitrile in the ratio of 80:15:5), suitable chromatographic parameters, appropriate retention time and good resolution between the peaks of baclofen and impurity A were achieved. In this condition the baclofen retention time was about 6 minutes (Figure 2) and the system suitability parameters such as theoretical plates (N = 6324), peak symmetry (A = 1.18), capacity factor (K '= 2.70) and resolution (R = 2.90) met the current recommendations.[4]

Selectivity

To evaluate selectivity, the ability of the method to analyze unequivocally the analyte among impurities that would be formed during the forced degradation studies and a known baclofen impurity, named impurity A, was evaluated.
For the forced degradation study, aliquots of the sample solution were exposed to various degradation conditions to obtain degradation products at the level of 15 to 20% (24,25), which is considered appropriate. Preliminary tests indicated the baclofen stability under all the stress conditions; therefore, sample solutions were maintained during 10 days under degradation, with strong reagents. Besides, temperature was associated in the alkaline and acidic hydrolyses, but even so, the degradation was lower than expected (Table 1). Under all other tested conditions – UVA radiation, 30% hydrogen peroxide oxidation and thermal degradation at 50°C, the drug level in the sample solution remained between 95 and 100% of the initial value. These results indicated the high baclofen stability, since even with the usage of severe conditions the degradation percentage was low. Besides, extra peaks were not observed under any forced degradation condition, in the range of 200–800 nm, even where there was minimal degradation. This may indicate that the degradation products had no chromophore groups or that baclofen decomposes to yield low molecular weight fractions.

Table 1. Baclofen residual content (%) after forced degradation study by HPLC method.

Stress condition	Baclofen residual content (%)	Peak purity
NaOH 5M, 10 days, RT	98.6	0.99998
NaOH 5M, 8 h, 80ºC	95.3	0.99998
HCl 5M, 10 days, RT	100.3	0.99998
HCl 5M, 8 h, 80ºC	96.2	0.99998
UVA radiation, 8 h	98.7	0.99999
UVA radiation, 10 days	95.5	0.99999
H_2O_2 30%, 8 h	100.5	0.99999
H_2O_2 30%, 10 days	98.2	0.99999
50°C, 8 h	99.9	0.99998
50°C, 10 days	99.9	0.99999

RT: room temperature

The purity of the peak under all conditions and the good resolution between the peaks of baclofen and impurity A (R > 2.0) suggested the suitability of the analytical conditions and therefore the method selectivity (Figure 2).

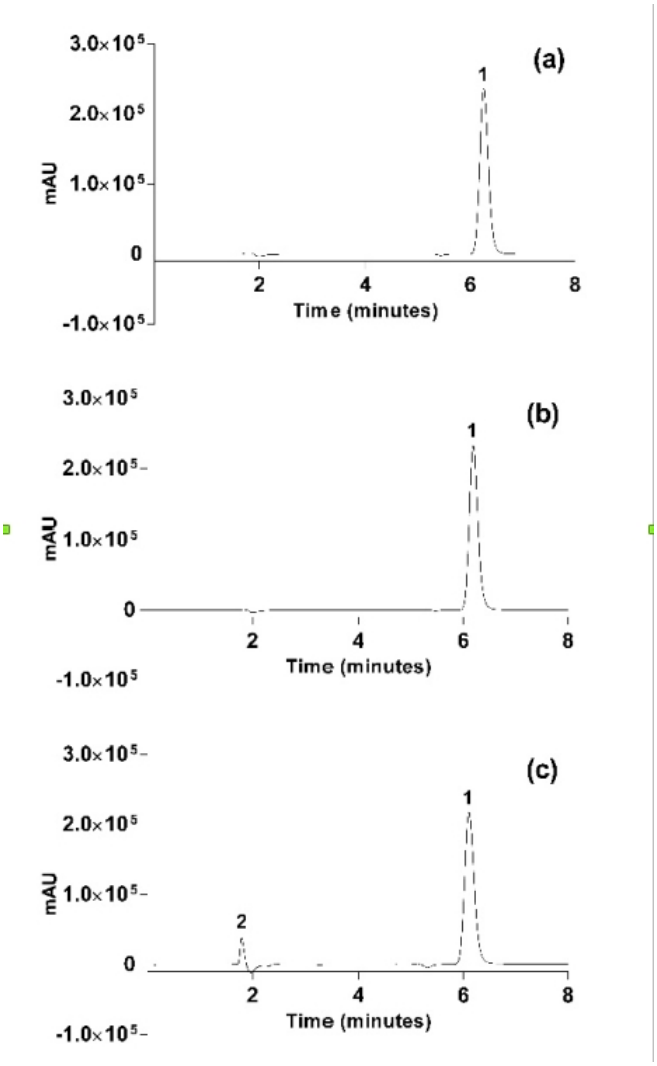

Figure 2. Chromatograms of baclofen SQR at 50 µg mL^{-1} (a), baclofen tablets at 50 µg/mL (b) and baclofen tablets 50 µg mL^{-1} plus impurity A at 250 µg mL^{-1} (c). (1) baclofen; (2) impurity A.

Linearity

Satisfactory linearity was observed over the concentration range of 5 to 100 µg mL^{-1}, with a correlation coefficient of 0.9999 and the linear regression equation y = 55052x + 9241, where x is the concentration and y is the absolute peak area (mAU). Normality of residuals distribution was proved by the Ryan-Joiner test (p>0.05). Independence of residuals was verified using the Durbin-Watson test. There was no correlation between the residuals and there was no effect of any treatment on the subsequent treatment (p>0.05). Homoscedasticity was proved by the Cochran test (p>0.05) indicating that the residual variance across all levels was constant. The statistical analysis by ANOVA showed that there is a linear regression (p<0.05) and no deviation from linearity (p> 0.05) at a statistical significance of 95%. The calculated results for LOD and LOQ were 0.34 µg mL^{-1} and 1.03 µg mL^{-1} respectively, suggesting good method sensitivity.

Accuracy

Accuracy was assessed by the recovery test at concentrations corresponding to 70, 100 and 130% of the usual concentration (50 µg mL^{-1}). The average recovery obtained was 99.27 ± 0.44%, and all the individual values were within 98-102% confirming the method accuracy (Table 2).

Table 2. SQR baclofen recovery percentage added to sample obtained by HPLC method.

Level (%)	Added concentration (µg.mL^{-1})	Recovery concentration (µg.mL^{-1})	Recovery (%)	Mean (%) ± SD[a]
70	5.0	4.98	99.54	99.78 ± 0.72
		4.96	99.21	
		5.03	100.59	
100	20.0	19.69	98.44	98.98 ± 0.96
		19.68	98.41	
		20.02	100.1	
130	35.0	34.73	99.23	99.05 ± 0.65
		34.42	98.33	
		34.86	99.60	
		Mean and RSD (%)[b]		99.27 ± 0.44

[a]n=3; [b] n=9.

Precision

The precision was evaluated at two levels, repeatability and intermediary precision (two days and two analysts). RSD values lower than the accepted value of 2% for all levels were obtained (Table 3), indicating the suitable inter and intra-day precision (26).

Table 3. Results of repeatability and intermediate precision to baclofen assay, by HPLC reverse phase method.

Sample	Repeatability (%)		Intermediate Precision mean (%) ± SD (RSD)%
	Day 1, Analyst 1	Day 2, Analyst 2	
1	97.57	96.61	97.52 ± 0.98 (1.01)
2	98.45	96.53	
3	99.19	97.95	
4	98.33	97.03	
5	98.74	96.49	
6	96.46	96.92	
Mean (%) ± SD	98.12 ± 0.97	96.92 ± 0.55	
RSD (%)	0.99	0.57	

Robustness

In the robustness study, we chose to use a full factorial design, which allows a faster and superior evaluation of the effect of isolated factors and their interaction. Factorial design is an important and simple statistical tool that enables the interpretation of results of several experimental conditions (27). Some of the factors which can be investigated by a HPLC method include mobile phase, rate of flow, column temperature, mobile phase proportion, column manufacturer, wavelength, among others (28). Thus, the effect of aqueous phase pH, methanol proportion and mobile phase flow rate on the sample assay at two levels, high and low, around the optimal condition, was studied.

These factors were selected because they are subject to changes in day-to-day work in the laboratory (22, 24, 29). A sample solution of 50 µg mL^{-1} was injected in a random sequence and the baclofen content was determined (Table 4).

Table 4. Drug content to different analysis conditions in HPLC method robustness study.

Sample	Order	Factor			Drug content (%)
		Aqueous phase pH	Flow (mL/min)	% Methanol	
1	10	7.0	1.0	15	97.91
2	5	7.0	1.0	15	98.00
3	11	6.5	1.2	10	98.51
4	6	7.5	0.8	10	98.75
5	4	6.5	0.8	20	98.57
6	9	6.5	1.2	20	98.50
7	7	7.5	0.8	20	98.82
8	8	6.5	0.8	10	98.65
9	1	7.5	1.2	20	98.71
10	2	7.0	1.0	15	98.80
11	3	7.5	1.2	10	97.97

The result was illustrated in a Pareto graph (Figure 3), which consists of bars with a length proportional to the absolute value of the estimated effect divided by the pseudo-standard error defined by Lenth (Lenth's PSE) (30). When an interaction between two or more factors occurs, this is indicated by a bar with their combination. The chart also includes a vertical line containing the t-critical value for α = 0.05. Smaller bars than the t-critical value indicate lack of significance of the factor considered (27).
As shown in Figure 3, the baclofen quantitation was not affected by the changes in the analytical parameters studied. Therefore, the results indicated the robustness of the developed method.

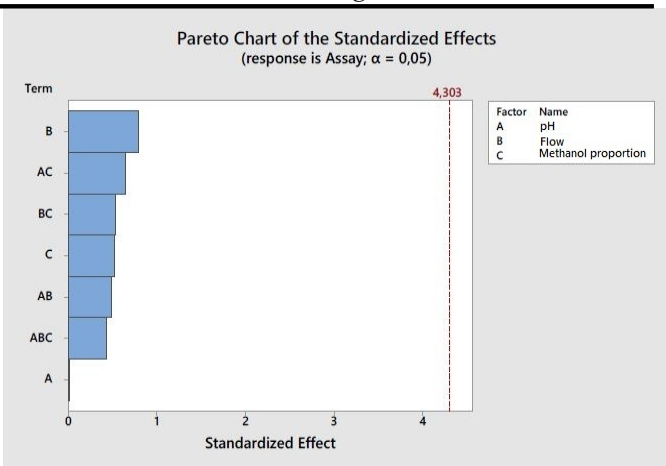

Figure 3. Pareto chart to robustness study of the HPLC method for baclofen tablets.

Application of the method to dissolution test

The official compendia (4, 5) recommend the same HPLC method used in the assay to quantify the dissolved amount of baclofen in the dissolution test. Thus, the dissolution test was carried out and the quantitation was performed by the proposed method. After 30 minutes of the dissolution test, the dissolved drug content was 84.75 ± 2.43% (n=6). Therefore the product in question meets the requirements recommended by the official compendia (4), which establish that after 30 minutes 75% of the drug should be dissolved.

Conclusions

The validated method showed to be specific, linear, accurate, precise and robust. In comparison to the pharmacopeial methods, the developed method uses smaller volumes of organic solvents and thereby reduces the waste generation. Additionally, this method does not require the column conditioning and the use of ion pairing reagents, reducing the cost of analysis. The results obtained in all the validation parameters confirmed the suitability of analytical conditions. Therefore, this method is a good alternative to assay baclofen tablets in quality-control analyses and stability studies.

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Conflict of interest

The authors declare no conflict of interest.

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