International Journal of Science and Research (IJSR)
ISSN: 2319-7064
SJIF (2022): 7.942

Analytical Method Development and Validation for Baclofen and its Stages by High Performance Liquid Chromatography

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Abstract: ***Objective: To develop and validate a rapid, precise, robust, cost-effective HPLC method for separating Baclofen and its impurities as per the latest regulatory requirement and regulatory expectations. Methods: The successful separation of the drug from its synthetic impurities was achieved byInertsil ODS 3V 150 X 4.6 mm, C18column using a mobile phase system containing 0.1% TFA: Acetonitrile (65: 35 v/v.) at detector wavelength 225 nm, with flow rate 0.6 ml/min and column temperature 27^{0}C. Results: The main Aim of the chromatographic method is to obtain separation of Baclofen from Impurity-A and Impurity-B. Impurities were separated using C18columns as well as different mobile phases. Good linearity was observed for Baclofen over the concentration range of 0.05mg/ml, with the linear regression (Correlation coefficient R = 0.999) and robust. Found the limit of detection and quantification of Baclofenwas 150 ng/ml and 500ng/ml, respectively, for a 10µL injection volume. Baclofen sample solution and mobile phase were stable for at least 48 hours. The % RSD for the assay content in six sample solutions is 0.25. Hence the method for the determination of Baclofen assay is precise. Conclusion: The developed HPLC method was simple, reliable, and cost-effective for Baclofen using the reverse mobile phase and validated as per ICH guidelines. Developed method can be used to quantitatively determine Baclofen in Bulk drug materials in the pharmaceutical industry.***

Keywords: Baclofen, Reverse phase, HPLC, Validation.

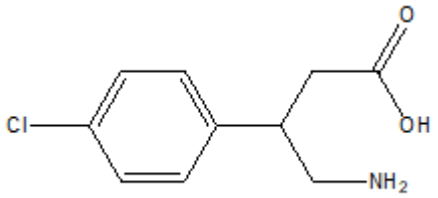
1. Introduction

Baclofen (Fig.1) is 4-amino-3-p-chlorophenyl butyric acid [1] [2] is a GABA-ergic agonist used to manage severe spasticity of cerebral, spinal origin in adult and pediatric patients. It is also widely used as a spasmolytic agent [3]**.** It is a skeletal muscle relaxant with its prime site of action in the spinal cord, where it binds to the inhibitory GABA-B receptor [4].

Certain methods have been reported for the qualitative and quantitative analysis for quantification of Baclofenin pharmaceuticals, techniques such as HPLC [5] [6], A rapid, sensitive, and economical RP-HPLC method for Determination Of Baclofen In Injections [7], Validated Stability-indicating HPTLC Determination of Baclofen in Bulk Drug, Pharmaceutical Formulations, and Real Human Urine and Plasma [8]. RP-HPLC method in Bulk Drug and Pharmaceutical Formulation [9], UPLC Separation and Quantification Of Baclofen And Its Potential Impurities In A Injection Formulation [10], where found use of costly solvent, lengthy analysis process for separation.

In the literature, there is no method for the separation and Validation of Baclofen and its impurities in Bulk drugs using reverse-phase containing buffer 0.1% TFA: Acetonitrile by high-performance liquid chromatography. This paper describes a reverse-phase LC method for the rapid separation and Assay of Baclofen and its impurities within 15 minutes of run time with proper separation, and no method reported less than 15 minutes of run time for analysis.

2. Structure



4-amino-3-(4-chlorophenyl)butanoic acid

Figure 1: Baclofenstructure

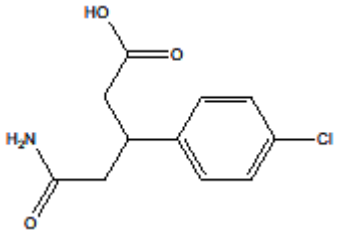


Figure 2: 3-(4-Chlorophenyl)glutamic acid (**Impurity-A)**

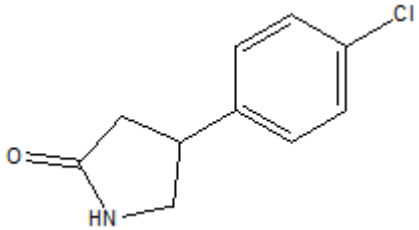


Figure 3: 4-(4-Chlorophenyl)-2-pyrrolidinone (**Impurity-B)**

3. Experimental

Chemicals
Baclofen kindly supplied by Lavender lab Pune, Maharashtra, India. HPLC grade Acetonitrile, TFA was purchased from Merck.

Equipment
For the Development and validation of the method, Agilent1260 series LC system was utilized with a UV detector and inbuilt auto-injector.

Preparation of Mobile Phase:
Take 1 ml TFA, carefully transfer into a 1000 ml measuring cylinder, add 650 mL of Milli-Q water, and dissolve. Makeup to the mark with acetonitrile to obtain the below ratio.
Mobile phase: 0.1% TFA: Acetonitrile (65: 35)

Stock and Sample Preparation
Weighed accurately about 25 mg of Baclofen sample and transferred it into a 50 mL volumetric flask, added about 30 mL of diluent, dissolved, and mixed. Makeup to the mark with diluent and mixed. Further diluted 5 mL of this solution to 50 ml with diluent. to obtain 0.05mg/mL

Diluent: Water: Acetonitrile (50: 50)

Chromatographic Conditions
The chromatographic conditions were optimized using Inertsil ODS 3V 150 X 4.6 mm, C18 column using a mobile phase system containing 0.1% TFA: Acetonitrile (65: 35 v/v.) at detector wavelength 225 nm with a flow rate of 0.6 ml/min and column temperature 27^0C. Diluents (50:50 v/v) acetonitrile: water. The injection volume was 10µl.

Validation of the method

Accuracy:
Prepared a series of Baclofen samples at 50%, 100%, and 150% in three triplicates concerning the target test concentration. Calculated the Recovery percentage from the slope and Y-intercept of the calibration curve obtained in the linearity section.

Precision
The Method repeatability was checked by injecting replicate injections of 500 µg/mL of the solution six times on the same day as the intraday precision study of Baclofen.

Limit of detection (LOD) and limit of quantification (LOQ) of Baclofen
The limit of detection, defined as the lowest concentration of analyte that can detect above the baseline signal, is estimated as three times the signal-to-noise ratio [11]. LOD and LOQ were achieved by injecting a series of dilute solutions of Baclofen and Impurity-A, and Impurity-B at the signal to noise ratios of 3: 1 and 10: 1

Linearity of Baclofen
Prepared a series of Baclofen samples at 500 ng/ml (LOQ) to 2500 ng/ml (500 ng/ml, 1000 ng/ml, 1500 ng/ml, 2000 ng/ml, and 2500 ng/ml), concerning the target test concentration of Baclofen. Injected each solution in triplicate and calculated the correlation coefficient for the sample. Calculated the percentage relative standard deviation of the slope and Y-intercept of the calibration curve obtained.

Robustness
To determine the robustness of the method, purposely altered experimental conditions such as mobile phase composition, flow rate, column temperature of Baclofen.

Solution stability and mobile phase stability

Prepared standard and Sample Solutions of Baclofen 0.05 mg/ml. The stability of Baclofen in solution at analyte concentration was Injected Blank, Standard Solution in five replicates, and Sample Solution at 0hrs, 6hrs, 24hrs, and 48hrs into the Chromatographic system**.** Content of Baclofen has been checked for six hours intervals up to the study period. Mobile phase stability carried out by evaluating the content of Baclofen sample solutions prepared freshly at six hours intervals for two days. The same mobile phase was used during the study period.

4. Results and Discussion

Method development
The main aim of the chromatographic method is to obtain the separation of Baclofen from Impurity-A and Impurity-B in a very short time and to reduce the cost of analysis. Impurities were separated using different stationary phases such as C18, C8, Phenyl, and cyano columns, as well as different mobile phases, were employed. Obtained the Accurate separation on Inertsil ODS 3V 150 X 4.6 mm, C18 column using a mobile phase system containing 0.1% TFA: Acetonitrile (65: 35 v/v.) at detector wavelength 225 nm with a flow rate of 0.6 ml/min, 15 minutes of run time with column temperature 27^0C and Diluents (50: 50 v/v) acetonitrile: water was used. Various experiments conducted to select the best mobile phases and stationery that would give optimum resolution and selectivity for separation within less time.

In the Presized method, the typical retention times of Baclofen and Impurity-A and Impurity-B were about 2.8 and 5.2, 10.8 minutes, respectively. The system suitability chromatogram shows the identical separation of Baclofen and its impurities (Figure No.4). A typical HPLC chromatogram of Baclofen Bulk sample (500 µg/ml) spiked with Impurities (1 %) is shown in (Figure No.5).

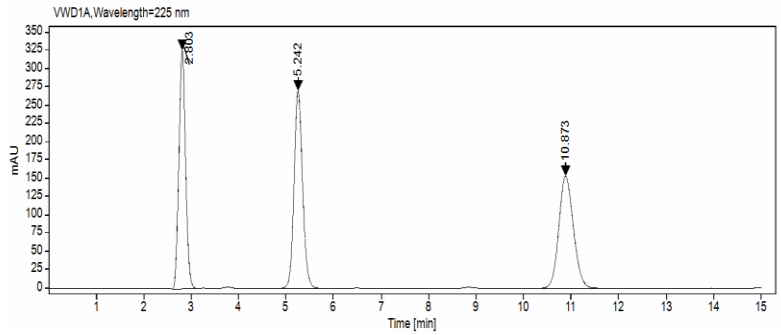


Figure 4: System suitability chromatogram

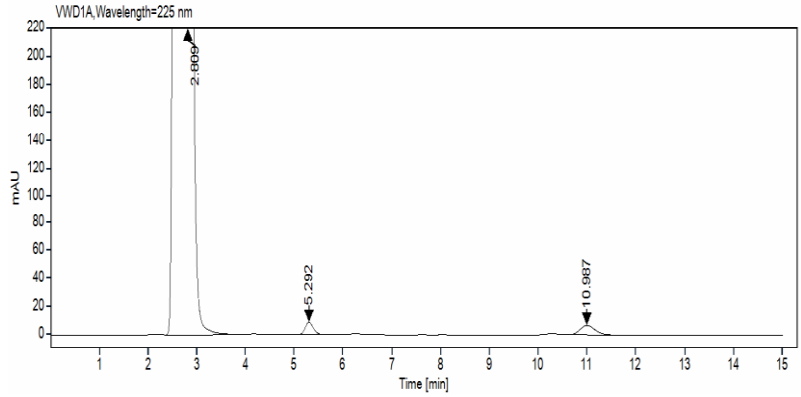


Figure 5: Baclofen bulk sample (500 µg/ml) spiked with Impurity-A and Impurity-B (1 %).

Validation results of the method

The system suitability test results are presented in (Table1). In the repeatability study, the relative standard deviation (RSD) was 0.25% for the Baclofen peak area (Table 2). In the intermediate precision study, results show that RSD values were in the same order of magnitude as those obtained for repeatability (Table 2).

The (LOD) and (LOQ) concentrations were estimated to be 150 and 500 ng/ml for Baclofen when a signal-to-noise ratio of 3 and 10 was used as the criteria. The method precision for Baclofen at the limit of quantification was less than 3 % RSD (Table 2).

Good linearity was observed for Baclofen over the concentration range of 500 - 2500 ng/ml, with the linear regression equation y = 14502X+117 (Correlation coefficient R = 0.999). Linearity was checked for Baclofen over the same concentration range for three consecutive days. The percentage relative standard deviation of the slope and Y-intercept of the calibration curve were 14502 and 117, respectively (Fig.6 and Table 2).

The standard addition and recovery experiments were conducted for Baclofen in Bulk samples in triplicate at 0.125, 0.250, and 0.375 percent of analyte concentration. Recovery was calculated from the slope and Y-intercept of the calibration curve obtained in the linearity study, and percentage recovery ranged from 98.0 to 102.0 (Table 3).
The chromatographic resolution of Baclofen peaks was used to evaluate the method's robustness under modified conditions. (Table 4), demonstrating sufficient robustness.

No significant change in the Baclofen content was observed in the sample during solution stability and mobile phase stability experiments. Hence Baclofen sample solution and mobile phase are stable for at least 48 hours.

Table 1: System-suitability report

Compound (n=3)	Rt	R_S	N	T
Baclofen	2.8	-	1859	1.07
Impurity-A	5.2	8.3	4119	1.1
Impurity-B	10.8	12.6	5919	1.12

n =3 determinations
R_S - USP resolution, N-number of theoretical plates (USP tangent method), T-USP tailing factor

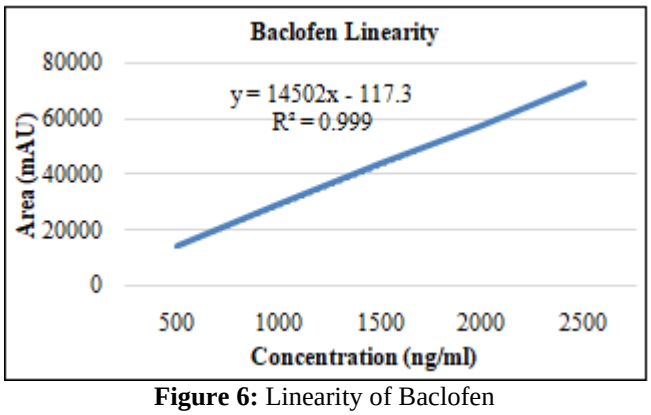


Figure 6: Linearity of Baclofen

Table 2: Validation results of the developed reverse phase method

Validation parameter	Results
Repeatability (n=6, % RSD)	
Retention time (Baclofen)	0.1
Area (Baclofen)	0.25

Intermediate precision (n=18, % RSD)	
Retention time (Baclofen)	0.1
Area (Baclofen)	0.18
LOD-LOQ (Baclofen)	
Limit of detection (ng/ml)	150
Limit of quantification (ng/ml)	500
Precision at LOQ (% RSD)	2.8
Linearity (Baclofen)	
Calibration range (ng/ml)	500-1500
Calibration points	5
Correlation coefficient	0.999
Slope (% RSD)	1.12
Intercept (% RSD)	0.9

Table 3: Recovery results of Baclofen in bulk drugs

Added (ng) (n=3)	Recovered (ng)	% Recovery	%RSD
1256	1274	99.5	0.41
2536	2512	98.9	0.45
3741	3750	100.2	0.4

n=3 determinations, RSD-Relative Standard Deviation

Table 4: Robustness of the method

Parameter	USP resolution between Baclofen and Impurity-A
Flow rate (ml/min)	
0.4	8.2
0.6	8.31
0.8	8.36
Column temperature (°C)	
22	8.22
27	8.31
32	8.38
Acetonitrile percentage in the mobile phase	
30	8.25
35	8.31
40	8.39

5. Conclusion

The developed HPLC method was simple, reliable, cost-effective, and rapid separation of Baclofen using a reverse mobile phase system containing 0.1% TFA: Acetonitrile (65: 35 v/v.) at detector wavelength 225 nm with a flow rate of 0.6 ml/min and column temperature 27^0Cand validated as per ICH guidelines. The validation of the method was carried out using Inertsil ODS 3V 150 X 4.6 mm, C18 column. The developed method can be successfully employed to determine Baclofen and its impurities in Bulk drug materials in the pharmaceutical industry.

Acknowledgments
We want to thank the management of Lavender Lab Pune, India, for donating drugs and providing the necessary facilities to develop and complete this work.

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