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RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE DETERMINATION OF BACLOFEN IN INJECTIONS

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ABSTRACT

A rapid, sensitive and economical RP-HPLC method has been developed and validated for quantification of baclofen in injections. A binary mixture of potassium dihydrogen phosphate, pentane 1-sulfonic acid sodium salt buffer (pH 3.0) and a mixture of acetonitrile, methanol in the optimized gradient mode was proved to be the most suitable mobile phase for the separation of baclofen on a non-polar Symmetry C18 column. The retention time for baclofen was at 5.538 ± 0.49 min. injected at a flow rate of 0.8 mL/min. and the detection wave length was fixed at 225 nm. The method obeyed linearity in the range of 0.0050 – 0.0150 mg/mL. The % RSD was found to be less than 2% in intra-day (0.5) and inter-day (0.1) variation studies indicating that the method is precise. The recovery of baclofen was found to be in the range of 98.6 - 100.5 % indicating that the method is accurate. The method also tolerated minor variations in chromatographic conditions indicating good robustness (% RSD < 1.0). The lowest values of LOD (4.46×10^{-5} mg/mL) and LOQ (1.35×10^{-4} mg/mL) as obtained by the proposed method indicate the sensitivity of the method. The marketed injection was found to contain an average of 101.45 ± 0.05 % w/v of baclofen as stated on the label claim and the excipients did not pose any interference at the retention time of the drug indicating specificity of the method. Experiments to study the effects of forced degradation on baclofen in injections were conducted in the validation study as per ICH guidelines. This method can be used for the regular quality control analysis of baclofen in API and injections.

KEYWORDS: Baclofen, RP-HPLC, Gradient, Symmetry C18 column, Validation.

INTRODUCTION

Baclofen[1,2] which is chemically known as (*RS*) - 4 – Amino – 3 - (4 – chloro phenyl) butanoic acid is a central nervous system depressant used as a skeletal muscle relaxant. It is primarily used to treat spasticity. It is a white to very faintly yellow crystalline powder which is slightly soluble in water, very slightly soluble in ethanol and practically insoluble in acetone. Baclofen (fig. 1) is a gamma amino butyric acid (GABA) derivative that stimulates GABA-B receptors leading to decreased frequency and amplitude of muscle spasms. It is especially useful in treating muscle spasticity associated with spinal cord injury. It appears to act primarily at the spinal cord level by inhibiting spinal polysynaptic afferent pathways and, to a lesser extent, monosynaptic afferent pathways (inhibitory neurotransmitter).

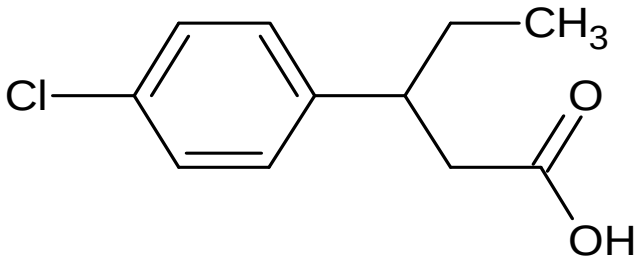

Fig. 1: Chemical structure of baclofen.

A survey of literature reveals various analytical methods reported for the determination of baclofen in biological fluids (human plasma, serum, urine, rat liver) and dosage forms. Few RP-HPLC[3-5] were reported for assay of baclofen in tablets along with several chiral HPLC[6-7] and bio analytical HPLC[8-15] methods. The reported methods also include diversified analytical techniques like LC-MS[16], capillary electrophoresis[17], potentiometry with ion selective electrode[18] and UV-Visible spectroscopy.[19-21] The aim of the present work is to develop and validate a new RP-HPLC method which could also be used as a stability indicating assay for baclofen in dosage forms.

MATERIALS AND METHODS

Drugs and Chemicals

The reference standard of baclofen (99.4% w/w purity) and baclofen related compound ‘A’ were supplied by Chandra Bhagat Pharma Pvt. Ltd., Hyderabad. The branded formulation of baclofen (LIORESAL lyophilized powder for injection, 2.0mg/mL, Novartis Pharmaceuticals Ltd.) was purchased from the local market. Acetonitrile and methanol (HPLC grade), potassium dihydrogen phosphate, pentane 1-sulfonic acid sodium salt and ortho phosphoric

acid (AR) were purchased from Merck Ltd. Ultra-pure water prepared from Milli-Q system (Millipore, Bedford, MA, USA) was used through the study. All other chemicals like sodium hydroxide, hydrochloric acid and hydrogen peroxide used in the study were of analytical grade.

Instrumentation

A Shimadzu HPLC (LC 2010 CHT) instrument equipped with quaternary gradient pump, UV/PDA detector, auto sampler and column heating oven was used for the study. A Symmetry C18 (250 x 4.6 mm, 5 µm) column was employed. Chromatographic analysis and data acquisition was monitored by using 'LC solutions' software. Degassing of the mobile phase was done using a PCI bath sonicator. A Sartorius SPA 225D electronic balance was used for weighing the materials. All pH measurements were made using a Metsar pH meter.

Mobile phase

Preparation of the buffer solution

Solution A: Potassium dihydrogen phosphate and pentane 1-sulfonic acid sodium salt buffer, pH 3.0. Accurately weighed about 1.38 gm. of potassium dihydrogen phosphate (0.01M) and 1.7 gm of pentane 1-sulfonic acid sodium salt (0.01M) was dissolved in 1000 mL of water. The pH of the solution was adjusted to 3.0 ± 0.2 with dilute phosphoric acid (10% w/v). The buffer was filtered through a 0.45 µm membrane and degassed before use.

Solution B: Acetonitrile and methanol were mixed in equal proportions.

The mobile phase consisted of solution 'A' and solution 'B' mixed in the optimized gradient mode.

Diluent

A mixture of solution 'A' and solution 'B' in the ratio of 65:35 was used as diluent.

Blank

The diluent solution was used as blank.

Preparation of stock and standard solutions of baclofen

About 25.0 mg of baclofen was weighed accurately and transferred into 50 mL volumetric flask, dissolved and diluted to volume with diluent to obtain a 0.5 mg/mL solution (stock). 1.0 mL of the above solution was pipetted out into a 50 mL volumetric flask and diluted to

volume with diluent to get about 0.01 mg/mL of baclofen solution (standard). Further dilutions were made from the stock solution in the required concentration range.

Preparation of solutions of baclofen formulation (injection)

Sample solutions were prepared by suitably diluting the baclofen injection (2.0 mg/mL). Accurately pipetted out 1.0 mL of baclofen injection was transferred into a 20 mL volumetric flask and diluted with diluent to get a solution of 0.1 mg/mL. From the above solution 2.0 mL was accurately pipetted out into a 20 mL volumetric flask and made up to volume with the diluent (sample).

Analytical Method Validation

The suitability of the developed method for intended purpose was proved by performing the analytical method validation in terms of linearity, specificity, precision, accuracy, limit of detection, limit of quantitation, robustness and system suitability testing as per the ICH guidelines.[22]

Standard solution of baclofen was prepared and injected five times before starting each validation parameter to check the system suitability.

Linearity and Range

Linearity of the method was determined by preparing six standard concentrations of baclofen in the working range of 0.005–0.015mg/mL in 10mL volumetric flasks. 20µL of each dilution was injected twice into the column and the drug in the eluents was monitored at 225nm. From the chromatograms obtained, mean peak area was noted and a plot of concentration vs. peak area was constructed.

Precision

Precision of the analytical method was established by injecting six preparations of baclofen sample (0.01 mg/mL), each injected twice on the same day (repeatability) and on a different day (intermediate precision). The % RSD for assay was calculated and analysed.

Accuracy

The accuracy of the method was determined by suitably diluting the sample (injection) solution to obtain concentrations corresponding to 50%, 100% and 150% levels of baclofen (0.0050mg/mL, 0.0100mg/mL and 0.0150mg/mL). Three preparations were made at each

level, each preparation injected twice and analyzed. The percent recovery was calculated from the average peak areas obtained.

Robustness

A study was conducted to determine the effect of deliberate variations in the optimized chromatographic conditions like flow rate (0.7 & 0.9 mL/min.), mobile phase composition (0.009 & 0.011M), pH (3.2 & 2.8) and column temperature (30 & 40 ^{o}C). Baclofen standard and sample solutions were evaluated at the altered conditions and the effect of these changes on the system suitability parameters like tailing factor and theoretical plates was studied.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

LOD and LOQ were calculated using residual standard deviation of the response and the slope of the regression line.

Analysis of baclofen from injections

Sample solutions were prepared by suitably diluting the baclofen injection (LIORESAL lyophilized powder, 2.0mg/mL). Accurately pipetted out 1.25 mL of baclofen injection was transferred into a 25mL volumetric flask and diluted with diluent to get a solution of 0.1 mg/mL (sample). The contents of the flask were sonicated and the mixture was filtered through 0.45µ membrane filter. From the above solution 1 mL was accurately pipetted out into a 10mL volumetric flask and made up to the volume with the diluent. 20µL of the above solution was then injected twice into the column. The mean peak area of the drug was calculated and the drug content in the formulation was calculated by the regression equation of the method.

Specificity

Specificity of the method can be studied in the presence of excipients, degradation products and impurities.

a. Interference from excipients and related compounds in baclofen injection

A mixture of all the commonly used excipients in injections like sodium chloride and water for injection were injected into the chromatograph as placebo. A blend of excipients spiked with pure baclofen and a small amount of baclofen related compound ‘A’ was also injected into the HPLC system to study any interference.

b. Forced degradation study on baclofen injection

The proposed method was applied on baclofen injection to observe the effective separation of baclofen and its forced degradation products at the retention time of baclofen. The forced degradation study was conducted by subjecting the samples of baclofen to acid/base hydrolysis, oxidative, photolytic and thermal stress conditions as per ICH guidelines.[23-24] Standard solution of baclofen was prepared and injected five times before starting the forced degradation study to check the system suitability. All sample solutions used in forced degradation studies were employed at an initial concentration of 0.1mg/mL and then diluted to give a final concentration of 0.01 mg/mL of baclofen.

Control sample

A 20µL of 0.01mg/mL of baclofen sample was injected into the chromatographic system and the obtained chromatogram was used as a control for the study of degradants in the further study.

Acidic Degradation

1.0 mL of baclofen sample solution was pipetted out into a 10 mL volumetric flask, 1.0mL of 1M hydrochloric acid was added, heated the solution to 60^{o}C for 3hr., cooled and immediately neutralized the solution using 1M sodium hydroxide solution. The stressed sample was diluted with the diluent, 20 µL was injected in duplicate and analyzed.

Alkaline Degradation

1.0mL of baclofen sample solution was transferred into a 10mL volumetric flask, 1.0mL of 1M sodium hydroxide was added, heated the solution to 60^{o}C for 3hr., cooled and immediately neutralized the solution using 1M hydrochloric acid solution. The stressed sample was diluted with the diluent, 20µL was injected in duplicate and analyzed.

Oxidative Degradation

Oxidative stress studies were conducted by treating 1.0 mL of baclofen sample solution with 2.4mL of 6% hydrogen peroxide in a 10mL volumetric flask. The solution was kept at room temperature for 2hr., diluted with the diluent and injected in duplicate into the chromatograph.

Photolytic Degradation

Dark Control

Dark control studies were carried out by transferring 1.0 mL of baclofen sample solution into a 10mL volumetric flask, stoppered with a lid and wrapped into an aluminium foil. The flask was subjected to an illumination of 1.2 million lux hours of cool fluorescent light and an integrated near UV energy exposure of 200 watt hours/m^2 simultaneously in a photo stability chamber maintained at 25°C. The stressed sample was diluted with the diluent and 20µL was injected in duplicate into the chromatograph.

Exposure to light

Accurately pipetted out 1.0 mL of baclofen sample solution into a 10 mL stoppered volumetric flask was subjected to an illumination of 1.2 million lux hours of cool fluorescent light and an integrated near UV energy exposure of 200 watt hours / m^2 simultaneously in a photo stability chamber maintained at 25°C. The stressed sample was diluted with the diluent and 20µL was injected in duplicate into the chromatograph.

Dry heat

Thermal stress was carried out by heating 1.0mL of baclofen sample solution in a controlled temperature oven at 80^{o}C for 7 days. The stressed sample mixture was cooled, diluted with diluent and injected in duplicate into the chromatograph.

RESULTS AND DISCUSSION

A new RP-HPLC assay method was developed and validated for the quantification of baclofen in injections as per ICH guidelines. The method was developed after a series of optimizations carried out with varied stationary and mobile phase conditions. A binary mixture of potassium dihydrogen phosphate, pentane 1-sulfonic acid sodium salt buffer (pH 3.0, Sol. A) and mixture of acetonitrile, methanol (Sol. B) in the optimized gradient mode was proved to be the most suitable of all combinations since the chromatographic peaks were better defined, resolved and almost free from tailing. The optimized chromatographic conditions and the gradient program are given in table 1.1 and table 1.2. Typical chromatogram of standard baclofen is given in fig. 2.

Table 1.1: Optimized chromatographic conditions.

Parameter	Value
Column	Symmetry C18 (250 x 4.6 mm, 5 µm) column
Mobile phase	Potassium dihydrogen phosphate and pentane 1-sulfonic acid sodium salt buffer (pH 3.0, Sol. A) and mixture of acetonitrile, methanol in equal proportions (Sol. B)
Elution mode	Gradient
Flow rate	0.8 mL/min.
Detection wave length	225 nm
Column temperature	35 ^{o}C
Volume of injection	20 µL
Run time	35.0 min.
Retention time obtained	5.538 ± 0.49 min.

Table 1: 2: Gradient Program.

Time (min.)	Solution A (%)	Solution B (%)
0	65	35
5.00	65	35
15.00	45	55
25.00	45	55
25.1	65	35
35.0	65	35

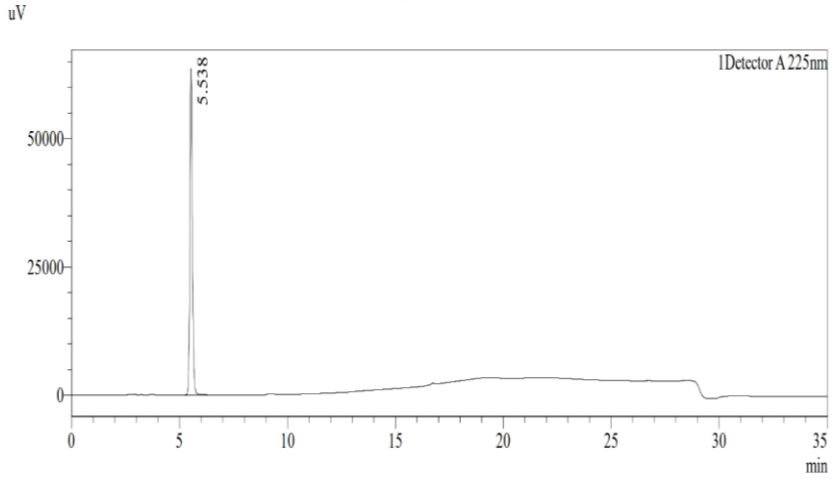

Fig. 2: Chromatogram of baclofen (standard).

Method Validation

Linearity and Range

The linear relation between the concentration and peak area of baclofen was obeyed in the range of 0.0050 – 0.0150 mg/mL as observed from the regression analysis and this equation was used to estimate the amount of baclofen in pharmaceutical dosage forms. The linearity data is reported in table 2 and the calibration plot is shown in fig. 3.

Table 2: Linearity data for baclofen.

Conc. (mg/mL)	*Peak area ± SD, % RSD
0.0050	264856 ± 64.35, 0.02
0.0080	428355 ± 211.42, 0.05
0.0090	481846 ± 137.89, 0.03
0.0100	530784 ± 301.93, 0.06
0.0110	582616 ± 1712.61, 0.29
0.0120	637663 ± 383.96, 0.06
0.0150	790251 ± 977.22, 0.12

* Mean of two replicates.

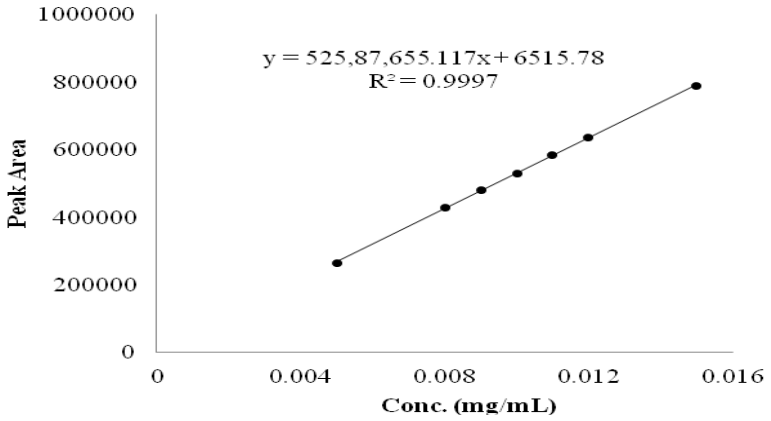

Fig. 3: Linearity plot for baclofen.

Precision

The repeatability and intermediate precision were studied by analyzing the sample solutions of baclofen. The low coefficient of variation obtained for assay of baclofen in the intraday (0.5) and inter day precision (0.1) study as given in table 3 is indicative of the precision of the method.

Table 3: Repeatability and intermediate precision study for baclofen.

Preparations	Repeatability		Intermediate precision	
	*Sample peak area	Assay (%)	*Sample peak area	Assay (%)
Preparation-1	535730	101.9	542984	101.0
Preparation-2	534982	101.8	542276	100.9
Preparation-3	536083	102.0	543015	101.0
Preparation-4	534632	101.7	542303	100.9
Preparation-5	529588	100.8	542528	100.9
Preparation-6	535659	101.9	543224	101.0
Average		101.7		101.0
SD		0.464		0.075
% RSD		0.5		0.1

* Mean of two replicates.

Accuracy

The method was proved to be accurate as the percentage recovery of baclofen from injections was within the range of 98.6 – 100.5% as given in table 4.

Table 4: Accuracy data.

Level (%)	Standard peak area	*Sample peak area	*Amount added (mg)	*Amount found (mg)	*Mean recovery ± SD, % RSD
50	534847	268807	0.005	0.005	100.5 ± 0.1, 0.09
100	534847	535879	0.010	0.010	100.2 ± 0.1, 0.06
150	534847	791204	0.015	0.0148	98.6 ± 0.1, 0.07

* Mean of six injections.

Robustness

The deliberately varied chromatographic conditions like flow rate, column oven temperature, buffer concentration and pH also gave acceptable system suitability parameters indicating the robustness of the method as given in table 5.

Table 5: System suitability parameters for robustness study.

Parameter	Condition	*Assay (%)	Tailing factor	Theoretical plates	Mean assay ± SD, % RSD
Flow rate (± 0.1 mL/min.)	0.7	100.1	1.2	10433	101.1 ± 0.9, 0.86
	0.8	**101.5**	**1.1**	**9850**	
	0.9	101.7	1.2	9039	
Column oven temperature (± 5 ^{o}C)	30	100.1	1.1	9465	100.7 ± 0.7, 0.72
	35	**101.5**	**1.1**	**9850**	
	40	100.4	1.2	9273	
Buffer Concentration (± 0.001M)	0.009	101.2	1.2	9638	101.1 ± 0.5, 0.51
	0.01	**101.5**	**1.1**	**9850**	
	0.011	100.5	1.2	9792	
Buffer pH (± 0.2 pH)	2.8	101.1	1.2	9740	100.9 ± 0.6, 0.57
	3.0	**101.5**	**1.1**	**9850**	
	3.2	100.3	1.2	9542	

* Mean of two injections.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD for baclofen in the present method was found to be 4.46×10^{-5} mg/mL and LOQ was found to be 1.35×10^{-4} mg/mL as calculated from the calibration curve.

Assay of baclofen from injections

The proposed method was applied for the quantification of baclofen in commercial injections and the assay obtained is given in table 6.

Table 6: Assay of baclofen injection.

Sample	Labeled amount (mg/mL)	Amount found ± S.D.	*Assay (%) ± S. D.
LIORESAL	2.0	2.029 ± 0.001	101.45 ± 0.05

*Mean of two injections.

Specificity

a. Interference from excipients and related compounds in baclofen injection

An observation of the placebo and spiked chromatograms for retention time and peak purity index indicates absence of excipient peaks/related compound 'A' near the drug peak in the study runtime. The peak for baclofen related compound 'A' (retention time at 17.055 min. which was confirmed by injecting a standard solution of baclofen related compound 'A' alone) was well resolved from the baclofen peak as shown in fig. 4a. This clearly shows that baclofen peak is well resolved in the presence of excipients/related compounds which depict the specificity of the method.

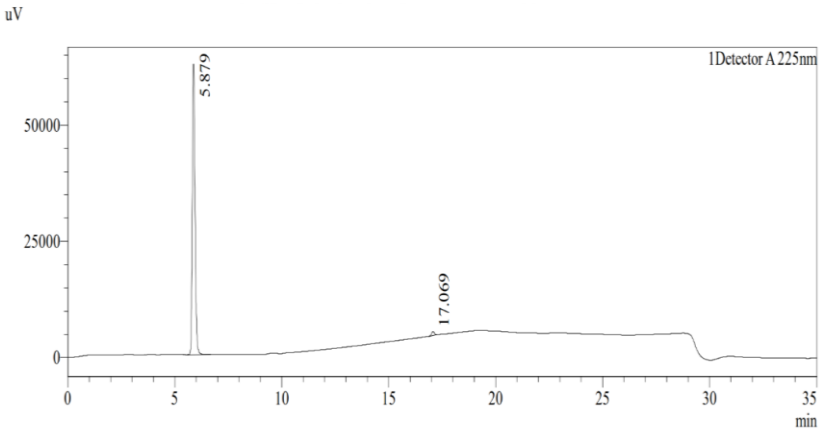

Fig. 4a: Chromatogram for specificity with baclofen related compound 'A' and excipients.

b. Forced degradation study on baclofen injection

The drug was found to be highly stable to all the stress conditions except peroxide stress and no major degradants were found in acid, base, thermal and photolytic degradation studies. A separate peak eluted before 3.5min. in peroxide stress which was well resolved from the main peak as observed from the resolution between the two peaks. The baclofen peak in these degradations was found to be homogenous and no other peaks merged with it which could be confirmed from the peak purity index (1.0000) and similarity index values (> 0.99989). No major degradants were found in photo stability and heat degradation studies. The overlain

chromatograms obtained in the forced degradation study are shown in fig. 4b and the data is given in table 7.

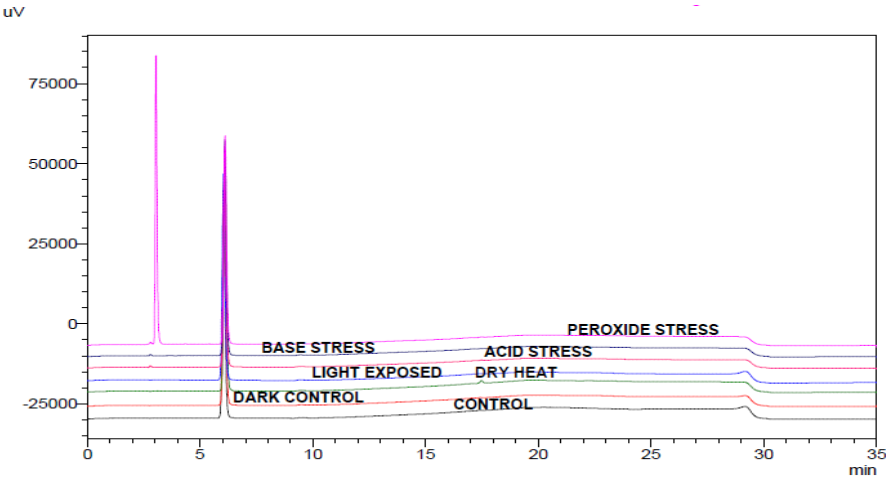

Fig. 4b: Overlain chromatograms for forced degradation study of baclofen.

Table 7: Forced degradation data.

Stress condition	Standard average area	*Sample average area	Assay (%)
Control	470756	479334	101.6
Acid stress	470756	481097	102.0
Alkaline stress	470756	480199	101.6
Peroxide stress	470756	464769	98.5
Dark control	534247	535674	99.8
Exposure to light	534247	536840	100.0
Dry Heat	534247	533065	99.3

* Mean of two injections.

CONCLUSION

A sensitive, precise and accurate RP-HPLC method was developed and validated for the analysis of baclofen which also proved to be stability indicating. The method uses a mobile phase which is robust and able to completely resolve all the related compounds and degradation products within the said runtime. The method can be conveniently used for quality control analysis of baclofen in bulk drug and injections without any interference from degradants or excipients.

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