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April-June 2016

International Journal of CHEMICAL AND PHARMACEUTICAL ANALYSIS

eISSN: 2348-0726 ; pISSN : 2395-2466

Research Article | Volume-3 | Issue-3 | Article ID: 980

DEVELOPMENT AND VALIDATION OF ANALYTICAL ASSAY METHOD FOR SEPARATION OF A SERIES OF SUBSTITUTED BENZ-1,3-OXAZINE DERIVATIVES BY LC/ LCMS

Santosh Kumar Bhardwaj*[1,2] K. Dwivedi[1] , D. D. Agarwal[1]

[1]*School of Studies in Chemistry, Jiwaji University, Gwalior-474011, Madhya Pradesh, India.*

[2]*Shimadzu Analytical India Pvt. Ltd, Delhi, India.*

***Corresponding Author:** Email: sbhardwaj81@yahoo.com*

Received: 16 March 2016 / Revised: 9 April 2016 / Accepted: 20 April 2016 / Available online : 30 June 2016

ABSTRACT

The paper reports a recent efforts to develop and validate an efficient and rapid analytical assay method by LC/ LCMS for a series of 2H-benzoxazinone based 3-aryl-3,4-dihydro-2H-benz[e]-1,3-oxazines. Such compounds posse's potent pharmacological importance, and are of the class of efavirenz, a non-nucleoside reverse transcriptase inhibitor (NNRTI). Series of seven compounds were separated using ODS , 250×4.6mm, 5 µm column with a mobile phase -A consist of 90:10 (v/v) of 0.05M ammonium acetate buffer with pH 5.60 ± 05, (pH adjusted with acetic acid) and acetonitrile. mobile phase -B consisting 90:10 .05M ammonium acetate buffer with pH 5.60 ± 05, pH adjusted with ortho phosphoric acid with a timed gradient program. Detection was carried out at 245 nm and the flow rate 1.0 ml/min. The method has been validated in terms of suitability, specificity, linearity, intra and interday Precision, limits of detection and quantification The method found to be accurate in the concentration range of 1.0–10.0 µg mL-1 for all compounds. LOD and LOQ were from 0.024 to 0.048 µg mL-1 and 0.075 to 0.147 µg mL-1. Method is linear over concentration range 0.1 to 100 µg mL-1. The proposed method was found to be accurate, precise, specific, linear, rugged, robust, and stability indicating for the determination of a series of 3-aryl-3,4-dihydro-2H-benz[e]-1,3-oxazines and has shown to be convenient for routine analysis of 3-aryl-3,4-dihydro-2H-benz[e]-1,3-oxazine derivatives.

Keywords *– LC/ LCMS, Method development, Validation, Liquid chromatography, 2H-benz[e]-1,3-oxazines.*

1. INTRODUCTION

Oxazinone, benzoxazinone and their derivatives are an important class of heterocyclic compounds. These heterocyclic systems exhibit biological activities, such as HIV-1 reverse transcriptase inhibitors. Among a wide variety of heterocycles that have been explored for developing pharmaceutically important molecules, 1,3-benzoxazines constitutes an important class due to their wide variety of biological activity.[1,2] 1, 3-benzoxazine are a group of compounds possessing a wide spectrum of biological activities such as antimicrobial, anti-inflammatory. From the therapeutic point of view benzoxazones derivatives have been prepared by various groups.[3] A considerable number of reports concerning 1,3-oxazine[5]derivatives which have undergone their greatest development in the last few years came in to the notice and occupied an unique place in material and medicinal chemistry due to their diverse physical and biological properties.[4-6] One of the most recent and most important examples is the 3,1-benzoxazine derivatives efavirenz, which has

recently been approved as an anti-HIV drug.[7] Efavirenz (Sustiva), a non-nucleoside reverse transcriptase inhibitor (NNRTI), which is used as a part of highly active antiretroviral therapy (HAART) for the treatment of human immunodeficiency virus (HIV) type 1, explored a new dimension for 2H-benzoxazin-2-ones in the field of medicinal world.[8] Derivatization of benzoxazinone has got a new pace to counter HIV in recent times [9-11].

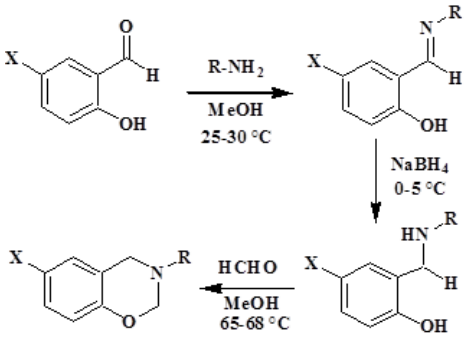
A number of analytical methods[12-16] has been reported for the separation and quantification of 1,4-benzoxazin-3-ones and benzoxazolin-2-ones by HPLC. Lemmer *et al.,*[17] has reported an accurate, selective, and sensitive method for the determination of the non-nucleoside reverse transcriptase inhibitors (NNRTIs) nevirapine (nvp) and efavirenz (efv) in human plasma using gas chromatography-mass spectroscopy in selected ion monitoring mode (GC/MS-SIM). Baumeler, *et al*,[18] has reported an improved method of sample preparation and simultaneous HPLC separation that allowed the separation of 2,4-dihydroxy-1,4-benzoxazin-3(4H)-one (DIBOA), 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3(4H)-one (DIMBOA), 2-hydroxy-1,4-benzoxazine-3(2H)-one (HBOA), 2-hydroxy-7-methoxy-1,4-benzoxazine-3(2H)-one (HMBOA) and their corresponding glucosides. Hemvichian, *et al* [19] has reported different aromatic amine-based polybenzoxazines which are subjected to thermal decompositions in a thermo gravimetric analyzer. The degradation products, which were volatile compounds evaporating out of the furnace as gases, are trapped and analyzed further by a gas chromatograph which is coupled with a mass selective detector (GC-MS). Sindhura *et al* [20] have reported a simple rapid, accurate, precise and reproducible validated reverse phase HPLC method was developed for the determination of Lamivudine, Zidovudine and Efavirenz in bulk and pharmaceutical dosage forms. Garg et al [21] have reported a new analytical method using reversed phase high performance liquid chromatography for analysis of four antiretroviral molecules lamivudine, abacavir, zidovudine and effavirenz.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

Acetonitrile (LC Grade), methanol (LC Grade), water (LC Grade), ammonium acetate, and acetic acid were obtained from Merck.

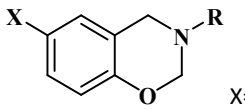
A series benz-1,3-oxazine derivatives were synthesized using literature method (Figure-1 and Table-1). Standards were prepared by purifying and recrystallizing benz-1,3-oxazine derivatives. Structures of benz-1,3-oxazine derivatives have been established by spectroscopic analysis.



R	X
$-C_6H_5$	-H
$-4-CH_3C_6H_4$	-H
$-4-ClC_6H_4$	-H
$-4-BrC_6H_4$	-H
-2-Aminopyridyl	-H
-3-Aminopyridyl	-H
$-4-CH_3C_6H_4$	-Br

Figure 1 : Chemical reaction scheme for synthesis of benz-1,3-oxazine derivatives

Table 1: Details of 3-Aryl-3, 4-Dihydro-2*H*-benz[*e*]-1,3-oxazine derivatives



X=H

S No.	ID	R	Name	Mass by LCMS (M+1)
1	2A	-C_6H_5	3,4-Dihydro-3-phenyl-2*H*-benz[*e*]-1,3-oxazine	212.15
2	2B	-4-$CH_3C_6H_4$	3,4-Dihydro-3-(4-methylphenyl)-2*H*-benz[*e*]-1,3-oxazine	226.15
3	2C	-4-ClC_6H_4	3,4-Dihydro-3-(4-chlorophenyl)-2*H*-benz[*e*]-1,3-oxazine	246.10
4	2D	-4-BrC_6H_4	3,4-Dihydro-3-(4-bromophenyl)-2*H*-benz[*e*]-1,3-oxazine	290.00
5	2E	-2-Aminopyridyl	3,4-Dihydro-3-pyridin-2-yl-2*H*-benz[*e*]-1,3-oxazine	213.10
6	2F	-3-Aminopyridyl	3,4-Dihydro-3-pyridin-3-yl-2*H*-benz[*e*]-1,3-oxazine	213.10
7	2G	-4-$CH_3C_6H_4$	6-Bromo-3,4-dihydro-3-(4-methylphenyl)-benz[*e*]-1,3-oxazine	304.30

2.2 Standard preparation

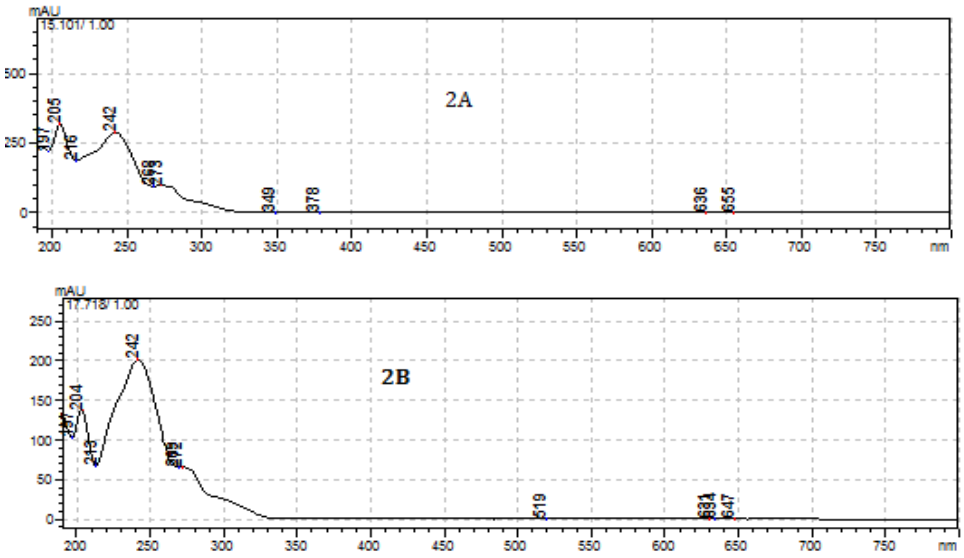
Stock solutions were prepared 1mg mL-1 of all standards and one mixed standard was prepared by weighing 100mg of all standards in 100 ml volumetric and making up with 100% HPLC grade methanol.

2.3 Sampling and sample preparation

Five set of samples of different conc. 1, 2 5, 10, 25, 50, 75 and 100 mg mlL-1has been prepared from the material obtained after the reaction without further purification so as to ensure the exact composition of the substance.

2.4 Instrumentation and separation conditions

LC analysis were carried out using LC-2010CHT (Shimadzu, Japan) and LC prominence (Shimadzu, Japan) advanced instruments equipped with UV-visible and PDA detector in series. The chromatographic condition optimized were ODS , 250×4.6mm, 5 µm column with a mobile phase -A consist of 90:10 (v/v) of 0.05M ammonium acetate buffer with pH 5.60 ± 05, (pH adjusted with glacial acetic acid) and acetonitrile in ratio 90/10 (v/v). mobile phase -B consisting 90:10 (v/v) of acetonitrile and 0.05M ammonium acetate buffer with pH 5.60 ± 05, pH adjusted with glacial acetic acid acid with a timed gradient program of T/%B:0/50,3/60,9/60,16/90,20/60,21/50 .Detection was carried out at 245 nm (figure-2) and the flow rate 1.0 ml/min.



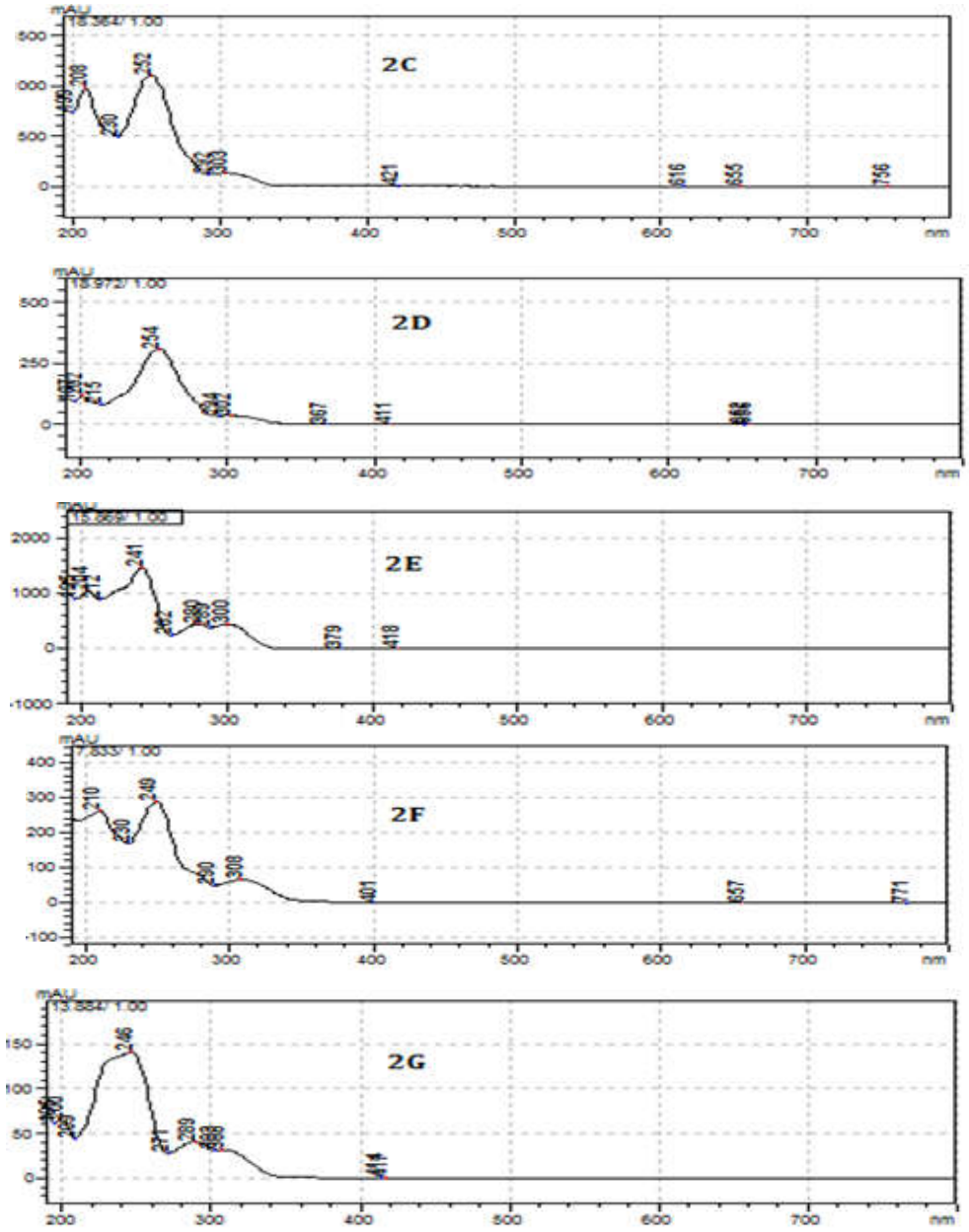


Figure 2 : UV spectrums of benz-1,3-oxazines derivatives for selection of wavelength

2.5. Molecular mass determination

LCMS analysis were carried out using LCMS-2020 and LCMS-8030 (Shimadzu, japan) with Lab Solution software ver 5.41. analysis were carried out by using C18 column (50mm length, 3 mm Id and 2.2µm particle size) with Mobile phase mobile phase -A consist of 90:10 (v/v) of 0.05M ammonium acetate buffer with pH 5.60 ± 05, pH adjusted with acetic acid and acetonitrile. Mobile phase -B consisting 90:10 (v/v) of acetonitrile and 0.05M ammonium acetate buffer with pH 5.60 ± 05, pH adjusted with acetic acid with a timed gradient program of T/%B:0/50,/60,9/60,16/90,20/60,21/50. Samples were injected with SIL-30AC auto sampler. Mass spectra were obtained in ESI as well as APCI in positive and negative modes between 50-500amu mass range and 0.5 second scan (event) time. (figure-3)

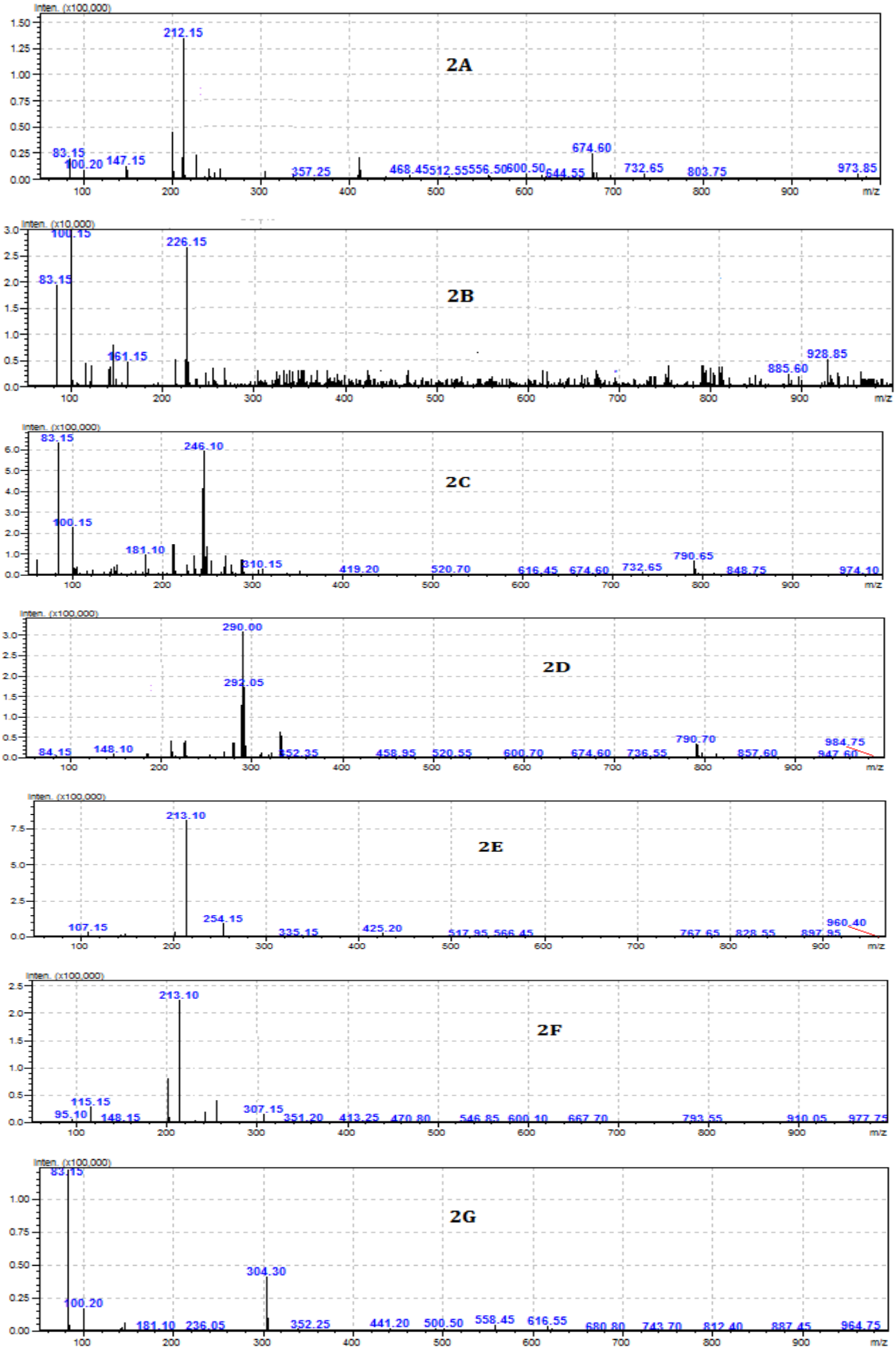


Figure 3: Mass spectrums of benz-1,3-oxazines derivatives

3. RESULTS AND DISCUSSION

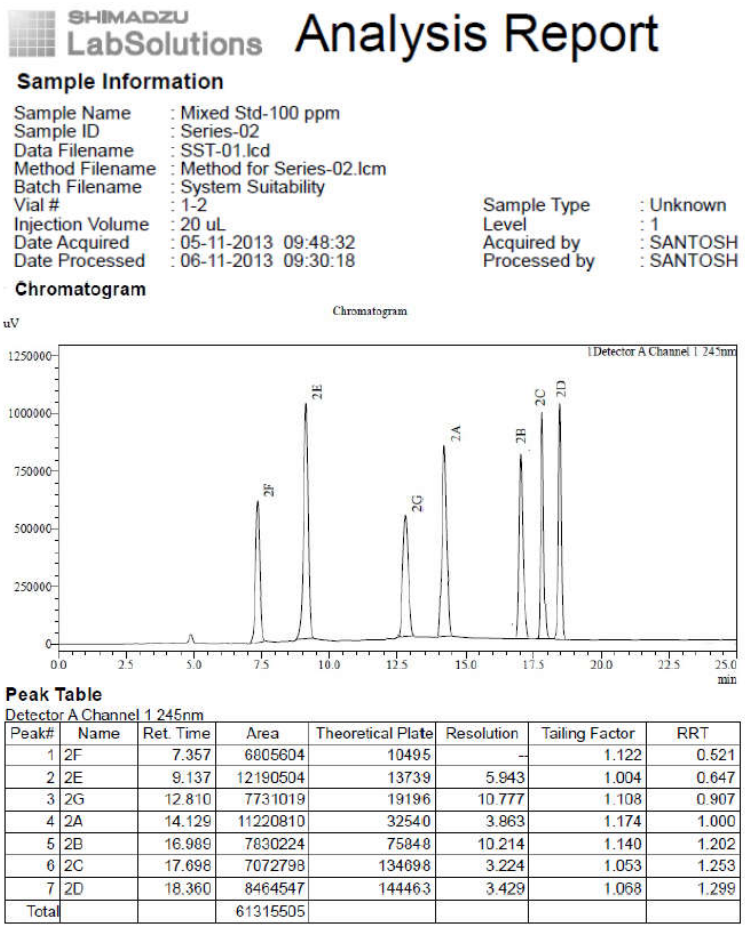
3.1. Method validation

3.1.1 System suitability

The system suitability and precision was calculated by computing the resolution between all analytes of a series of 3-aryl-3,4-dihydro-2*H*-benz[e]-1,3-oxazine derivatives and relative standard deviation for retention times and area response of all peaks. Resolution between peaks 2A and impurity of 2E of 3-aryl-3,4-dihydro-2*H*-benz[e]-1,3-oxazine was critical. Chromatographic conditions were optimized to achieve these system suitability parameters. In optimized chromatographic conditions resolution between peaks were not less than 2.0. In optimized chromatographic conditions, minimum resolution between peak were 3.22 (NLT- 2.0), maximum relative standard deviation of peaks for retention times and area were 0.024 and 0.39 (NMT – 2.0), maximum tailing factor 1.17 (NMT 2.0) and minimum theoretical plate counts in standards solution 10495 which meet the ICH requirement (NLT 2000). These results (given in table-2 and figure-4) conclude that method confirm system suitability criteria mentioned in the ICH and reported literature values. [23, 24]

Table 2: System Suitability results of benz-1,3-Oxazine derivatives

S.#	Parameter	Results	Acceptance criteria
1	Minimum resolution between adjacent peaks	3.224	NLT 2.0
2	Maximum relative standard deviation (%RSD)	0.39	NMT 2.0
3	Maximum tailing factor for all analytes	1.174	NMT 2.0
4	Minimum theoretical plate count	10495	NLT 2000



Peak Table

Detector A Channel 1 245nm

Peak#	Name	Ret. Time	Area	Theoretical Plate	Resolution	Tailing Factor	RRT
1	2F	7.357	6805604	10495	–	1.122	0.521
2	2E	9.137	12190504	13739	5.943	1.004	0.647
3	2G	12.810	7731019	19196	10.777	1.108	0.907
4	2A	14.129	11220810	32540	3.863	1.174	1.000
5	2B	16.989	7830224	75848	10.214	1.140	1.202
6	2C	17.698	7072798	134698	3.224	1.053	1.253
7	2D	18.360	8464547	144463	3.429	1.068	1.299
Total			61315505				

Figure 4 : HPLC Chromatogram of system suitability of bennz-1,3-oxazines

3.1.2 Specificity

Specificity and selectivity were studied for the examination of the presence of interfering components, stress studies were performed for a series of 3-aryl-3,4-dihydro-2*H*-benz[e]-1,3-oxazine to provide an indication of the stability indicating property and specificity of the proposed method. International degradation was attempted to stress condition of heat (80°C,120hr) acid (1.0 M HCl,15 min),base (5 M NaOH, 120 min) oxidation (3.0% H_2O_2 ,10 min) and photolytic degradation (10K Lux, 48hr) to evaluate the ability of proposed method to separate 3-aryl-3,4-dihydro-2*H*-benz[e]-1,3-oxazine from its degradation product. The purity of all peaks were checked by using Lab solution software and PDA detector, SPD-M20A (Shimadzu, Japan) and found within acceptance criteria NLT 0.990. Interference of peaks were also checked with LCMS and no interference was observed. Separation between all peaks including, diluents peak, un-reacted raw materials, unknown impurities and degradation peaks were good. These results (Table-3 and Figure-5) conclude that method confirm specificity criteria mentioned in the ICH and reported literature values.[23,24]

Table 3: Specificity results

S.#	Name of Compound	RT (Min)	Area	Resolution (USP)	Peak Purity Index
1	Unk-1	4.908	250101	--	0.9998
2	2F	7.43	6685326	9.169	0.9999
3	2E	9.243	9748505	6.01	1.0000
4	Unk-2	9.797	132500	2.086	1.0000
5	2G	13.003	6363659	11.218	0.9999
6	2A	14.284	8772744	3.912	1.0000
7	Imp-2E	15.303	3780426	4.143	1.0000
8	Unk-3	15.915	2354049	2.929	1.0000
9	2B	17.122	5603795	5.192	1.0000
10	2C	17.83	4710881	3.598	1.0000
11	2D	18.491	7222290	3.774	1.0000
Acceptance Criteria				NLT 2.0	NLT 0.99

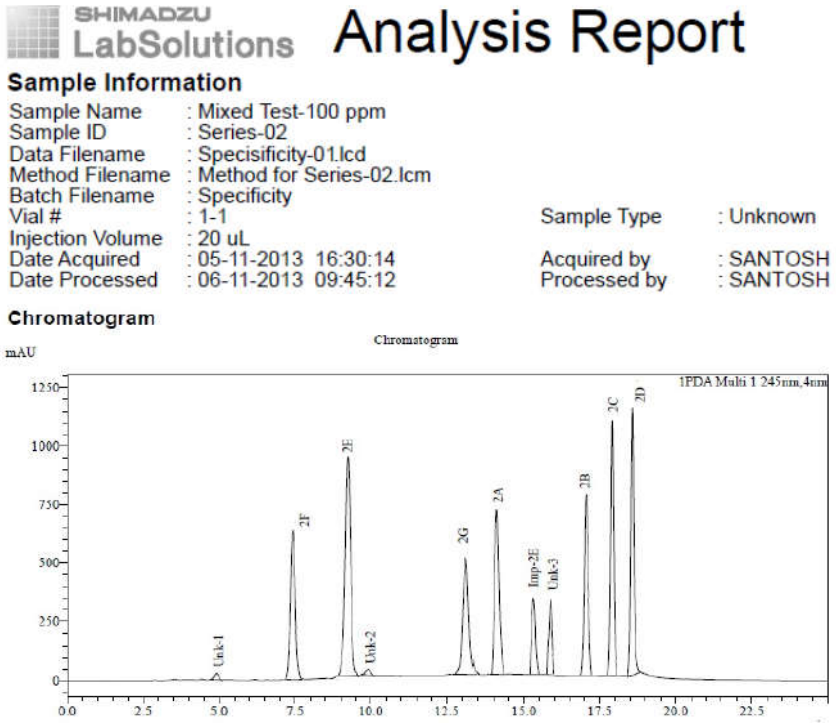


Figure 5: HPLC Chromatogram of Specificity of bennz-1,3-oxazine derivatives

Peak Table

PDA Ch1 245nm

Peak#	Name	Ret. Time	Area	Area%	Resolution	Peak Purity Index
1	Unk-1	4.908	254782	0.408	--	0.999806
2	2F	7.430	6685326	10.707	9.169	0.999886
3	2E	9.243	11393690	18.248	6.010	1.000000
4	Unk-2	9.797	290532	0.465	2.086	1.000000
5	2G	13.003	6363659	10.192	11.218	0.999859
6	2A	14.284	8772744	14.050	3.912	1.000000
7	Imp-2E	15.303	2736835	4.383	4.143	1.000000
8	Unk-3	15.915	2677148	4.288	2.929	1.000000
9	2B	17.122	7560309	12.108	5.192	1.000000
10	2C	17.830	7562390	12.112	3.598	1.000000
11	2D	18.491	8142284	13.040	3.774	1.000000
Total			62439698	100.000		

3.1.3 Limit of detection & Limit of quantitation

LOD and LOQ for 3-aryl-3,4-dihydro-2*H*-benz[e]-1,3-oxazine were calculated on the basis of signal to noise ratio using Lab Solution software at 245nm where all analyte have good absorbance. The value of LOD and LOQ for 3-aryl-3,4-dihydro-2*H*-benz[e]-1,3-oxazine were less than 0.1µg/ml (0.1ppm) with optimized method, these values are better than reported values for similar compounds either as API or in bulk formulations with UV-visible and PDA detectors. % RSD was in the range of 1.21-1.82 % (NMT 5.0%) for LOQ precision. (table-4 and figure-6) These results conclude that method confirm LOQ precision criteria mentioned in the ICH and reported literature values. [23-25]

Table 4: LOD & LOQ results of Benz-1,3-oxazine derivatives

Entry	R	LOD (ppm)	LOQ (ppm)
2a	$-C_6H_5$	0.031876	0.096595
2b	$-4-CH_3C_6H_4$	0.037555	0.113803
2c	$-4-ClC_6H_4$	0.039935	0.121014
2d	$-4-BrC_6H_4$	0.039506	0.119714
2e	-2-Aminopyridyl	0.024751	0.075002
2f	-3-Aminopyridyl	0.0431	0.130605
2g	$-4-CH_3C_6H_4$	0.048632	0.147368

Chromatogram

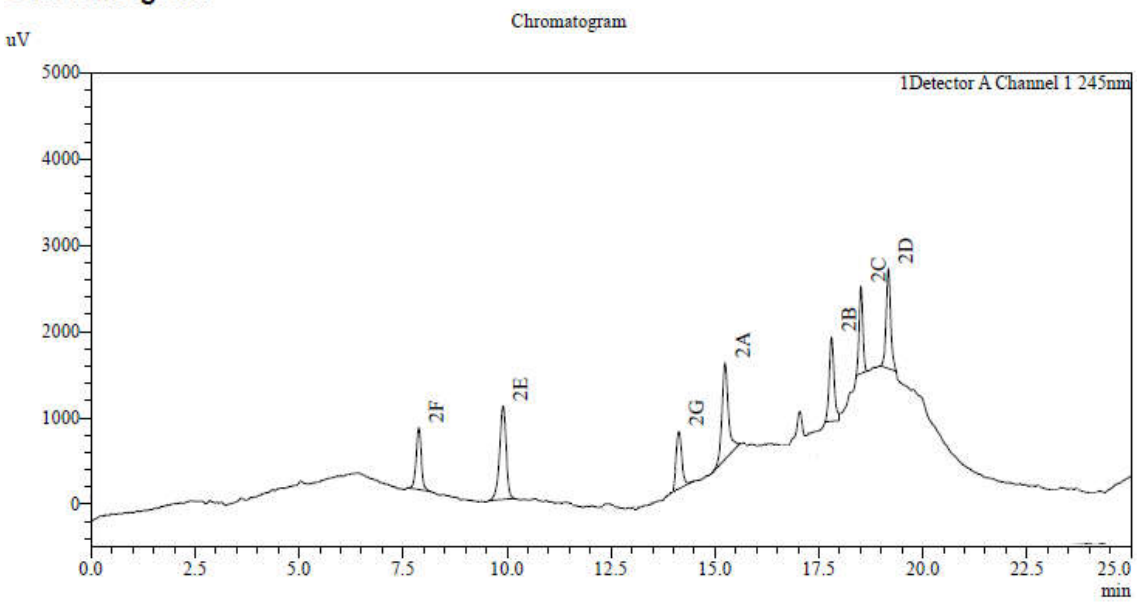
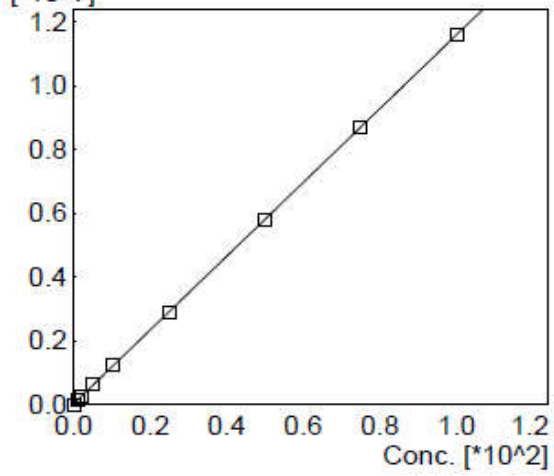


Figure 6: HPLC Chromatogram of LOD/LOQ of bennz-1,3-oxazine derivatives

3.1.4 Linearity

Linearity of peak area response versus concentration was studied over the calibration range 0.1µg/ml to 100µg/ml for all 3-aryl-3,4-dihydro-2*H*-benz[e]-1,3-oxazine.The correlation co-efficients obtained were 0.9999-0.99999 (NLT-0.990) (figure-7).The above results shows that an excellent correlation existed between the peak area and the concentration of all analytes. These results conclude that method confirm linearity criteria mentioned in the ICH and reported literature values.[24,25]

ID# : 4
Name : 2A
Quantitative Method : External Standard
Function : f(x)=115712*x+24696.5
Rr1=0.9999760 Rr2=0.9999520
Curve Type : Linear



#	Conc.(ppm)	Area
1	0.1	9171
2	1	121194
3	2	252852
4	5	630266
5	10	1240806
6	25	2883147
7	50	5811579
8	75	8685722
9	100	11609883

Figure 7: Linearity curve of 3,4-Dihydro-3-phenyl-2*H*-benz[e]-1,3-oxazin-2-one (2a)

3.1.5 Precision

The precision of the assay method was checked by injecting six standard preparations of 3-aryl-3,4-dihydro-2*H*-benz[e]-1,3-oxazine .The % RSD of the area for 3-aryl-3,4-dihydro-2*H*-benz[e]-1,3-oxazine was calculated .The % RSD of six measurement of test sample was 0.19-0.645% . [24]

3.1.5 Intermediate Precision

The intermediate precision of the method was evaluated using different analysts and different instruments in the same laboratory. The % RSD of six measurement of test sample of analyst -1 and analyst-2 was 0.19-0.64% and 0.20-0.59% respectively. [25]

3.1.6 Accuracy

Accuracy study was conducted by using standard addition method. A known concentration of standard substance (analyte) was added to blank preparation of sample matrix and recovery of analyte is calculated on the basis of area obtained in the chromatogram The result shows that best recoveries between 99-101 % of the spiked samples were obtained at each added concentration, indicating that the method was accurate.(table-5)[25]

Table 5: Accuracy results of 3, 4-dihydro-3-phenyl-2H-benz[e]-1,3-oxazine (2a)

S.#	Accu. Level (ppm)	Area of Standards	Area of Spiked	Amount Added (ppm)	Amount Recovered (ppm)	% Recovery	Average Recovery (%)
1.	LOQ	25789	25683	0.20	0.199	99.589	99.330
2.		25897	25737		0.199	99.382	
3.		25756	25503		0.198	99.018	
1.	5	245451	242436	5	4.94	98.772	98.958
2.		244638	242194		4.95	99.001	
3.		244638	242436		4.95	99.100	
1	10	466237	465346	10	9.98	99.809	99.878
2		465256	464038		9.97	99.738	
3		465142	465542		10.01	100.086	
Average of average %recovery							99.388

3.1.7 Robustness

Robustness was investigated by varying the conditions w.r.t change in flow rate, mobile phase pH and wavelength. The study was conducted at different flow rate 0.9-1.1 ml/min. The effect of the pH of the mobile phase on the resolution was studied by varying pH from 5.60 to 5.80and 5.40, while other mobile phase compositions were held constant. Resolution was also studied by changing column and instruments. The wavelength change at 243 nm to 247 nm. The method was found to be robust with respect to flow rate, mobile phase pH, Column and wavelength without any changes in system suitability parameters such as resolution, tailing factor and theoretical plate. Resolution is 2.987-3.423, tailing factor is 1.236- 1.319 and theoretical plate is 9806-10065 which is within acceptance criterion (table-6). [23-25]

Table 6: Variable conditions of robustness method and results

S. #	Parameter	Normal condition	Altered Parameter	Minimum Resolution	Maximum Tailing factor	Minimum Theoretical plates count
1	Flow rate	1.0 ml/min	0.9 ml/min	3.050	1.236	10675
			1.1 ml/min	3.423	1.293	10337
2	Buffer pH	Ammonium acetate buffer (pH 5.60)	buffer pH 5.80	3.132	1.319	11172
			buffer pH 5.40	3.293	1.269	9806
3	Column	Luna C18	Column-1	3.246	1.273	10573
			Column-2	2.987	1.255	11060
4	Wavelength	245nm	243nm	3.162	1.235	10140
			247nm	3.152	1.239	10065
Acceptance criteria				**NLT 2.0**	**NLT 2.0**	**NMT 2000**

3.1.8 Solution stability

The cumulative %RSD for analytes in test solution were calculated. The results obtain in the solution stability study at different time intervals for test preparation. It was concluded that the test preparation solution was found stable up to 72 h at 2 – 8 ˚C and ambient temperature, as during this time the result was not decrease below the minimum percentage.[25]

4. CONCLUSION

The results obtained for the HPLC assay method validation meet the system suitability requirement which indicate that the system is suitable and precise for analysis. Experiments concludes that method is specific for process impurities and degradant product. So it can be used as a stability indicating method for stability studies. Based on results it can be concluded that method is having a capability to detect analytes in lower concentration from 0.024 to 0.048 µg mL-1 and quantification limit is 0.075 to 0.147µg mL-1 for different derivatives of benz[e]-1,3-oxazines. Experiments concludes that the response of analytes peaks is linear over concentration range 0.1

µg mL-1 to 100 µg mL-1. Based on the results it can also be concluded that this method is precise and rugged .The % RSD of test result proves the ruggedness of test method for the variability's like two different instruments, two different analysts, two different column of same specification and two different days. The method is accurate over range LOQ to 10µg mL-1 of nominal concentration. Experiments concludes that method is robust, system suitability were checked at each variable condition and results found to be within acceptance criterion. Sample solution is stable for 72 hours without any significant change. The proposed method was found to be accurate, precise, specific, linear, rugged, robust, and stability indicating for the determination of a series of 3-aryl-3, 4-dihydro-2*H*-benz[e]-1,3-oxazines.

5. ACKNOWLEDGMENT

We sincerely thanks Shimadzu Analytical India Pvt Ltd New Delhi for using sophisticated Analytical Instrumentation Facility,for analytical method development and Advanced instrumentation Research Facility (AIRF) of Jawahar Lal Nehru University (JNU) , New Delhi, India for Spectral analytical data.

6. ABBREVIATIONS

ACN	Acetonitrile
HPLC	High Performance Liquid Chromatography
ICH	International conference on Harmonization
Id	Internal Diameter
LCMS	Liquid Chromatography- Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantitation
m	Meter
MeOH	Methanol
mg	Milligram
ml	Mili litre
mm	Mili meter
nm	Nano meter
ODS	Octyl decyl silane
PDA	Photo diode array
UV-Vis	Ultra Violet-Visible
µg	Microgram
µl	Microlitre
µm	Micron
µg mL-1	Microgram per mili litre

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