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Research Article

Validated HPLC method for Identification and Quantification of 4-Hydroxy Benzoic Acid in Levetiracetam Oral Solution Drug Product Formulation

MSN Reddy, Gopalakrishnan Venkatasami*, Venkata R Kotta, Muruganantham Ramamoorthy, Shreya Patel

Department of Chemistry, MSN Pharmaceuticals Inc, USA.

ABSTRACT

A simple, selective, sensitive, accurate and precise RP-HPLC method was developed for the identification and quantification of 4-hydroxy benzoic acid which is the main degradation product from parabens in Levetiracetam Oral solution. The separation was performed on a C18-column using a mobile phase 0.1% Phosphoric acid buffer as mobile phase A and 100% Acetonitrile as a mobile phase-B in a gradient elution. The detection was performed at a fixed wavelength (λ=230 nm), with a flow rate of 1.0 ml/min. The retention time of 4-Hydroxy Benzoic acid and Levetiracetam were found to be 11.78 min and 9.03 minutes, respectively. After developing the method, it was assured for the intended use by validation of the analytical parameters like linearity, accuracy, precision, limit of detection, limit of quantitation and robustness. The results of all the parameters of the method were found to be within the acceptance criteria as per the International Council for Harmonization (ICH) guidelines. The detector response was linear in concentrations ranging from 0.5033 µg/mL (0.05%)-4.0264 µg/mL (0.4%) of 4-Hydroxy benzoic acid. The Limit of Detection (LOD) and Limit of Quantification (LOQ) of p-Hydroxy Benzoic acid were found to be 0.1007 µg/mL and 0.5033 µg/mL. The intra and inter day variations were found to be less than 2%. The analytical recoveries of 4-hydroxy benzoic acid are ranges from 94.6% to 107.2%. The proposed method was used to monitor the degradation of the parabens in Levetiracetam Oral solution drug product USP100 mg/5 mL formulation.

Keywords: Levetiracetam; 4-Hydroxybenzoic acid; RP-HPLC; Validation

INTRODUCTION

Levetiracetam is an important antiepileptic drug that is used as a monotherapy or in combination with other drugs in patients who suffered from partial and secondary generalized seizures [1]. Despite its wide use, few HPLC procedures have been developed for Levetiracetam determination in bio and formulation samples using either mass spectrometry or UV detection or capillary electrophoresis [2-8]. Parabens or 4-Hydroxybenzoates are derivatives of 4-hydroxybenzoic acid (p-hydroxybenzoic acid) and are used in industry, particularly in pharmaceutical, cosmetics and food, due to their appealing characteristic of acting as preservatives and antimicrobial compounds. Liquid preparations are particularly susceptible to microbial growth because of the nature of their ingredients. Such preparations are protected by the addition of preservatives, which prevent the alteration and degradation of the product formulation. The simplicity and effectiveness of parabens as preservatives, in combination with their low cost and the long history of their use probably explains why they are so commonplace, exposing human population to parabens from a wide variety of sources on a daily basis. The most common parabens are methyl, ethyl, propyl and butyl parabens [9]. The Levetiracetam Oral Solution formulation uses methyl and propyls parabens as preservatives.

These appreciated preservatives are fairly degradable, which can lead to the accumulation of toxic products. It is reported [10]. That conversion of parabens into 4-hydroxy benzoic acid or p-Hydroxy benzoic acid which possesses a weaker preservative activity may be observed in presence of microbes in solutions of parabens. Degradation of parabens results in loss of the prescription quality. To the best of information available in the literature, there is no specific validated HPLC method reported

Correspondence to: Gopalakrishnan Venkatasami, Department of Chemistry, MSN Pharmaceuticals Inc, USA; E-mail: gopal.venkat@msnpi.com

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for the determination of 4-Hydroxy benzoic acid in Levetiracetam Oral solution. Keeping that in view, the present HPLC method was developed and validated specifically for the determination of 4-Hydroxy benzoic acid in Levetiracetam Oral Solution.

MATERIALS AND METHODS

Chemicals and drugs

Levetiracetam Oral Solution was manufactured at MSN Pharmaceuticals Inc, (New Jersey, United States of America). 4-Hydroxy Benzoic acid impurity was procured from Supelco (Sigma-Aldrich), Levetiracetam Active Pharmaceutical Ingredient (API), Levetiracetam related compounds were manufactured by MSN Life Sciences Pvt Ltd (Hyderabad, India). Phosphoric acid of ACS grade was purchased from Merck and HPLC grade solvents like Methanol and Acetonitrile were purchased from Fisher.

Equipment

It consists of High Performance Liquid Chromatograph of Waters (Maryland, United States of America) equipped with Alliance e2695 separation module, UV/Visible detector (2998 and 2489). The output signal was monitored and processed by waters Empower 3 software. The stationary phase employed was Phenomenex Kinetex C18 (150 × 4.6 mm; 5 µm particle size) column.

Standard solution preparation

Stock standards solution was prepared by dissolving about 25 mg of p-Hydroxybenzoic Acid (4-Hydroxybenzoic Acid) standard into a 250 mL volumetric flask. Add diluent to about 60% volume of the flask, sonicate to dissolve with intermittent shaking, dilute to the volume with diluent and mix well. The stock standard concentration is about 100 µg/mL of p-Hydroxybenzoic Acid. From the stock standard solution pipetted 2.0 mL into a 100 mL volumetric flask, dilute to the volume with diluent, mix well. The concentration is about 2.0 µg/mL of p-Hydroxybenzoic Acid.

Sample preparation

Sample solution was prepared by weighing about 2.3 g of Levetiracetam Oral Solution, USP, 100 mg/mL into a 200 mL volumetric flask with aid of the diluent. Added diluent to about 60% volume of the flask, sonicate for 5 minutes with intermittent shaking. Allowed the solution to room temperature, diluted to the volume with diluent and mixed well. The concentration is about 1.0 mg/mL of Levetiracetam.

Hplc method development

0.1% phosphoric acid: acetonitrile (95:5%v/v) was chosen ad diluent. Multiple trials were done using 0.1% phosphoric acid solution as mobile phase –A and 100% acetonitrile as mobile phase-B for the development of an HPLC method for estimation of p-Hydroxybenzoic acid impurity in Levetiracetam oral solution. Various gradient programs were evaluated. Trials were continued till the method was optimized.

Determination of 4-hydroxybenzoic acid in levetiracetam oral solution sample

Standard solution of 4-Hydroxybenzoic acid and sample solution of Levetiracetam oral solution were injected and chromatograms were recorded at 230 nm with UV detector. The determination of percentage of 4-Hydroxybenzoic acid is given by the following formula:

$$\%of\ 4-\text{Hydroxybenzoic acid (HBA)} = \frac{A_{HBA}}{A_{Std}} \times \frac{Wt_{std}}{250\,mL} \times \frac{2.0\,mL}{100\,mL} \times P \times \frac{200\,mL}{Wt_{Spl}} \times \frac{D}{LC} \times 100$$

Where:

AHBA: Peak area of 4-Hydroxybenzoic Acid in the sample solution

Astd: Average peak area of 4-Hydroxybenzoic Acid in the standard solution

Wtstd: Weight of standard, in mg

Wtspl: Weight of Sample, in gram

D: Density of Oral Solution, in g/mL

P: Potency of standard

LC: Label Claim, 100 mg/mL as Levetiracetam

Method validation

Participants' Analytical method validation was performed as per the ICH guidelines [11]. The developed method was validated for the various parameters like selectivity, linearity, Limit of Detection (LOD), Limit of Quantification (LOQ), accuracy, robustness and precision.

Selectivity

Selectivity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically, these might include impurities, degradants, matrix, etc. Selectivity of the test method was evaluated by placebo, active and other related impurities interference study.

Linearity

Linearity study was covered the range of 0.05%-0.4% of the expected level of the analyte. Different concentration solutions of 4-Hydroxybenzoic acid solution (0.5 µg/mL, 1.0 µg/mL, 2.0 µg/mL, 3.0 µg/mL and 4.0 µg/mL) were prepared from the stock solution using diluent. The calibration curve was obtained by plotting peak area vs. concentration, using the least squares method. ICH recommends that, for the establishment of linearity, a minimum of five concentrations should normally be used. Acceptance criteria is correlation coefficient must be not less than 0.98.

Limit of Detection (LOD)

Determination of the signal to noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. Acceptance limit for detection limit for 4-Hydroxybenzoic acid peak was calculated using signal to noise ratio (S/N) must be Not Less Than (NLT) 3 for Detection Limit (DL).

Limit of Quantification (LOQ)

Determination of the signal to noise ratio is performed by comparing measured signals from samples with known low

concentrations of analytic with those of blank samples and by establishing the minimum concentration at which the analyst can be reliably quantified. Acceptance limit for quantification limit for 4-Hydroxybenzoic acid peak was calculated using signal to noise ratio (S/N) must be Not Less Than (NLT) 10 for Detection Limit (DL).

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness. As per ICH guideline recommendation, the accuracy study was assessed by nine determinations i.e., three replicates at three concentrations across the specified range of the procedure. Acceptance criteria were kept as Individual % Recovery must be within 75.0%-125.0% at LOQ level (0.05% Level) and individual % Recovery must be within 80.0%-120.0% at other levels.

Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions on different days. ICH guidelines recommend that repeatability should be assessed by using a minimum of six determinations at 100% of the specification level test concentration at different days. Acceptance criteria are % RSD of the % recovery of 6 injections must be not more than 10.0%.

Robustness

Robustness was evaluated by slightly changing the chromatographic conditions which includes flow rate and column temperature.

Effect of slight change in flow rate: The solution was analyzed at 0.9 ml·min−1 and 1.1 ml per minute rather than optimized flow rate of 1.0 ml per minute. Chromatograms were collected to compare with optimized chromatographic conditions. The %RSD of 6 4-Hydroxy benzoic acid injections peak area was compared with normal condition.

Effect of slight changes in percent column temperature: The solution was analyzed by slightly varying the HPLC column temperature at 25°C and 35°C rather than optimized HPLC column temperature of 30°C. Chromatograms were collected to compare with optimized chromatographic conditions. The %RSD of 6 4-Hydroxy benzoic acid injections peak area was compared with normal condition.

RESULTS AND DISCUSSION

HPLC method development

The test method condition for the identification and quantification of 4-hydroxy benzoic acid in Levetiracetam Oral Solution drug product formulation was developed using Phenomenex Kinetex C18 (150 × 4.6 mm; 5 µm particle size) column using 0.1% Phosphoric acid buffer as mobile phase A and 100% Acetonitrile as a mobile phase-B at a different flow rate and with various gradient programs to separate 4-Hydroxybenzoic acid peak from the placebo, diluent, Levetiracetam active and related compounds. At last, an optimized method conditions were developed, which employs 0.1% Phosphoric acid buffer as mobile phase A and 100% Acetonitrile as a mobile phase-B at a flow rate of 1.0 mL/min. The optimized chromatographic conditions were shown in Table 1 and the standard chromatogram is shown in Figure 1.

Table 1: Chromatographic condition

Mobile phase	**Mobile Phase A: 0.1% Phosphoric Acid Solution**		
	Mobile Phase B: 100% Acetonitrile		
Column	Phenomenex Kinetex C18, 150 x 4.6 mm, 5 µm		
Elution	Gradient		
Flow Rate	1.0 mL/min		
Injection Volume	10 µL		
Autosampler Temperature	5°C		
Detection	230 nm		
Column Temperature	30°C		
Gradient Program	Time (Minutes)	Mobile Phase A (%)	Mobile Phase B(%)
	0	98	2
	14.5	90	10
	15	90	10
	18	25	75
	20	25	75
	20.1	98	2
	25	98	2
Run Time	25 minutes		
Retention Times	Levetiracetam: about 9.0 minutes		
	4-Hydroxybenzoic Acid: about 11.7 minutes		

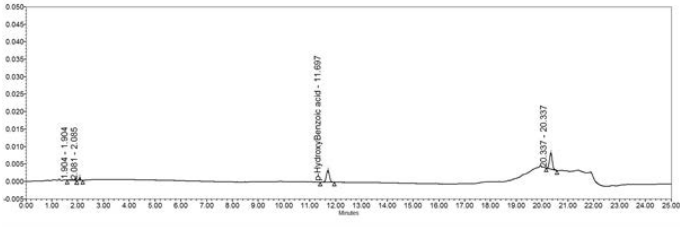

Figure 1: Chromatogram of 4-Hydroxybenzoic acid standard solution

Method validation

Analytical method validation was performed as per the ICH guidelines [11]. The developed method was validated for the various parameters like specificity, linearity, Limit of Detection (LOD), Limit of Quantification (LOQ), accuracy, robustness and precision.

Selectivity: Selectivity of the test method was evaluated by placebo, active and related impurities interference. 4-Hydroxybenzoic Acid was spiked at 0.2% of sample concentration, Levetiracetam related impurities were spiked at 0.1% of the sample concentration. Those were spiked in the sample containing Levetiracetam active and placebo for the selectivity study of the test method. The selectivity chromatogram is shown in Figure 2.

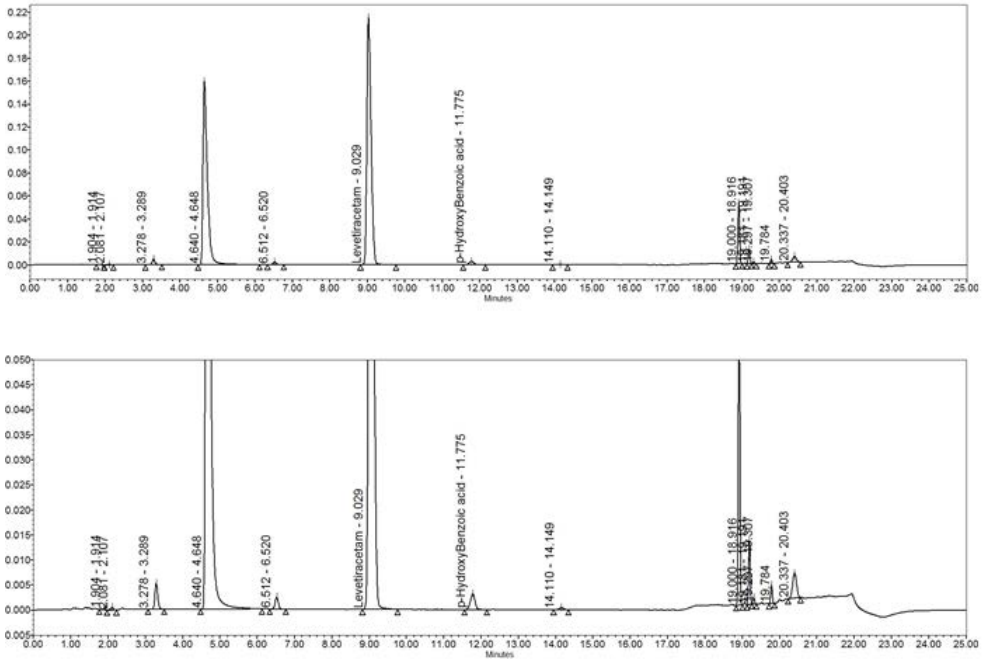

Figure 2: Full and expanded Chromatogram of Selectivity solution

Table 2: Accuracy study of p-Hydroxybenzoic acid

Recovery level	Amount of 4-Hydroxybenzoic Acid Added (µg/mL)	Amount of 4-Hydroxybenzoic Acid Found (µg/mL)	% Recovery	Mean (%) ± SD	% RSD
0.05%	0.5007	0.5368	107.2	101.2 ± 5.333	5.27
		0.4859	97		
		0.4974	99.4		
0.10%	1.0014	0.9844	98.3	98.8 ± 0.416	0.422
		0.99	98.9		
		0.992	99.1		
0.40%	4.0056	3.8119	95.2	94.8 ± 0.321	0.339
		3.792	94.7		
		3.7894	94.6		

Linearity: Linearity study was performed in the range of about 0.5 µg/mL to 4 µg/mL of 4-Hydroxybenzoic Acid, which was equivalent to the range of 0.05% to 0.40% of sample concentration. Calibration curves of 4-Hydroxybenzoic acid were plotted using peak area vs. concentration and were shown in Figure 3. Correlation coefficient values were found to be 0.9998. As per ICH guidelines these values were within acceptable limit and hence the method was found to be linear.

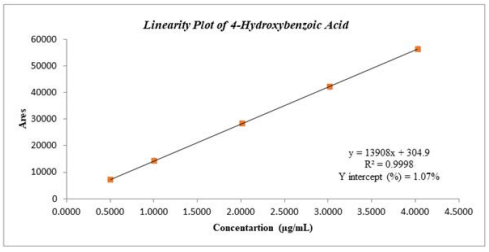

Figure 3: Calibration curve of p-Hydroxybenzoic acid

LOD and LOQ: The lowest possible concentrations of 4-Hydroxybenzoic acid that can be detected and quantified by the present method were found to be 0.1007 µg/mL and 0.5033 µg/mL. The low values of LOD and LOQ indicates that the method can be used for detection and quantification of p-Hydroxybenzoic acid on the Levetiracetam oral solution drug product formulation over a very wide range of concentrations.

Accuracy: Accuracy study results shown on the Table 2. Accuracy study was performed by spiking sample solution with 4-Hydroxybenzoic Acid at the concentrations of 0.5 µg/mL, 1.0 µg/mL and 4.0 µg/mL, which were equivalent to 0.05%, 0.10% and 0.40% of the sample concentration, respectively. Accuracy study sample solutions were prepared in triplicate. Mean percentage recovery values at QL level found to be within the acceptance limit of 75.0% to 125.0% and 80.0% to 120.0% level other levels. Hence, accuracy was established for the developed

method and was found to be accurate.

Precision: Intra-day precision study and inter day precision study results from 6 preparations at 0.10% level were shown on Table 3. The percentage RSD values of 4-Hydroxybenzoic acid obtained from both precision studies was found to be less than 1% which was well within the limits mentioned on the ICH guidelines. Hence, the method was found to be precise.

Table 3: Precision study of p-Hydroxybenzoic acid

Sample No	Intra-day % Recovery	Inter-day % Recovery
1	98.3	98.1
2	98.9	98.1
3	99.1	99.7
4	98.6	97.8
5	98	98.7
6	96.7	97.5
Mean (n=6)	98.3	98.3
% RSD (n=6)	0.9	0.8
Mean (n=12)	Not Applicable	98.3
% RSD (n=12)	Not Applicable	0.8

Range: The concentration range of the test method was determined by the common passing concentrations which met the acceptance criteria of both linearity and accuracy studies. The range of the test method is from 0.5007 µg/mL to 4.0056 µg/mL of p-Hydroxybenzoic Acid, which is equivalent to the range from about 0.05% to about 0.40% of the sample concentration.

Robustness: Mobile phase flow rate was slightly altered by 0.1 units (0.9 mL/min and 1.1 mL/min). HPLC column temperature was slightly altered by 5 units (25°C and 35°C) The %RSD of 4-Hydroxy benzoic acid peak areas from 6 injections from modified condition were calculated and shown in Table 4 the % RSD values were found to be not more than 2.0% for variation in flow rate and HPLC column temperature. According to ICH guidelines the %RSD should be less than 2% and hence the method was found to be robust.

Table 4: Robustness of the method

Parameters	Normal condition	Condition opted	%RSD of 4-Hydroxybenzoic acid peak area
Flow rate	1.0 mL/ min	0.9 mL/min	0.7
		1.1 mL/min	0.3
Column temperature	30°C	25°C	0.8
		35°C	0.8

CONCLUSION

The present work was planned for the identification and quantification of 4-Hydroxybenzoic acid in the Levetiracetam Oral solution drug product formulation. Reverse phase high performance liquid chromatographic method (RP-HPLC), which was developed and validated in the present work was found to be simple, linear, accurate, precise, robust, therefore, suitable for the intended use. Hence, this method is reliable and convenient for routine quality control analysis of 4-Hydroxybenzoic acid impurity in the Levetiracetam oral solution drug product.

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