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Method Development and Validation of A Novel Anti-Depressant Bupropion by RP-HPLC

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A B S T R A C T

A speedy, simple and precise RP-HPLC process was developed for the estimation of novel antidepressant drug bupropion with Waters X – Bridge C-18 5µm, 4.6 X 150 mm columnusing mobile phase Acetonitrile: Ammonium bicarbonate (5mM) pH-9 adjusted with 1% Ammonium hydroxide (50:50, %v/v).The flow rate was 1 ml/min and quantification was done by PDA detector at wavelength254nm.The Bupropion eluted from the column in 5.194 min. The validation was carried out in the light of ICH guidelines with respect to parameters linearity, specificity, accuracy, limit of detection (LOD) and limit of quantification (LOQ). The proposed method showed linearity in the concentration range of 50 to 250 ppm for Bupropion. The linear regression equation of Bupropion was found to be y = 6E+06x + 91344 and correlation coefficient value was found to be 0.997 indicating a high degree of linearity for the drug. The limit of detection (LOD) of bupropion was 0.5 ppm and limit of quantification (LOQ) was 2.0 ppm. The low values of %recovery and %C.V. showed that the method is precise within the acceptance limit of 5% (according to ICH guidelines).

Key words: Bupropion, RP-HPLC, PDA detector, ICH

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INTRODUCTION

Bupropion was first patented in 1974[1] and released onto the world market in 1985. It was briefly withdrawn due to seizures incidences but reintroduced in 1989 after the daily recommended dose was reduced to lower seizure likelihood. Bupropion is a dopamine and norepinephrine reuptake inhibitor [2].It is about twice as potent an inhibitor of dopamine reuptake than of norepinephrine reuptake. Besides reuptake inhibition of dopamine and noradrenaline, bupropion also causes the release of dopamine and noradrenaline[3]. Bupropion has numerous therapeutic indications including, depression[4] smoking cessation[5], sexual dysfunction[6], obesity[7], attention deficit hyperactivity disorder[8] and seasonal affective disorder[9]. It has recently been shown to have anti-inflammatory properties[10]. In 2007 it was the fourth-most prescribed antidepressant in the USA. Bupropion is the water soluble hydrochloride salt of an aminoketone[11], with a pKa of 7.9[12]. It is also known with the generic name of amfebutamone hydrochloride. Bupropion is a second-generation antidepressant agent that is also used in the management of smoking cessation [13]. CYP2B6 is a polymorphic hepatic enzyme[14]of potential importance in the metabolism of drugs such as Bupropion[15], efavirenz[16] and cyclophosphamide[17]. Wide interindividual variability in the hepatic expression of CYP2B6 has been reported, In humans, bupropion is extensively metabolized to three principal metabolites (Fig.1.) such as hydroxyl-bupropion or morphinol, erythrohydrobupropion, and threo-hydro-bupropion.The pharmacologically active metabolite hydroxyl-bupropion appears to be the major metabolite, since the plasma levels of hydroxybupropion greatly exceeds with respect to those of the parent drug. The cytochrome P450 (CYP) enzyme system, especially CYP2B6, has an important role in bupropion hydroxylation. Also product labeling have indicated that bupropion or hydroxybupropion inhibits CYP2D6. The the present study the in vitro hydroxylation of bupropion by the CYP enzyme system was investigated. CYP2B6 was identified to have the major role in hydroxybupropion formation. In addition, we have also investigated the possibility of CYP2D6 inhibition by bupropion or hydroxybupropion [15].

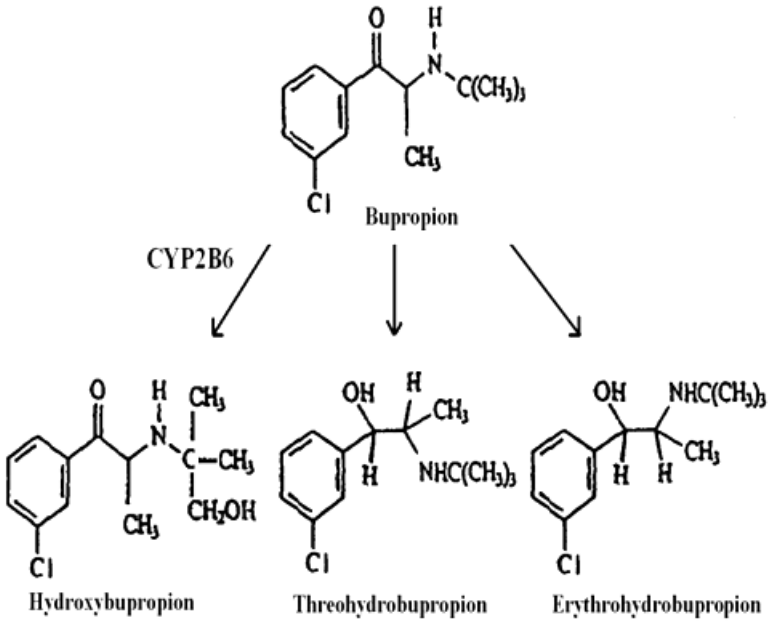

Figure: 1 Principal Metabolites of bupropion in humans

Analytical Method Validation

Method validation is defined as the process of defining and proving an analytical method acceptable for its intended use. Recent guidelines for methods development and validation for new noncompendial test methods are provided by the FDA draft document, "Analytical Procedures and Methods Validation Chemistry, Manufacturing, and Controls Documentation". In recent years, a great deal of effort has been devoted to the harmonization of pharmaceutical regulatory requirements in the United States, Europe, and Japan. As part of this initiative, the International Conference on Harmonization (ICH) has issued guidelines for analytical method validation[16].

Method validation is a continuous process. The goal is to ensure confidence in the analytical data throughout product development. Another challenge encountered early in the development of methods intended to support stability studies is ensuring that the method is stability indicating. This process is typically achieved by conducting forced-degradation studies. The design and execution of these studies requires thorough knowledge of the product being tested as well as a good understanding of the analysis technique[17].

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice. Analytical methods need to be validated or revalidated before introduction into routine use.

- Whenever the conditions change for which the method has been validated (e.g., an instrument with different characteristics or samples with a different matrix); and
- Whenever the method is changed and the change is outside the original scope of the method.
- When quality control indicates an established method is changing with time
- In order to demonstrate the equivalence between two methods (e.g., a new method and a standard).

INSTRUMENTATION

HPLC System	Alliance Waters e2695 Separations Module[1]
HPLC Pump	Waters 2774 pump[2]
HPLC Detector	Waters 2998 Photodiode Array Detector[3]
Software	Empower Pro[4]
Column	Waters X – Bridge C-18 5µm, 4.6 X 150 mm column[5]
Injector Loop	Rheodyne, Model No. 2767, Made in USA
Syringe	Waters (100 µl)
Balance	Electronic Balance Mettler Toledo
Sonicator	Ultrasonic Aarkey Labtronix Industries PVT Ltd India
pH meter	Thermo Electron Corporation (digital)
Centrifuge machine	Spinwin

CHEMICALS AND REAGENTS

Working standards of Bupropion were obtained from Analytical Testing Service (unit-II) Okhla New Delhi, India having purity >98%.

Table 1: Description of reagents

S.No.	Reagents	Manufacturer	Grade	Batch No.
1.	Acetonitrile	Sigma Aldrich	HPLC	MMBB2792
2.	Ammomium Bicarbonate	Merck Ltd, Mumbai, India	AR	MK6M552979
3.	Ammonium Bicarbonate	Merck Ltd, Mumbai, India	LR	MA1M610077
4.	Methanol	Sigma Aldrich	HPLC	MMBB2881
5	Formic acid	Sigma Aldrich	HPLC	94318-F
6	Triflouroacetic	Merck Ltd, Mumbai, India	HPLC	S6225762
7	Ammonium Hydroxide	Sigma Aldrich	HPLC	47626512-S
8	Sodium Hydroxide	Finar Reagent	AR	9652333-S3
9	Hydrochloric acid	Finar Reagent	AR	19085524
10	Hydrogen	Qualigens Pvt. Ltd.	AR	44273-F
11	Milli Q water	Millipore (India) Ltd. Bangalore	HPLC	

METHOD DEVELOPMENT[6]

Method Development for the Assay of Bupropion:

Chromatographic experiment:

Different chromatographic conditions were tried to optimize the method, which include the following:

Column: - X- BRIDGE C-8 3.5µ, 4.6 X 50 mm

Flow: - 1 ml/min

Detector U.V.:- 214 nm

Injection Volume: - 20µl

Run time:- 10 min

Column temp.:- 30^0C

Buffer:- 0.5 mm Ammonium acetate

Mobile Phase:- ACN : 0.5 mm Ammonium acetate (50:50 % v/v)

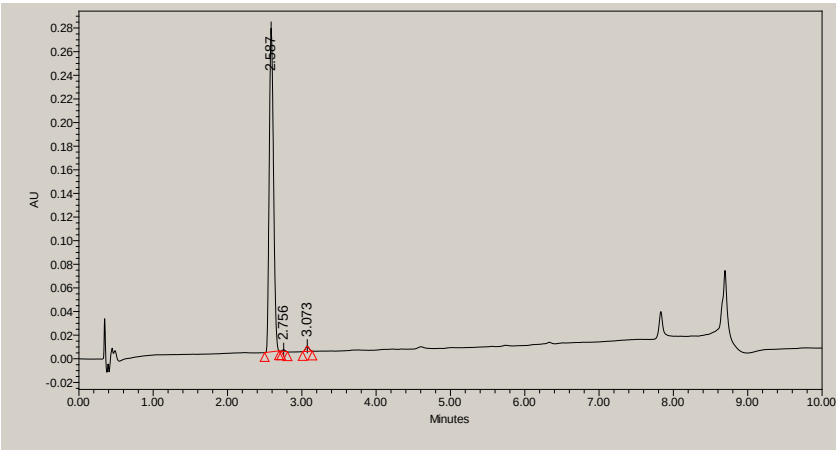

Figure: 2 Chromatogram of Trial-1

S.No.	RT	Area
1	2.507	1743456
2	2.758	149821
3	3.073	729856

Observation - In this condition Bupropion peak not eluted well so change the buffer.

Final Method for Assay of Bupropion

Preparation of Buffer

Accurately weigh and transfer about 450mg ammonium bicarbonate to 1000 ml water, dissolve and adjust the pH to 9.00 ± 0.05 with ammonium hydroxide solution (1%v/v). Filter through 0.45µ or finer porosity membrane filter.

Diluents

Use water: ACN (1:1) as diluent.

Standard stock solution of Bupropion

Accurately weigh and transfer about 10 mg of Bupropion working standard to a 100 ml volumetric flask. Add 50 ml of diluent, sonicate to dissolve and make up the volume with the same upto 100 ml. It become 100 ppm solution.

Optimised Chromatographic Conditions

Table: 2 Optimized Conditions

S. No.	Parameter	Optimized Condition
1	Instrument (HPLC)	Alliance Waters e2695 Separations Module
2	Column	Waters X – Bridge C-18 5µm, 4.6 X 150 mm column
3	Mode	Gradeint
4	Mobile phase	(ACN: Ammonium bicarbonate buffer (5mM) pH-9 adjusted with Ammonium hydroxide)
5	Column Oven temperature	30^0C
6	Flow rate	1 ml/min
7	Detector	Photodiode array
8	Sample tray temperature	Ambient room temperature
9	Detection wave length	214nm
10	Injection volume	3µL
11	Retention time (R_t)	5.191
12	Run time	10 min

Table: 3 Flow Gradient used during development

TIME (min)	FLOW ml/min	BICARBONATE	ACETONITRILE
00.00	1.00	50%	50%
1.00	1.00	50%	50%
6.00	1.00	10%	90%
8.5	1.00	10%	90%
9.00	1.00	50%	50%
10.00	1.00	50%	50%

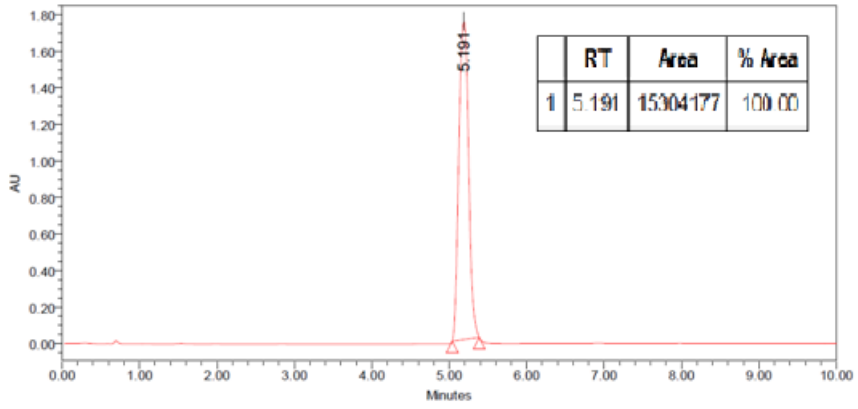

Figure: 3 Chromatogram shows the response of Bupropion after analytical method development.

Purity Data

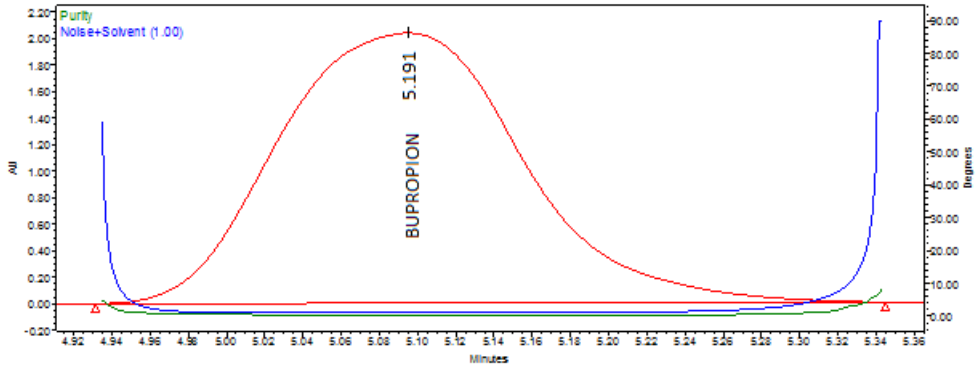

Figure: 4 Purity plot of Bupropion after development

Table: 4 Purity data

Name	Retention Time (Min)	Purity Angle	Purity Threshold	% Area	Height
Bupropion	5.191	0.257	1.025	100%	700956 µV

METHOD VALIDATION[7]

Specificity[8]

Specificity of the method was done by comparing the chromatogram of drug with the chromatogram of blank (mobile phase). The chromatograms of blank, and drugs are given below:

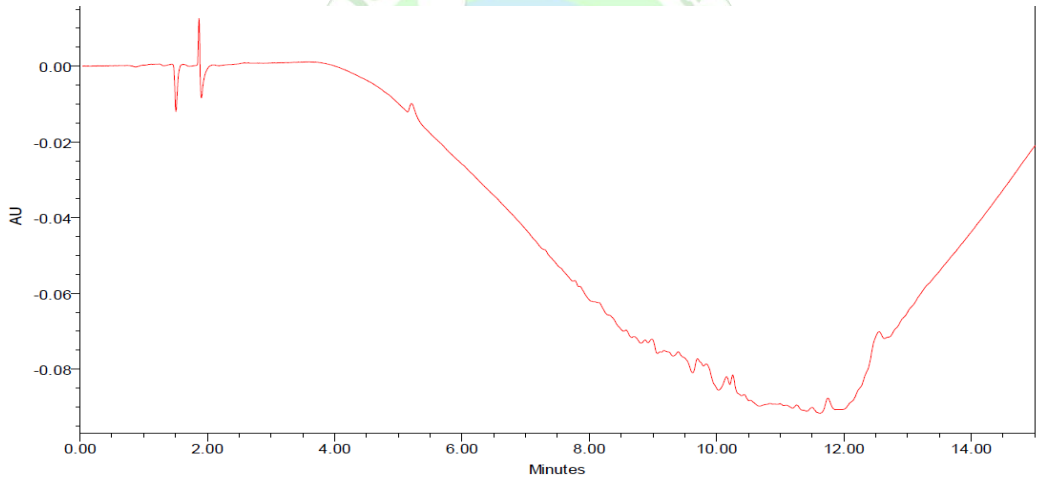

Figure: 5 Chromatogram of Blank (Mobile Phase)

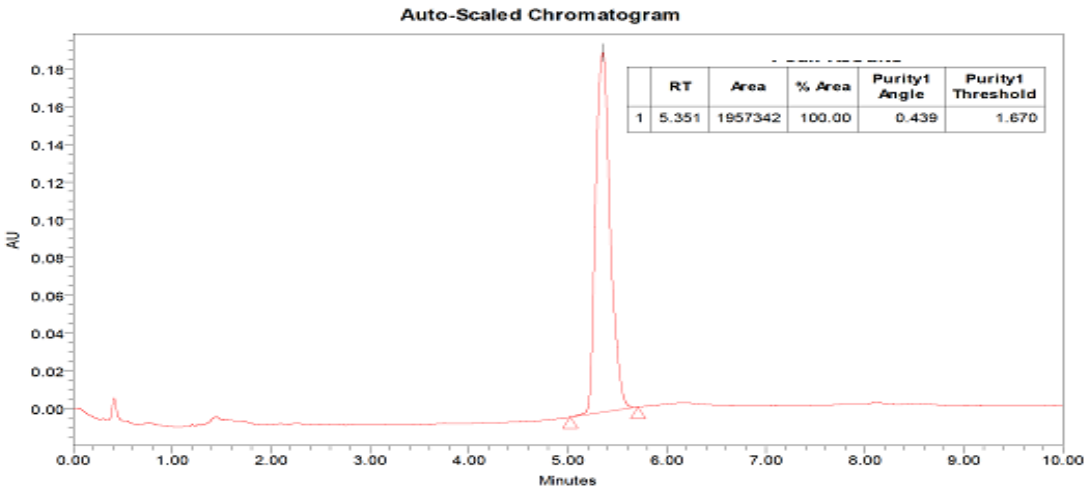

Figure: 6 Chromatogram of Bupropion

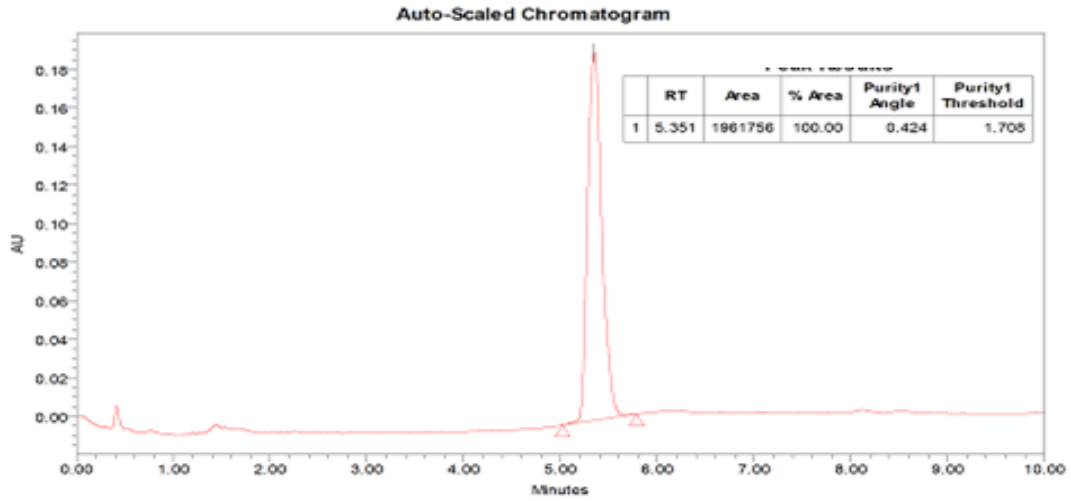

Figure: 7 Chromatogram of standard sample of Bupropion

LINEARITY[9]

Six different concentration of the drug were prepared for linearity studies. Response was measured as peak area. The calibration curve was obtained by plotting peak area (mv.s) against concentration (µg/ml). The standard curves of all three drugs in the mixture obtained from analysis at 1st, 3rd and 8th days are given below:

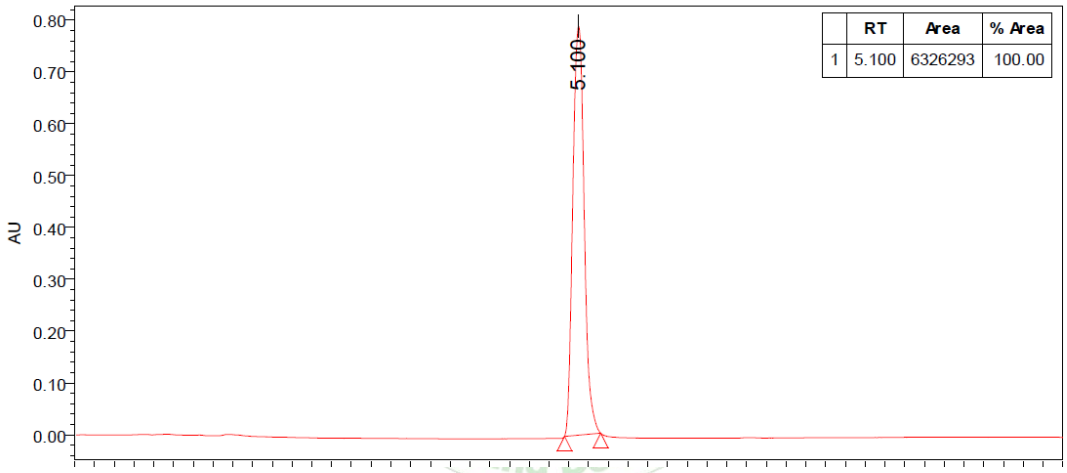

Figure: 8 Chromatogram of 50 ppm concentration of Bupropion

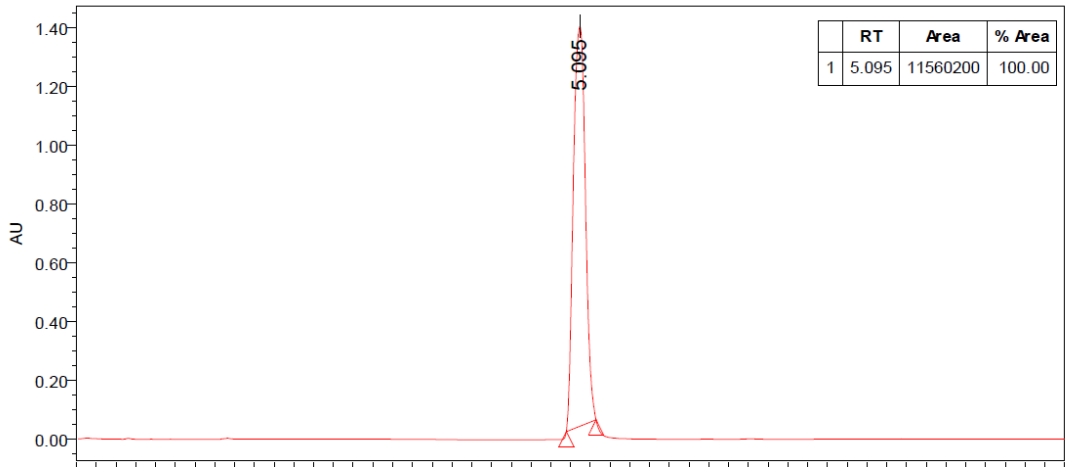

Figure: 9 Chromatogram of 100 ppm concentration of Bupropion

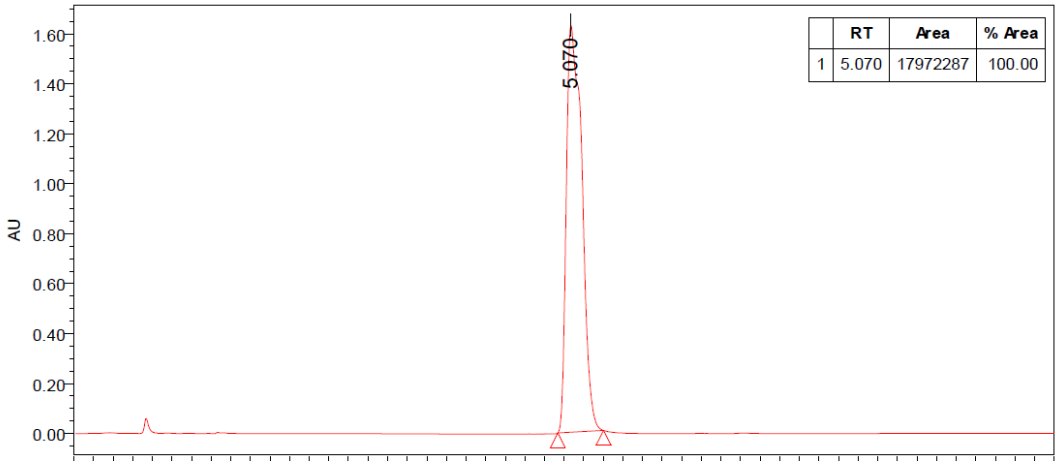

Figure: 10 Chromatogram of 150 ppm concentration of Bupropion

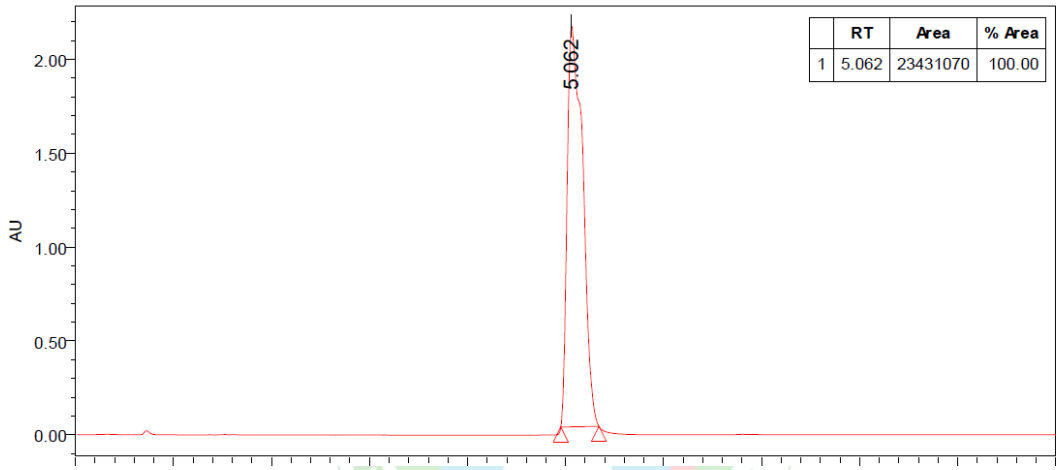

Figure: 11 Chromatogram of 200 ppm concentration of Bupropion

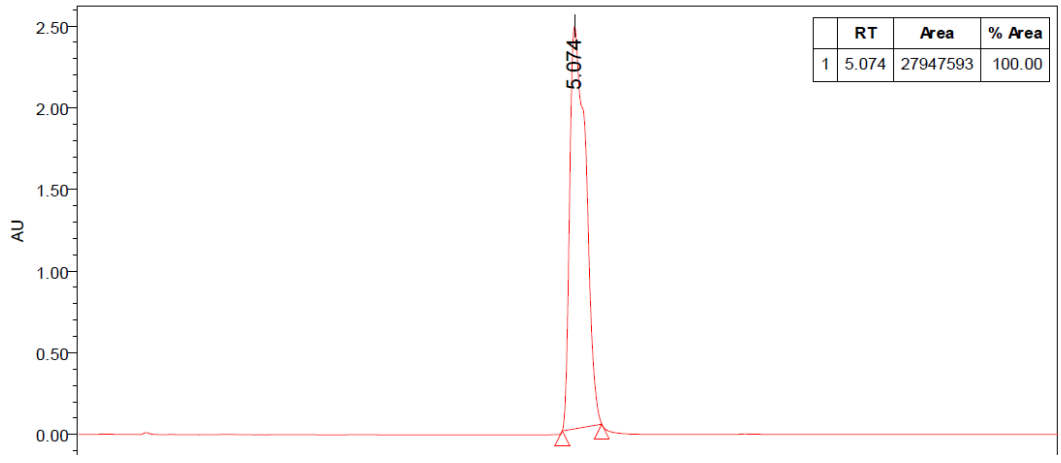

Figure: 12 Chromatogram of 250 ppm concentration of Bupropion

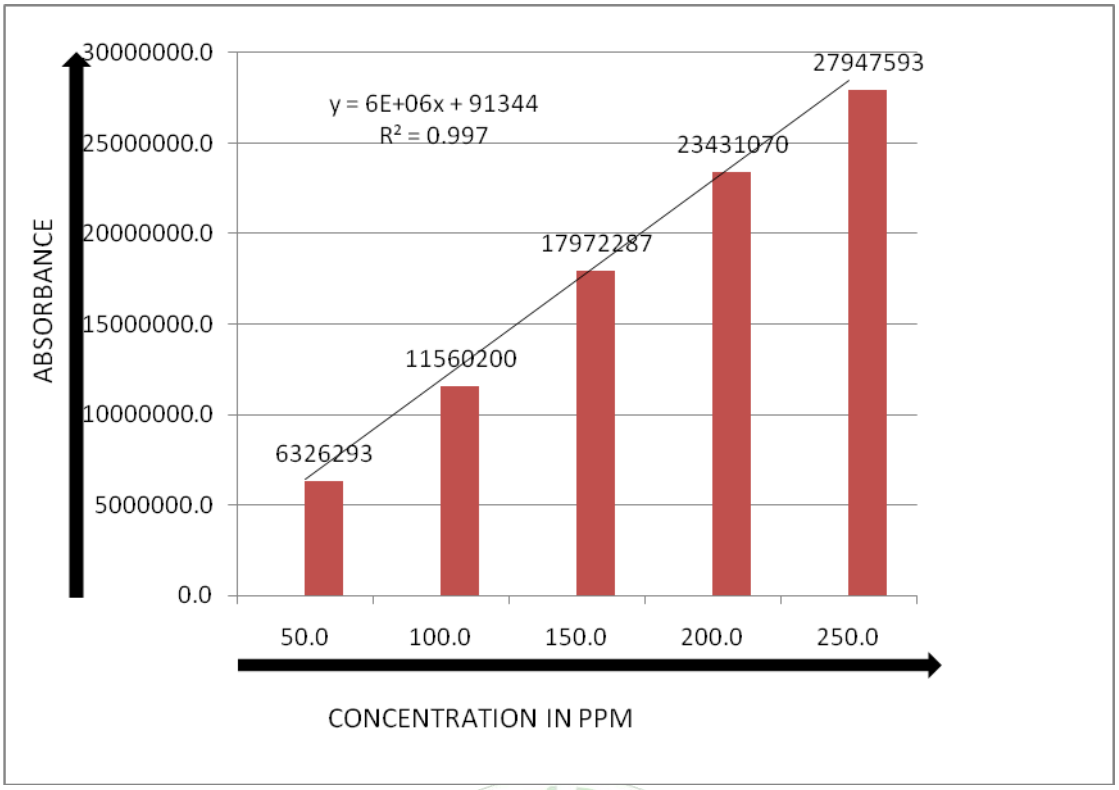

Figure: 13 Linearity graph b/w absorbance & concentration

Table: 5 Linearity data

CONC IN PPM	Area	$_R2$
50.0	6326293	0.997
100.0	11560200	
150.0	17972287	
200.0	23431070	
250.0	27947593	

Analysis of Quality Control Solutions

Quality control solutions for the Bupropion in the concentration range of low (50ppm), medium (100ppm), high (250ppm) was prepared and three injections of each concentration was made quality control. The purpose of quality control solutions was to check the performance of the instrument before analysis of test solution, or to confirm whether the instrument gave constant results or not, by comparing the data of standard solutions with that of quality control solutions.

Chromatogram of low concentration (50 ppm solution) of Bupropion

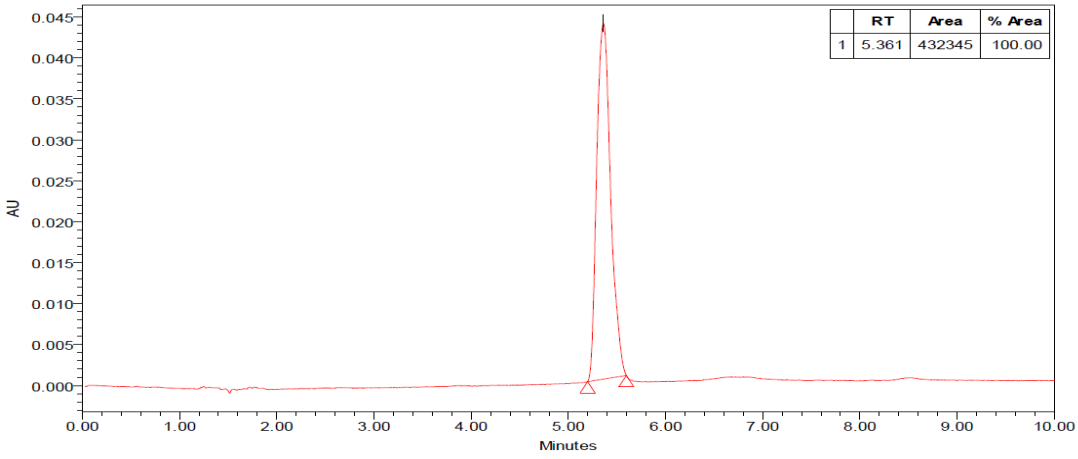

Figure: 14 Chromatogram of I injection of 50 ppm solution

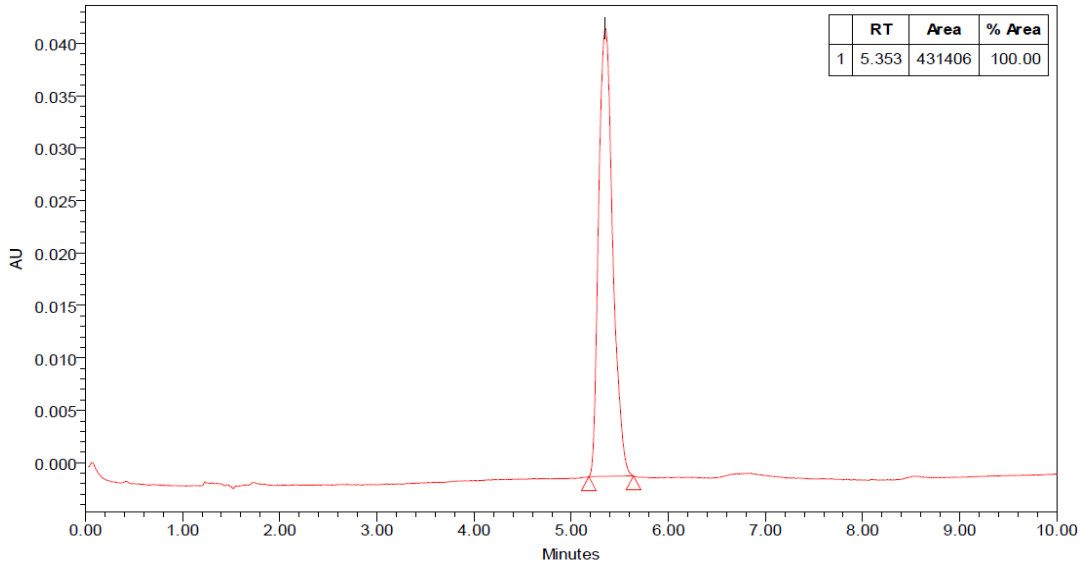

Figure: 15 Chromatogram of II injection of 50 ppm solution

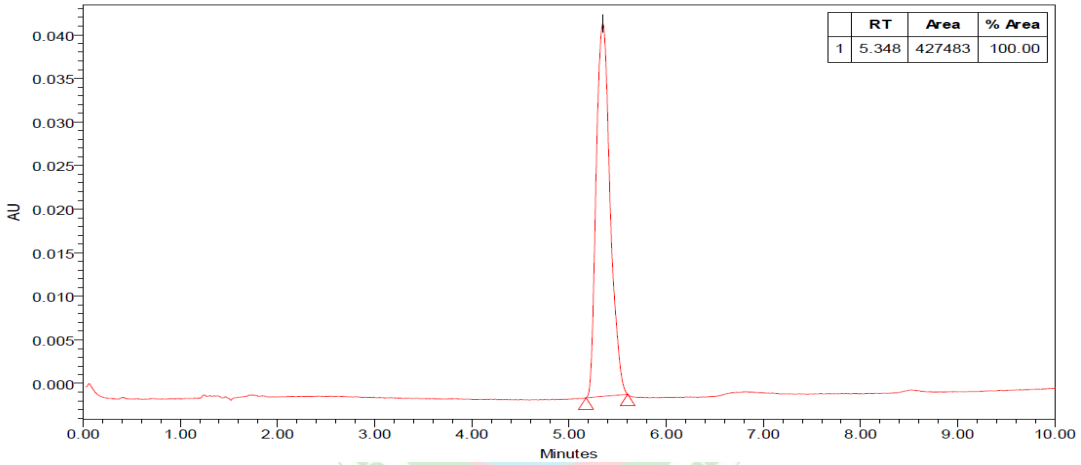

Figure: 16 Chromatogram of III injection of 50 ppm solution

Chromatogram of low concentration (150 ppm solution) of Bupropion

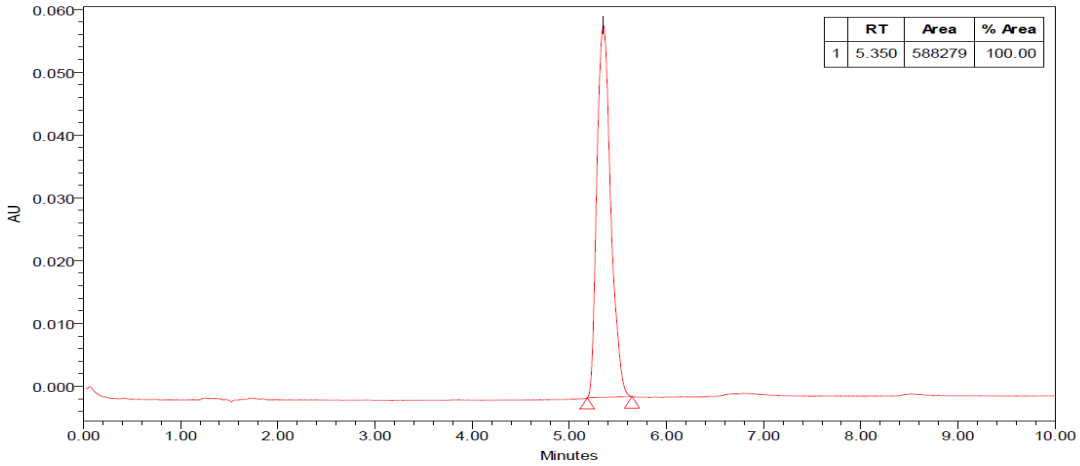

Figure: 17 Chromatogram of I injection of 150 ppm solution

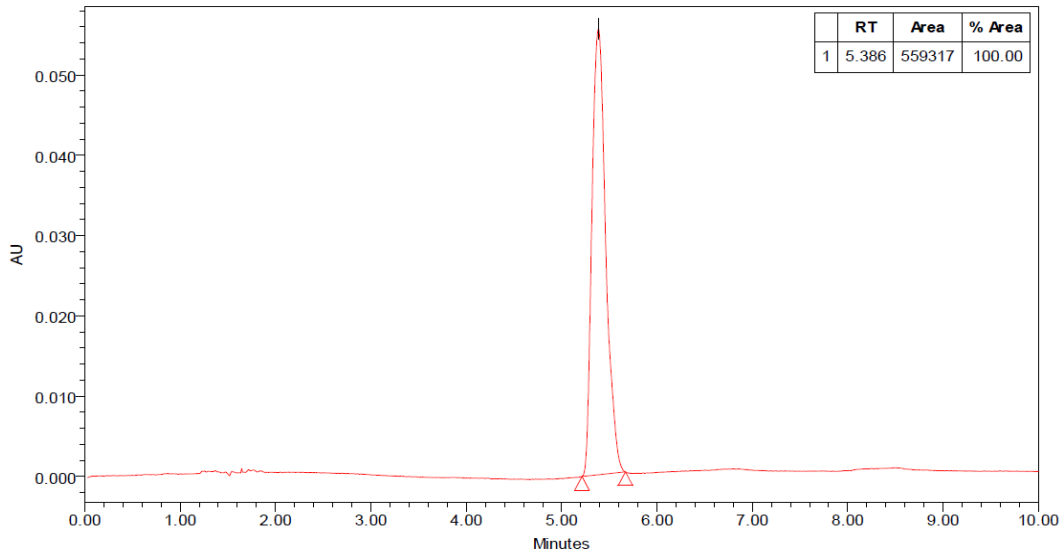

Figure: 18 Chromatogram of II injection of 150 ppm solution

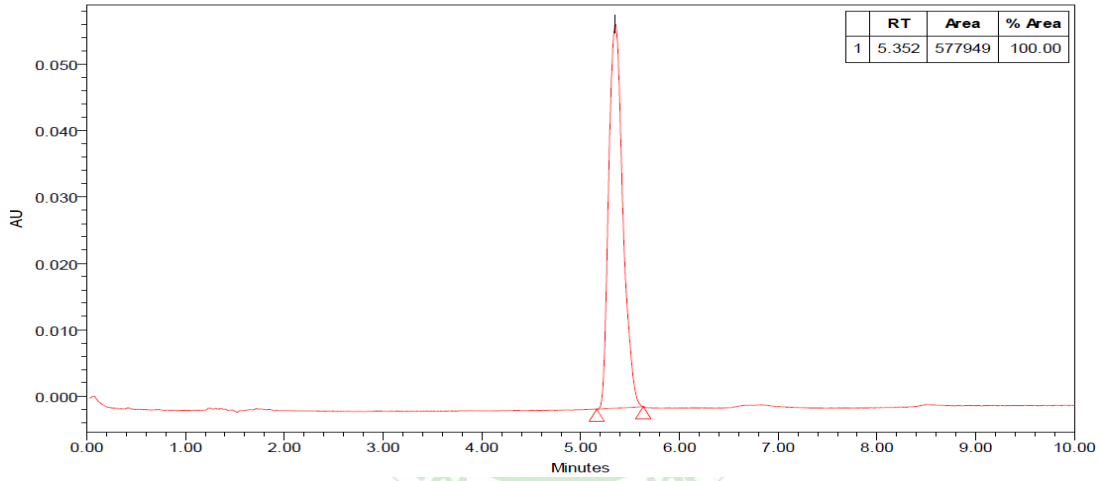

Figure: 19 Chromatogram of III injection of 150 ppm solution

Chromatogram of low concentration (200 ppm solution) of Bupropion

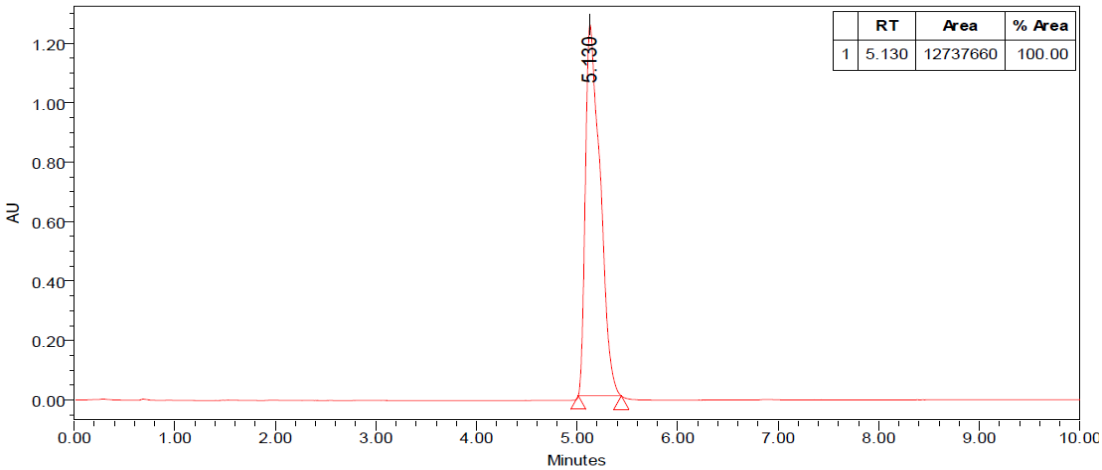

Figure: 20 Chromatogram of I injection of 200 ppm solution

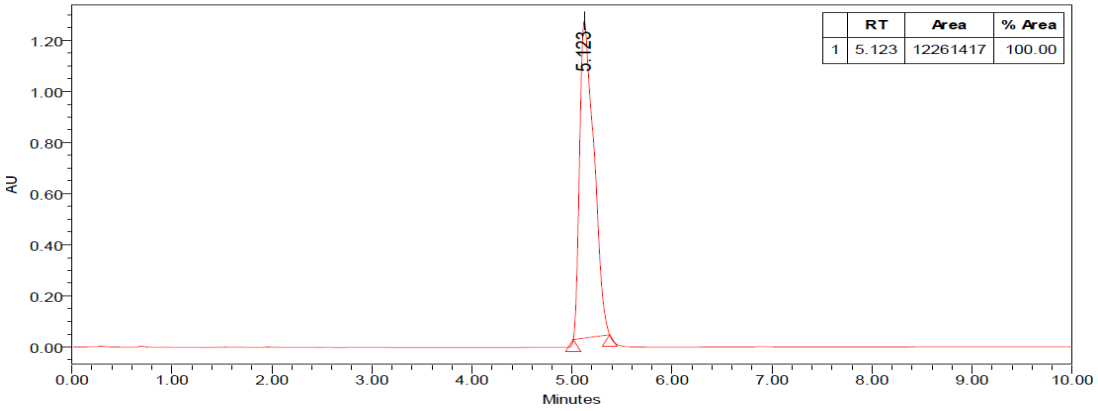

Figure: 21 Chromatogram of II injection of 200 ppm solution

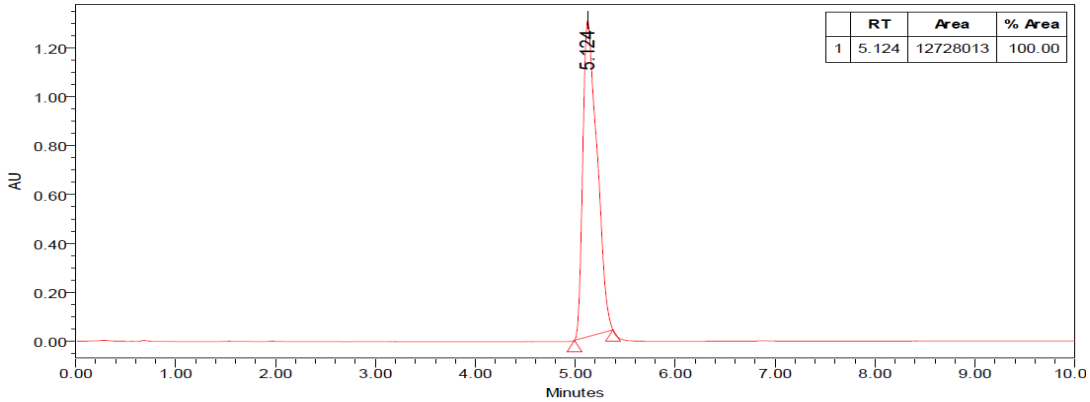

Figure: 22 Chromatogram of III injection of 200 ppm solution

Table: 6 Data Analysis of Quality Control Solutions of Bupropion

Conc.(ppm)	Area			Area mean	±S.D.	%CV	Difference (standard vs. QC)
	Injection						
	1st	2nd	3rd				
50	32345	431406	427483	430411.3	2579.105	0.5%	-4.432
150	588279	559317	577949	575181.7	14677.98	2.05%	-0.284
200	12737660	12261417	12728013	12575697	272216.9	2.1%	+2.73

RESULT AND DISSCUSSION

This study describes a highly sensitive, accurate and reproducible HPLC method for the determination of Bupropion because no such method was developed.

Instrumentation

A method has been developed by experimentation based on the literature survey and ascertained by statistical parameter of sampling using a Alliance Waters e2695 Separations Module from USA which is equipped with a Waters 2774 pump, Waters 2998 Photodiode Array Detector and a injector loop made of Rheodyne, Model No. 2767, Made in USA having a injection volume of 20 µl.

Drug's identification and characterization

Identification and purity of drug obtained by using UV, IR, NMR spectroscopy, mass spectroscopy and melting range determination.

Bupropion

^{1}H NMR: δ

IR:

ESI-MS: 240 (M+1).

Melting range: 270-272 ^{0}C

λmax: 242.6 nm.

Method Development

Drugs Solubility[17]

Solubility in different organic and aqueous solvents determined the best composition of the sample solvent. Bupropion was freely soluble in methanol, soluble in ACN and in mobile phase and slightly *soluble* in water.

Mobile Phase Selection

Different mobile phase were tested but adequate separation of drugs was found in acetonitrile: 5mM Ammonium bicarbonate buffer (50:50 %v/v).

pH Selection

By altering the pH of mobile phase separation of peak was observed. The pH of mobile phase was adjusted to 7.0, 8.0, 9.0 and 10.0. At pH 9.0 satisfactory separation of the drug with good resolution and short run time was achieved. At pH 7.0, 8.0 and 10.0 low retention time of Bupropion and poor separation. So mobile phase acetonitrile: 5 mM

Ammonium bicarbonate buffer (50:50% v/v) pH 9.0 was selected for method development.

Wavelength Selection

Maximum absorbance of Bupropion at 242.6nm was determined in mobile phase by utilizing Waters 2998 Photodiode Array Detector. Maximum peak height of drug was obtained at 242 nm by injecting the 3µg/ml concentration of sample of the drug and allows to run at different wavelength.

Flow rate programming

For the purpose of rapid analysis of the drug flow rate programming was used which result in shorter run time. Best results were obtained with flow rate programming of selected mobile phase. Mobile phase was started at a flow rate of 1.0 ml/min which was continued for 1.0 min to 10.00 min.

Optimized Chromatographic Conditions

As a result of several above experiment steps optimized conditions were selected and a simple, rapid and sensitive method was developed.

Table: 7 Optimized Chromatographic Conditions

S. No.	Parameter	Optimized Condition
1	Instrument (HPLC)	Alliance Waters e2695 Separations Module
2	Column	Waters X – Bridge C-18 5µm, 4.6 X 150 mm column
3	Mode	Gradeint
4	Mobile phase	(ACN: Ammonium bicarbonate buffer (5mM) pH-9 adjusted with Ammonium hydroxide)
5	Column Oven temperature	30^0C
6	Flow rate	1 ml/min
7	Detector	Photodiode array
8	Sample tray temperature	Ambient room temperature
9	Detection wave length	214nm
10	Injection volume	3µL
11	Retention time (R_t)	5.191
12	Run time	10 min

Method Validation

Validation of the developed and optimized HPLC method was carried out with respect to the parameters such as specificity, linearity, stability, accuracy, precision and limit of quantification (LOQ), limit of detection (LOD) in the light of internationally accepted ICH guidelines.

Specificity

The specificity of the method was determined by comparing the chromatograms obtained from the sample containing Bupropion standard stock with those obtained from test sample of Bupropion and blank of that. The specificity study revealed at the absence of interference of impurities with the drug since no extra peak appeared at the Rt of drug.

Linearity

For linearity studies, even different concentrations of the drug were prepared. The response was measured as peak area. The calibration plot was generated by replicate analysis at five concentration levels and the linear regression equation were calculated using the least square method within Microsoft Excel® program. The calibration curve obtained by plotting the peak area (y) versus analyte concentration (x) in µg/ml showed linearity in the concentration range of50 to 250 ppm. The linear regression equation is y = ax ± b, where a, is slope of the curve and b is the intercept. On the basis of following result typical linear regression equation was found to be y = 6E+06x + 91344. A correlation coefficient value was found to be 0.997 for the drug indicates a high degree of linearity.

Accuracy

The accuracy of method was determined by calculation of % recovery. Recovery is typically determined by comparing the response of the method to a reference material with the known value assigned to the material. If the recovery of the assay was poor (e.g. less than 90%) it would be a good indication that there is a problem with the method. The developed method for Bupropion is a valid method.

Precision

The precision of method was established by carrying out analysis of the analyte based on standard deviation or relative standard deviation of result from six injections at two different concentrations of drug and working standard solutions. If % RSD of the assay is > 2% then the developed method is not a presided method.

Stability

The stability of the analyte solution was determined by treating the analyte in different conditions (alkali, acid, peroxide & elevated temp) at interval of 1st day, 2nd day, 3rd day, 4th day and 5th day.

The stability of solution was determined by comparing the peak area of the chromatogram of analyte on the 1st day and 5th day with that of the freshly prepared solution on the 1st day. After 5 days study in different conditions (alkali, acid peroxide & elevated temperature) it was observed that the drug Bupropion was not degraded (stable) under acid and peroxide condition and also in elevated temperature (60^0C)

upto 5 days study but it was degraded in basic condition in just 3 day.

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

Table 8: LOD and LOQ of Drug

Drugs	LOD (ppm)	LOQ (ppm)
Bupropion	0.5	2.00

Robustness

Robestness is the degree of reproducibility of results obtained by the analysis of the same sample under a variety of normal test conditions i.e. different analysts, laboratories, instruments, reagents, assay temperatures, small variations in mobile phase, different days etc. (i.e. from laboratory to laboratory, from analyst to analyst.)

Table: 9 Robustness Data

Robustness data table				
S. No.	**Temp**	**Flow**	**R.T. (min)**	**Area**
1	25^0C	0.9ml/min	5.526	6985492
2		1.0 ml/min	5.346	3938026
3		1.1 ml/min	4.817	11994681
4	30^0C	0.9ml/min	5.545	9668377
5		1.0 ml/min	5.199	12316977
6		1.1ml/min	4.803	12508314
7	35^0C	0.9ml/min	5.611	5457399
8		1.0 ml/min	5.187	10490406
9		1.1ml/min	4.803	12373100

After changing the temperature and flow rate it was observed that as flow rate increases from 0.9ml/min to 1.1ml/min retention time decreases and area increases in an acceptable limit of 0.8ml to 1.2 ml.

CONCLUSION

Developed assay method is simple, rapid, accurate, precise, economical, specific and reproducible for the qualitative and quantitative determination of Bupropion with good resolution in short time and high sensitivity.

In the present work the analyte was separated in a short run. Optimization of the method showed that apart from the mobile phase pH and composition, flow rate is an important crucial parameter.

The selection of gradient mobile phase and flow rate, cut down over all time of sample analysis and thereby made the method most cost effective and rapid. Wavelength selection made the method more sensitive.

It was concluded that the developed method offers several advantages such as rapidity, simple mobile phase and sample preparation step, improved sensitivity makes it specific and reliable for its intended use. This method can be applied to analysis of pharmaceutical dosage forms.

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