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Development and Validation of Stability Indicating UFLC Method for the Estimation of Anti-spasmodic Drug - Valethamate Bromide

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Authors' contributions

This work was carried out in collaboration between all authors. Author RSA designed the study and performed the statistical analysis. Author AT wrote the protocol and wrote the first draft of the manuscript. Author Ramesha provided active pharmaceutical ingreadent to carry out the work. Author RSC managed the analyses of the study. Authors KA and BMG managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

A stability indicating Ultra-Fast Liquid Chromatography (UFLC) method has been developed to estimate valethamate bromide (V&B) in tablet dosage forms and to separate analyte from other degradants and to carryout forced degradation studies. The chromatographic separation was achieved by using phenomenex kinetex 5 µ C_{18} 100A (250x 4.6 mm) column with a flow rate of 1 mL/min in an isocratic mode with the mobile phase consisting of 0.05 M potassium dihydrogen phosphate buffer pH 7.5 (adjusted with potassium hydroxide) and acetonitrile in the ratio of 50:50. The eluents were monitored with PDA detector at 227 nm. In this developed method valethamate bromide was eluted at a retention time of 4.4 ± 0.1 min. The proposed method is having linearity in

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the concentration range of 2-12 µg/mL. The present method was successfully validated with respect to system suitability, linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ) and robustness as per international council for harmonization (ICH) guidelines. The drug was subjected to different degradation conditions like acid and alkali hydrolysis, oxidation, thermal and UV radiation etc. Peak purity of the drug was passed in all degradation conditions which demonstrated the specificity of assay method for it in presence of degradation products. The proposed method can be applied for determination of valethamate bromide in pure drug and in pharmaceutical formulation.

Keywords: Valethamate bromide; UFLC; validation; stability indicating assay.

1. INTRODUCTION

Valethamate bromide is chemically, Diethyl (2-hydroxyethyl) methyl ammonium 3-methyl-2-phenyl valerate bromide (Fig. 1). Valethamate bromide is a quaternary ammonium anti-muscarinic agent and exerts a preferential parasympatholytic or anti-cholinergic effects on smooth muscles. It has less undesirable anti-cholinergic effect as compared to atropine and other quaternary ammonium compounds. It hastens cervical dilation and reduces the duration of the 1st stage, when administered after initiation of the 1st stage of labor. In medical termination of pregnancy, it is used locally (intracervical) for smooth cervical dilation reduces pain and decreases blood loss [1]. It is also used in painful menstruation periods, during the birth. It is official in Indian national formulary (INF) 13th edition [2].

Stability indicating assay method (SIAM) is an estimative analytical method used to detect residual levels of the active pharmaceutical ingredient (API) present due to degradation. Stability studies provide evidence on how the quality of a drug varies with time under the influence of a variety of environmental factors such as temperature, humidity and light. Lack of drug substance stability may affect the purity, potency and safety of the drug product. It provides information on drug characteristics, identification of potential degradants [3]. ICH defines SIAM as quantitative analytical methods that are based on the characteristic structural, chemical or biological properties of each ingredient of a drug product and that will distinguish each active ingredient from its degradation product so that the active ingredient content can be accurately measured [4].

As per the literature, various methods like HPTLC [5], RP-HPLC [6-12] have been reported for the determination of valethamate bromide. The existing methods have some drawbacks like more retention time, complex mobile phases. From the above mentioned literature; it is also known that no methods have been reported for the estimation of valethamate bromide in tablet dosage form by ultra-fast liquid chromatography and no stability indicating assay method was developed.

The main objective of this work is to develop a simple, rapid and precise stability indicating assay method for the estimation of valethamate bromide in bulk and tablet dosage forms using ultra-fast liquid chromatography and to validate the developed method. The method was thoroughly tested for its specificity and stability indicating properties by resolution of the drug form its hydrolytic, oxidative and thermal degradation products.

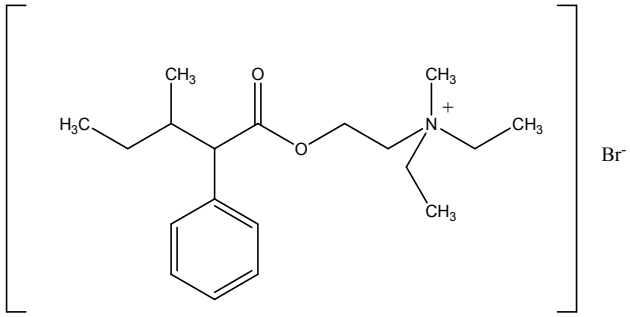

Fig. 1. Structure of valethamate bromide

2. EXPERIMENTAL

2.1 Method Development

2.1.1 Materials

A pure reference standard of valethamate bromide was procured from R L Fine Chemicals, Bangalore. Valethamate bromide formulation (Epidosin) was obtained from local pharmacy. HPLC grade water and acetonitrile were obtained from Merck Pvt. Ltd, Mumbai. The chemicals used were of analytical reagent grade (AR grade).

2.1.2 Instrumentation

The current research was carried out on UFLC from of Shimadzu Prominence LC-20AD equipped with a 1260 binary pump VL (35 MPa), Prominence SIL-20ACHT auto sampler and prominence SPD-M20A diode array detector connected to a computer loaded with LC solution software. Weighing of the samples was performed on a Shimadzu electronic analytical balance AY-220. The degassing of solvents was done by using an ultrasonic bath (Mark ultrasonic sonicator).

2.1.3 Selection of chromatographic technique

The selection of the method depends on the physico-chemical properties of the drug substance that is on the nature of the sample, molecular weight and solubility.

As the drug is polar, the present technique was selected as reverse phase chromatography based on properties of the compound and reverse phase chromatographic conditions needs to be optimized.

2.1.4 Selection of diluents

According to literature available, as the drug was easily soluble in water, methanol and acetonitrile, extraction of the drug was done using two ratio of acetonitrile and buffer (pH 7.5). Finally, the ratio of ACN: Buffer potassium dihydrogen phosphate pH 7.5 (50:50) as diluents was selected for the complete extraction of the drug.

2.1.5 Selection of detection wavelength

The selection of wavelength depends on absorbance of the drug. A UV spectrum of methanol was recorded by scanning from 190 to 400 nm using methanol as blank. From this spectrum λ_{max} at 227 nm was selected for the proper study.

2.1.6 Chromatographic conditions

The chromatographic separation was attained using C18 column (Phenomenex kinetex 5 µ C18 100A 250 X 4.6 mm). The UV wavelength was set at 227 nm for quantification of valethamate bromide. Isocratic elution was adopted with an injection volume of 10 µL and flow rate of 1 mL/min. The mobile phase consists of phosphate buffer (pH 7.5) and acetonitrile (50:50 v/v). The contents of mobile phase were filtered before use through membrane filter (0.45 µ).

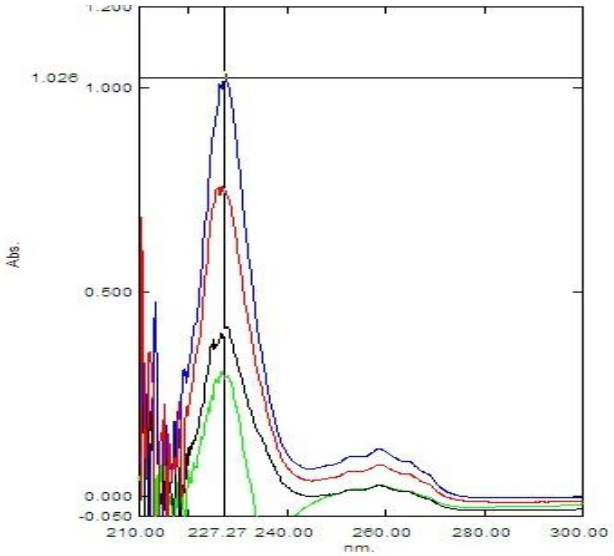

Fig. 2. Overlay spectra of valethamate bromide

2.2 Preparation of Solutions

2.2.1 Preparation of buffer solution

Accurately weighed 16.8 g of potassium dihydrogen phosphate was transferred into 1000 mL volumetric flask, 100 to 150 mL of Millipore water was added and mixed well with intermittent shaking and the volume was made up to the mark with same water (pH 7.5±0.05). The solution was then filtered through 0.45 micron filter.

2.2.2 Preparation of mobile phase

Mobile phase composed of acetonitrile and potassium dihydrogen phosphate buffer pH 7.5 (50:50). Measured 500 mL of acetonitrile and 500 mL of potassium dihydrogen phosphate buffer (pH 7.5) into a 1000 mL volumetric flask, mixed well with intermittent shaking, sonicated and filtered through 0.45 micron filter.

2.2.3 Preparation of standard stock solution

A stock solution of 1000 µg/mL was prepared by weighing 100 mg of valethamate bromide into 100 mL volumetric flask. To this 10 mL of HPLC grade acetonitrile was added to dissolve and the volume was made upto the mark using millipore water. The solution was filtered through 0.2 µ syringe filter and stored at 4ºC.

2.2.4 Preparation of solutions for linearity

From the stock solution (1000 µg/mL) 1 mL is transferred into a 100 mL flask and diluted with diluent to get a sub stock of 100 µg/mL. From this 100 µg/mL solution 0.2, 0.4, 0.6, 0.8, 1 and 1.2 mL were pipetted out separately into 10 mL volumetric flask, then diluted up to the mark with millipore water to get 2, 4, 6, 8, 10 and 12 µg/mL solution. Triplicate injections were injected for each solution and were chromatographed under the chromatographic conditions specified. Drug peak areas and concentration were plotted for each drug and linearity was achieved. The calibration graph of valethamate bromide was shown to be Fig. 3.

2.2.5 Assay sample preparation

Average weight is calculated by weighing 20 tablets (Epidosin). The tablets were crushed into homogenous powder. A quantity of powder equivalent to one tablet containing 10 mg of valethamate bromide was transferred into a 100 mL volumetric flask. To this 10 mL of acetonitrile was added and sonicated for 20 minutes by shaking continuously. The solution was cooled to ambient temperature and volume was made up to the mark with Millipore water to get 100 µg/mL solutions and sonicated for 15 minutes. The solution was filtered using 0.45 µm nylon filters. From the filtered solution, aliquots of appropriate volume were transferred to 10 mL volumetric flasks and diluted to volume with acetonitrile to furnish the concentration range listed in Table 1.

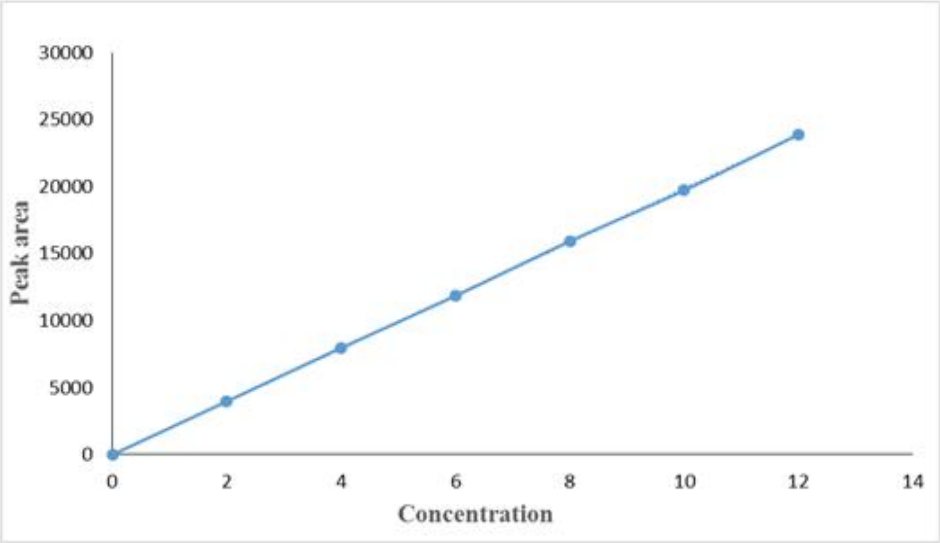

Fig. 3. Linearity graph

Table 1. Optimization of the UFLC method using different mobile phase

Trail no.	Mobile phase composition	Peak quality
1	Water (100)	Peak was not eluted
2	Water: Methanol (50:50)	Peak was not eluted
3	Potassium dihydrogen phosphate buffer (pH 7): ACN (80:20)	Peak was eluted at 7.5 min, with a broad peak. Hence to improve the peak shape pH of the buffer was changed
4	Potassium dihydrogen phosphate buffer (pH 7.5): ACN (80:20)	Peak was eluted at 9 min and the shape of the peak was good but the retention time was long. To reduce the retention time the ratio of the mobile phase was changed in the next trays
5	Potassium dihydrogen phosphate buffer (pH 7.5): ACN (50:50)	Peak was eluted at 4.4 min with a good shape. The desired objectives were achieved and hence this is considered as an optimised method.

2.3 Method Validation

2.3.1 Linearity

The linearity of the method was established by taking a series of concentration mixtures of valethamate bromide (2-12 µg/mL). A graph of concentration versus peak area was plotted. The regression line obtained was linear. From the data obtained, co-relation coefficient, slope and Y-intercept were calculated.

2.3.2 Accuracy

Accuracy was performed at levels (50%-150%) for valethamate bromide using standard addition method. A known quantity of sample was spiked with the standards. Samples were analysed in triplicate for each level. From the results % recovery was calculated.

2.3.3 Precision

The precision of the method was assessed by studying intra-day and inter-day variation. In the intra-day studies, standard and sample solutions were analysed in triplicate on the same day and percentage relative standard deviation (RSD) was calculated. In the inter-day studies, standard and sample solutions were analysed in triplicate on five consecutive days and percentage RSD were calculated.

2.3.4 Limit of detection (LOD) and Limit of quantification (LOQ)

LOD and LOQ were calculated by using the following formula LOD = 3.3(SD)/S and LOQ = 10(SD)/S, where SD = standard deviation of response and S = slope of the calibration curve. The values obtained are given in Table 6.

2.3.5 Robustness

Robustness is a measure of capacity of analytical methods to remain unaffected by small but deliberate variation of the operating conditions. This was tested of by studying the effect of by small but deliberate variations in method parameters such as column temperature (± 0.5ºC), pH (± 0.2%), flow rate (± 0.1 mL) and wavelength (± 2 nm). The temperature of the column was increased to 25.5ºC and decreased to 24.5ºC from the standard 25ºC. The flow rate of the mobile phase was increased to 1.1 mL/min and decreased to 0.9 mL/min from 1.0 mL/min. The wavelength was varied from 227 nm to 225 nm and 229 nm. The final variation was done by changing the pH to 7.3 and 7.7 from 7.5.

2.3.6 System suitability

Five replicate injections of standard solutions were injected and the chromatograms were recorded. The system is suitable for analysis if the theoretical plates in 5 replicate injections should be not less than 2000.

2.4 Forced Degradation Studies

2.4.1 Preparation of solutions for degradation

2.4.1.1 Preparation of 0.1N Hydrochloric acid solution

Measured accurately 4.25 mL of concentrated HCl (33%) analytical grade into a 500 mL volumetric flask and diluted up to the mark with

Millipore water mixed thoroughly, sonicated and filtered through 0.45 µ filter.

2.4.1.2 Preparation of 0.1N Sodium hydroxide solution

Accurately weighed 2 gm of NaOH into 500 mL volumetric flask and 50 mL of Millipore water was added to the flask and sonicated to dissolve. The resulting solution was diluted with milliporewater and filtered through 0.45 µ filter.

2.4.1.3 Preparation of 3% Hydrogen peroxide solution

Measured accurately 50 mL of 30% H_2O_2 into 500 mL volumetric flask and diluted up to the mark with milliporewater mixed thoroughly, sonicated and filtered through 0.45 µ filter.

2.4.2 Preparation of samples for degradation studies

2.4.2.1 Acid degradation sample

1 mL of valethamate bromide was pipetted out from 1000 µg/mL sample solution in a 10 mL volumetric flask, 1 mL of 0.1N HCl was added and kept aside for 24 hrs. It was then neutralised using 0.1N NaOH and volume was made upto 10 mL using diluent. Further dilutions were made to obtain a concentration of 4 µg/mL.

2.4.2.2 Base degradation sample

1 mL of valethamate bromide was pipetted out from 1000 µg/mL sample solution in a 10 mL volumetric flask, 1 mL of 0.1N NaOH was added and kept aside for 24 hrs. It was then neutralised using 0.1N HCl and volume was made upto 10 mL using diluent. Further dilutions were made to obtain a concentration of 4 µg/mL.

2.4.2.3 Oxidative degradation sample

1 mL of valethamate bromide was pipetted out from 1000 µg/mL sample solution in a 10 mL volumetric flask and was stressed with 3%H_2O_2 and kept aside for 24 h and after which the volume was made up to 10 mL using diluent. Further dilutions were made to obtain a concentration of 4 µg/mL.

2.4.2.4 Thermal degradation sample

Powdered tablet equivalent of 10 mg was taken and kept in oven at 70ºC for 24 h. Then after 24 h, the powder was transferred to a 10 mL volumetric flask and made the volume with diluent. The solution was filtered and further diluted to obtain a concentration of 4 µg/mL.

The following formula was used for calculation of % degradation

$$\%\ degradation = \frac{\%assay\ of\ untreated\ sample - \%assay\ of\ degraded\ sample}{\%assay\ of\ untreated\ sample}$$

3. RESULTS AND DISCUSSION

In the present study we developed a simple and sensitive analytical stability indicating UFLC method for the valethamate bromide in pure and pharmaceutical dosage form.

3.1 Optimization of the Mobile Phase

Based on sample solubility, stability, suitability, various mobile phase compositions were tried to achieve good separation with sharp peaks. Due to difficulty in separation, insufficient number of theoretical plates various ratio of different organic solvents like acetonitrile and methanol were being tried in which gradual proportions of acetonitrile was used. The potassium dihydrogen phosphate buffer was used. The different chromatographic conditions such as mobile phase, temperature, flow rate, and solvent ratio were maintained for the drug. The resulting chromatograms were recorded and the chromatographic parameters were calculated. Table 1 tabulates the various trays by using mobile phase of different solvent composition.

3.2 Method Validation

3.2.1 Linearity and range

The linearity for valethamate bromide was checked in the concentration range of 2-12 µg/mL respectively. The concentration ranges were found to be linear when a graph was plotted with peak area against concentration. The acceptance criterion for linear range is that the regression co-efficient value should not be less than 0.9991 with regression equation Y= 1836.3x+ 295.76 were a. x is independent variable ie concentration and y dependent variable ie. area. The results of regression parameters are shown in Table 2.

Table 2. Regression parameters

Parameters	Valethamate bromide
Linearity Range	2-12 µg/mL
Regression equation	Y= 1836.3x+ 295.76
Regression coefficient (R^2)	0.9991

3.2.2 Accuracy

The Individual % recovery should be between 97.0 and 103.0. The average % recovery of each level should be between 98.0 and 102.0 and % RSD for recovery at each level should not be more than 2.0. The % recovery of valethamate bromide was found to be 98.86%, 101.46% and 100.03%. The values were found to be within the acceptable limits. The Table 3 provides results of recovery study.

3.2.3 Precision

3.2.3.1 System precision

The system precision is to ensure that the analytical system is working properly towards the selected method. It is done by injecting the six samples and checking the reproducibility in retention time and the area of the samples. The results of system precision are given in the Table 4.

Table 3. Accuracy data of the method

Name of the drug	Actual concentration (µg mL^{-1})	Intra-day			Inter-day		
		Found concentration (µg mL^{-1}) ± SD	RSD (%)	Mean % recovery	Found concentration (µg mL^{-1}) ± SD	RSD (%)	Mean % recovery
Valethamate bromide	2	1.93 ± 0.03	0.618	98.96	1.92 ± 0.072	0.688	98.82
	4	3.97 ± 0.06	0.829	101.46	3.96 ± 0.081	1.103	99.97
	6	5.99 ± 0.13	1.364	100.03	5.98 ± 0.181	1.433	99.94

S. no.	Actual concentration (µg/ mL)	Standard (µg/ mL)	Amount Found ± SD (µg/ mL)	% RSD	Mean % recovery
1	2	4	5.93 ± 0.03	0.618	98.86
2	4	4	8.03 ± 0.06	0.829	101.46
3	6	4	10.00 ± 0.13	1.364	100.03

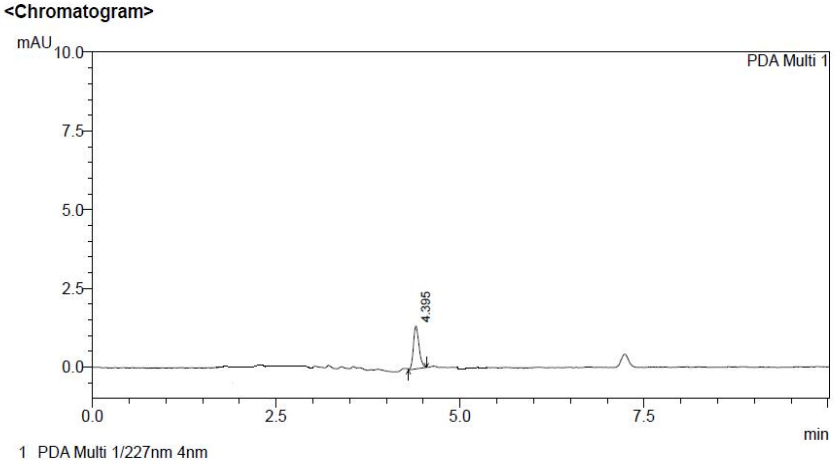

Fig. 4. Chromatogram of sample solution

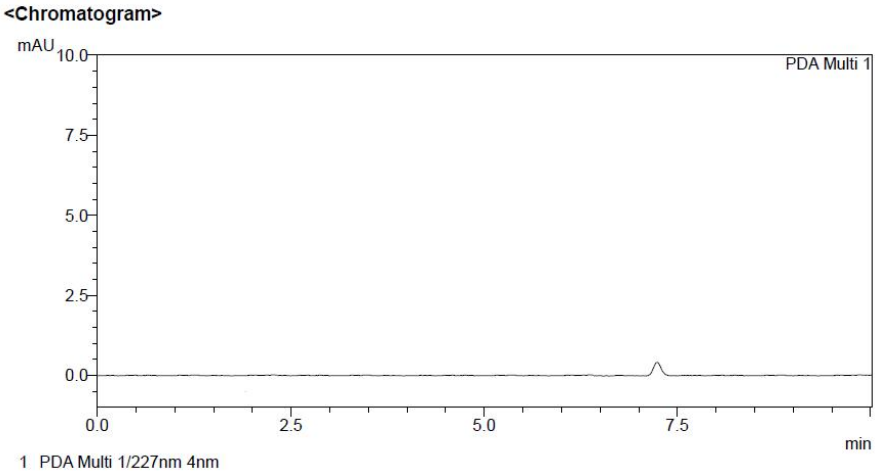

Fig. 5. Chromatogram of blank

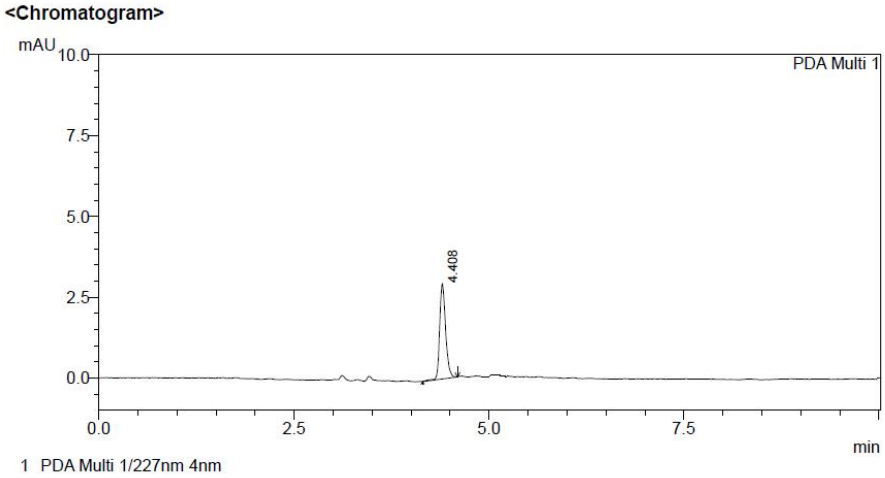

Fig. 6. Chromatogram of standard valethamate bromide

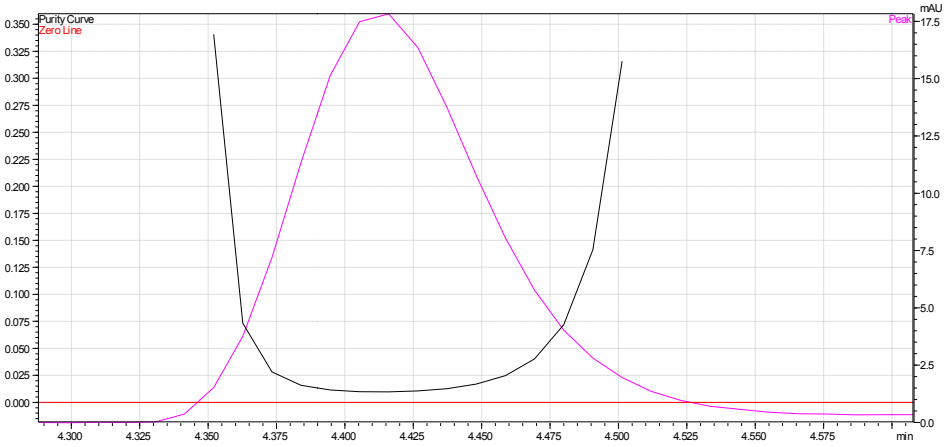

Fig. 7. Peak purity of valethamate bromide

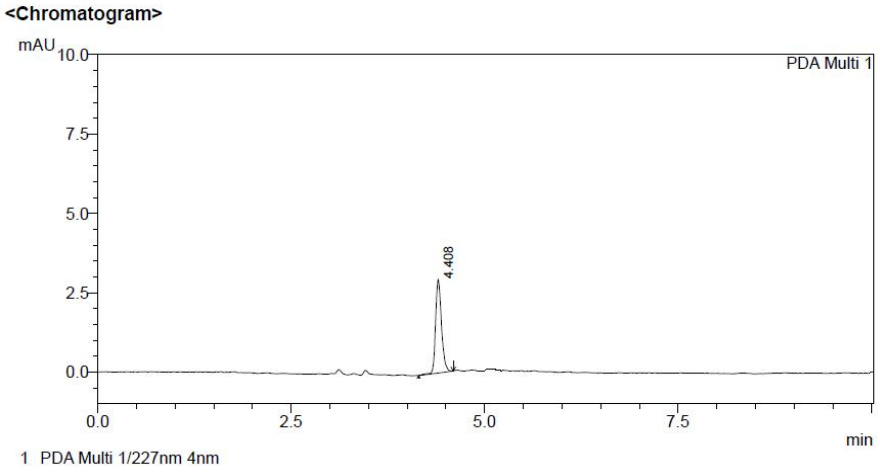

Fig. 8. Chromatogram of unstressed sample

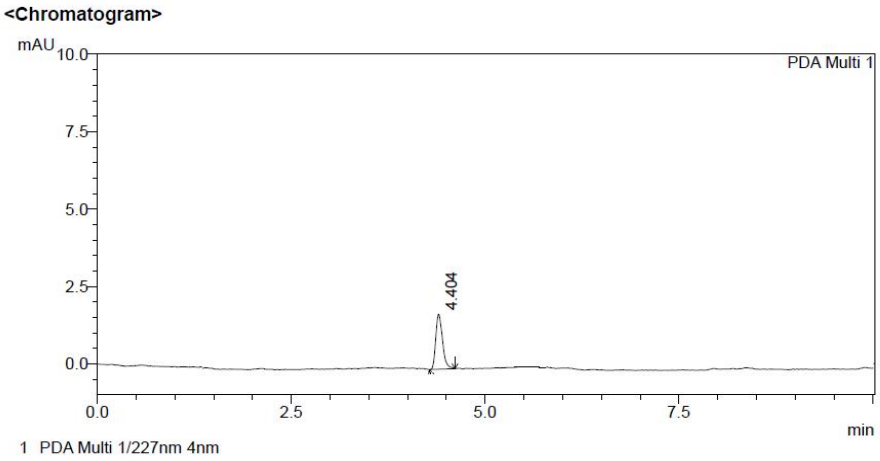

Fig. 9. Chromatogram of acid stressed sample

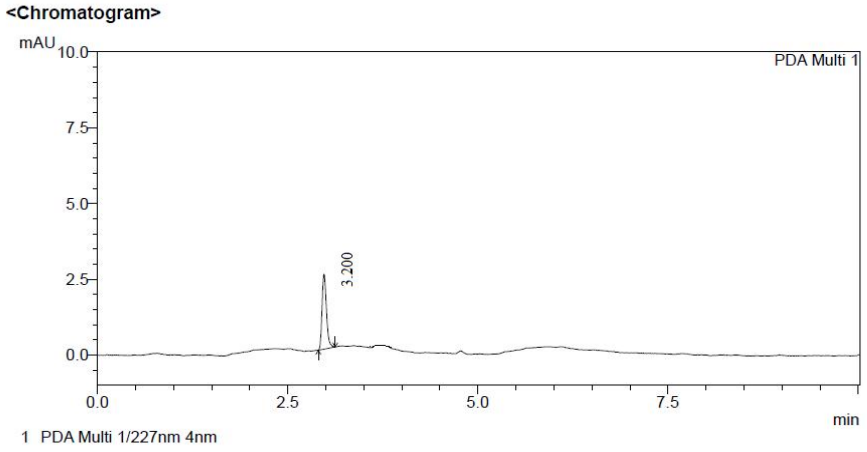

Fig. 10. Chromatogram of alkali stressed sample

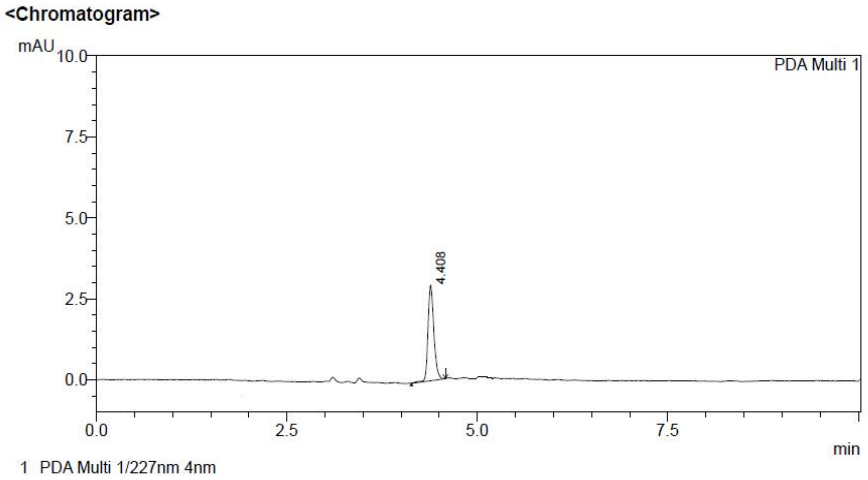

Fig. 11. Chromatogram of oxidative stressed sample

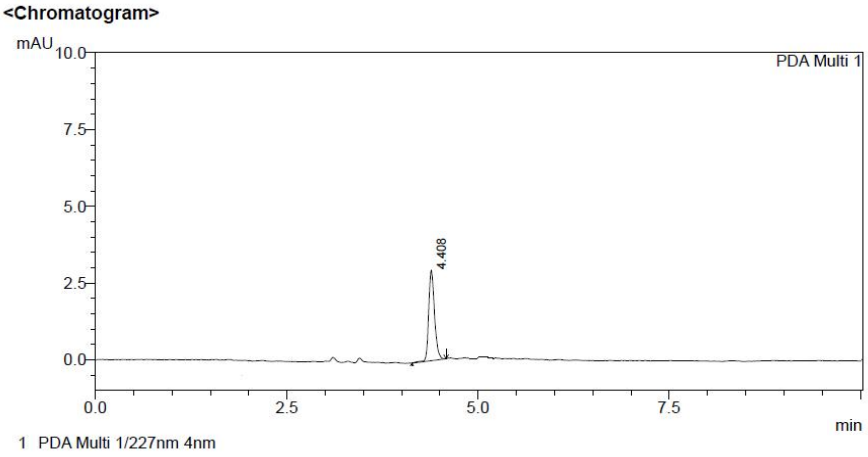

Fig. 12. Chromatogram of thermal stressed sample

Table 4. System precision of the method

Injection number	Peak area
1	7940
2	7968
3	7850
4	7972
5	7870
6	7907
Average	7917.83
RSD	0.0064
%RSD	**0.64**

The method was found to be precise with % RSD of valethamate bromide at 0.64%. The values were found to be within the acceptable limits. The interday and intraday precisions were performed on standard solutions and the results were tabulated as given Table 5. The %RSD of valethamate bromide for inter-day and intra-day precisions were found to be within the acceptable limits.

3.2.4 Limit of detection and limit of quantification

Limit of Detection is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions.

Limit of Quantitation is the lowest amount of analyte in a sample that can be quantitated with acceptable accuracy and precision, under the stated experimental conditions. The LOD and LOQ values were calculated using the equation method. The results of LOD and LOQ are given in Table 6.

Table 5. Method precision

S. no	Amount of drug taken (µg/mL)	Intraday			Interday		
		Peak area	Mean peak area	% RSD	Peak area	Mean peak area	% RSD
1		7943			7967		
2		7965			7990		
3	4	7989	7961	0.23	7965	7985	0.45
4		7974			7999		
5		7940			7982		
6		7956			7998		

Table 6. LOD and LOQ results

Drug	LOD (µg/mL)	LOQ (µg/mL)
Valethamate bromide	0.540	1.620

3.2.5 Robustness

To ensure the insensitivity of the HPLC method to minor changes in the experimental conditions it is important to demonstrate robustness of the method. The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters. The robustness was checked by varying different parameters like composition of mobile phase, wavelength, and column oven temperature and by different analysts and at different days. None of the modifications caused a significant change in the resolution between the drugs and IS, peak area RSD, USP tailing factor, peak width or theoretical plates.The method was found to be robust and rugged and the values of robustness are given Table 7.

3.2.6 System suitability

The results of system suitability parameters of valethamate bromide by UFLC method is given in the Table 8.

4. FORCED DEGRADATION STUDY

A study was conducted to demonstrate the effective separation of degradants from valethamate bromide peak in assay method. Separate portions of drug product and placebo

Table 7. Results for robustness of valethamate bromide

Valethamate bromide				
Condition		**Tailing**	**Theoretical plates**	**%RSD**
Optimized method		**1.308**	**12343.9**	**---**
Mobile phase ratio	48:52	1.307	13468.8	0.97
(ACN: Buffer)	52:48	1.452	13102.658	1.01
Buffer pH	Decreased (-0.5 units)	1.379	13474.437	0.59
	Increased (+0.5 units)	1.346	13894.356	1.11
Flow rate	Decreased (-0.1 mL/min)	1.385	13174.152	1.43
	Increased (+0.1 mL/min)	1.352	12506.373	0.99
Column temperature	Decreased (-5^0 C)	1.379	12291.447	1.33
	Increased ($+5^0$ C)	1.407	12886.440	1.78
Wavelength	Decreased 2nm	1.302	12354.84	0.44
	Increased 2nm	1.306	12358.99	1.25

Table 8. Results of system suitability

System suitability parameters	Observed value	Acceptance criteria
Tailing factor	1.308	NMT 2.0
% RSD	0.36	NMT 2.0
Plate count	12343.4	NLT 2000

All the values of the system suitability parameters were found to be within the acceptable limits

Table 9. Peak purity results from forced degradation studies

Stress conditions	Peak area		% degradation
	Unstressed sample	Stressed sample	
Degradation in acid	20524	14778	28.1
Degradation in base	20524	19149	6.7
Oxidative degradation	20524	17651	14
Thermal stress	20524	20409	0.56

were exposed to the following stress conditions to induce degradation. Stressed samples were analyzed as per test method with photo diode array detector. The chromatograms of the stressed samples of valethamate bromide were tested for purity of peak using LC Solutions. The peaks shape was not changed and no additional peaks were observed whereas peak area is altered. When subjected to acid, alkali, thermal and UV there is no change in RT except when subjected to alkali there is change in RT the drug is eluted at 3.2 min. This shows that there is no interference of main peak with degradants in quantification of the valethamate bromide in Tablets. The results are shown in Table 9. The chromatograms of stressed samples are compared with unstressed sample and purity peaks are shown in Fig. 7.

5. CONCLUSION

No method has been developed for the estimation of hence; an attempt has been made to develop a novel method for analyzing, valethamate bromide in pure drug and in tablet dosage form by ultra-fast liquid chromatography. Simple and accurate method has been developed for the estimation of valethamate bromide in tablet dosage form and was used to carry out forced degradation studies. Results of analysis of the samples revealed that the proposed method is suitable for the analysis and recovery was found to be acceptable. A system suitability test was established and related parameters were recorded. Hence this method stands validated and can be used for routine analysis in quality control laboratories.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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