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A facile and efficient analytical method development and validation for separation of a series of substituted 3aryl-3, 4-dihydro-2H-benz[*E*]-1,3-oxazin-2-ones by GC/ GCMS

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ABSTRACT

The paper reports a recent efforts to develop and validate an efficient and rapid analytical assay method by GC/ GCMS for a series of 2H-benzoxazinone based 3-aryl-3,4-dihydro-2H-benz[e]-1,3-oxazin-2-one. Such compounds possess potent pharmacological importance and are of the class of efavirenz, a non-nucleoside reverse transcriptase inhibitor (NNRTI). Chromatography was performed with a non-polar capillary column, Rtx-5 (length 30 meter, id 0.25mm and film thickness 0.25µm), carrier gas nitrogen/ helium used at flow rate 1.20 ml/minute with split/splitless injector and flame ionization detector. GC oven temperature was programmed from 80°C hold for 1.0 min. and then increase at rate 15°C/min to 160°C, held isothermal at 160°C for 1.0 minute, temperature again increased at rate 20°C/min.to 260°C, held isothermal at 260°Cfor 5 minute and increase to final temperature at rate 20°C/min. to 280 °C held isothermal at 280 °C for 5 minute. Injector and detector temperatures were optimized 260°C and 280 °C respectively. Each analysis required 20minutes for separation of a series of all seven analytes. The method was validated for linearity, accuracy, precision, specificity, system suitability, and robustness. The method proved to be accurate, precise, specific, rapid, and reproducible according to ICH standards. The method showed good recoveries (99.50 to 100.50 %) for all analytes, and the relative standard deviations of intra- and inter-day were <2.0%. LOD and LOQ were from 0.17 to 0.34µg mL-1 and 0.51 to 1.02µg mL-1 for all analytes. This method has shown to be convenient for routine analysis of3-aryl-3,4-dihydro-2H-benz[e]-1,3-oxazin-2-one.This method is novel since there has been no report about analysis of benz-1,3-oxazine-2-ones derivatives or its class at µg mL−1 levels using a simple GC method

Key words: GC/GCMS, Method development, validation, gas chromatography, 1,3-oxazin-2-ones.

INTRODUCTION

Benzoheterocycles particularly benzoxazine and benzthiazine are an important class of N-containing heterocycles as they exhibit interesting biological activities and are used as key structural motifs for the synthesis of various pharmaceutical agents and natural products [1].Many important medicines, dyes, pesticides, etc, are found in the series ofheterocyclic compounds, called oxazines and thiazines, they are found mainly in the polycyclic divisions in which other rings, such as the benzene ring, are fused to the oxazines or thiazine ring [2,3]. 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 [4]. A considerable number of reports concerning 1,3-oxazinederivatives 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[5-7].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 methodshas been reported for the separation and quantification of 1,4-benzoxazin-3-ones and benzoxazolin-2-ones by HPLC [12-16]. After going through detailed literature, it is observed that only few methods are reported so far the analysis of benzoxazin-2-one by gas chromatography. Lemmer *et al.*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)[17]. Baumeler*et al*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 [18]. Hemvichian, *et al*has reported different aromatic amine-based polybenzoxazines which are subjected to thermal decompositions in a thermo gravimetricanalyser [19]. 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. This is the first reported method for the analysis of 3-aryl-3,4- dihydro-2*H*-benz[e]-1,3-oxazin-2-one e.g. for 3,4-Dihydro-3-phenyl-2*H*-benz[*e*]-1,3-oxazin-2-one or of its class using Gas Chromatography (Figure-1).

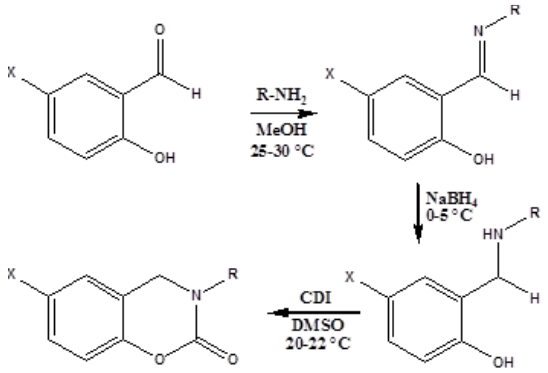


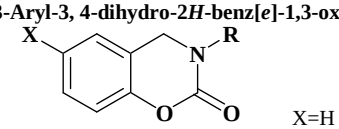
Figure-1 : Chemical reaction for synthesis benz-1,3-oxazin-2-one derivatives

EXPERIMENTAL SECTION

Working standards of 1,3-oxazin-2-ones were prepared by the literature method has been re-crystallized several times and then purified with methods like column chromatography and preparative HPLC and their structure has been established by spectroscopic analysis[20]. Results of spectroscopic analysis were compared with literature values.(Table-I)

Methanol (HPLC grade) was purchased from Fisher Scientific.

Table-1 : Details of 3-Aryl-3, 4-dihydro-2*H*-benz[*e*]-1,3-oxazin-2-one derivatives



X=H

Sample Id	R	Name of Compound	MassGCMS
1A	-C_6H_5	3,4-Dihydro-3-phenyl-2*H*-benz[*e*]-1,3-oxazin-2-one	225.2
1B	-4-$CH_3C_6H_4$	3,4-Dihydro-3-(4-methylphenyl)-2*H*-benz[*e*]-1,3-oxazin-2-one	239.2
1C	-4-ClC_6H_4	3,4-Dihydro-3-(4-chlorophenyl)-2*H*-benz[*e*]-1,3-oxazin-2-one	259.6
1D	-4-BrC_6H_4	3,4-Dihydro-3-(4-bromophenyl)-2*H*-benz[*e*]-1,3-oxazin-2-one	304.1
1E	1-Naphthyl	3,4-Dihydro-3-(1-naphthyl)-2*H*-benz[*e*]-1,3-oxazin-2-one	275.3
1F	-2-Aminopyridyl	3,4-Dihydro-3-pyridin-2-yl-2*H*-benz[*e*]-1,3-oxazin-2-one	226.2
1G	-3-Aminopyridyl	3,4-Dihydro-3-pyridin-3-yl-2*H*-benz[*e*]-1,3-oxazin-2-one	226.2

Standard preparation:

3-aryl-3,4-dihydro-2*H*-benz[e]-1,3-oxazin-2-ones were prepared by the literature method has been re-crystallized several times and then purified with methods like column chromatography and preparative HPLC and their structure has been established by spectroscopic analysis [20].Solutions were prepared 1 µg mL−1 of all standards and one mixed standard was prepared by weighing 10milligram of all standards in 100millilitre volumetric and making up with 100% HPLC grade methanol.

Sampling and sample preparation

Samples of 3-aryl-3,4-dihydro-2*H*-benz[e]-1,3-oxazin-2-ones were prepared of from the material obtained after the reaction without further purificationas well as after purification so as to ensure the exact composition of the substance.

Instrumentation

GC analysis were carried out using a GC-2010(Shimadzu, Japan) and GC-2014 (Shimadzu, Japan) advanced instruments equipped with flame ionization detector (FID) system, split/splitless injection system and fused-silica capillary columns (30 m, 0.25 mm i.d., film thickness 0.25 µm, Restek, France), Rtx-5Sil MS(95% dimethyl, 5% diphenyl polysiloxane).

Method Development

Proper selection of the methods depends upon the nature of the sample (volatile or nonvolatile molecule), its molecular weight, solubility and melting point. 3-aryl-3,4-dihydro-2H-benz[e]-1,3-oxazin-2-onesare soluble in polar solvents (methanol, chloroform) hence gas chromatography was selected to estimate them. To develop a rugged and suitable GC method for the quantitative determination of 3-aryl-3,4-dihydro-2H-benz[e]-1,3-oxazin-2-ones, the analytical conditions were selected after testing the different parameters such as diluents, melting point and other chromatographic conditions

Chromatgraphic Parameters

GC oven temperature was programmed from 80°C hold for 1.0 min. and then increase at rate 15°C/min to 160°C, held isothermal at 160°C for 1minute. Temperature again increased at rate 20°C/min.to 260°C, held isothermal at 260°Cfor 5 minute and increase to final temperature at rate 20°C/min. to 280 °C held isothermal at 280 °C for 5 minute. Injector and detector temperatures were kept 260°C and 280°Crespectively. Samples were injectedin defined concentration with the help of auto injector and auto sampler (Shimadzu, AOC 20i & AOC 20s) in the split mode with split ratio of 1:10, using helium as carrier gas constant flow rate of 1.20ml/min. Relative amounts of components were calculated based on GC peak areas obtained without using FID response factor correction. Retention indices (RI) of compounds were determined relative to the retention times of series of n-alkanes (C7–C33),with linear interpolation, using Van den Dool and Kratz (1963) equation and software GCMS Solution. A homologous series of n-alkanes, (C7-C33) custom retention time index standard (Restek, France, Catalogue: 560295, Lot#A079729) was used as standard (Table-2).

Molecular mass determination by GCMS

Determination of the molecular mass of the chromatographic peaks was performed by GC coupled with electron impact mass spectrometry (GCMS) on a Shimadzu GCMS QP 2010 Ultra (Shimadzu, Japan) equipped with split/splitless liquid injector and fused-silica capillary columns (30 m, 0.25 mm id, film thickness 0.25 µm, Restek, France), Rxi-5Sil ms(95% dimethyl/ 5% diphenyl polysilarylene).Injector, ion source and interface temperature were set at260°C, 220°C and 250°C respectively, oven temperature programmed 80°C hold for 1.0 min. and then increase at rate 15°C/min to 160 °C held isothermal at 160 °C for 1 min. Temperature again increase at rate 20°C/min to 260 °C held isothermal at 260 °C for 5 min. and increase to final temperature at rate 20°C/min. to 280 °C held isothermal at 280 °C 5 min.Samples were injected in defined concentration with the help of auto injector (Shimadzu, AOC 20i) in the split mode with split ratio of 1:20, using helium as carrier gas constant flow rate of 1.20ml/min.Mass spectra were obtained in EI-mode with 70 eV ionization energy between 50-500amu mass range and 0.5 second scan (event) time , analysis details of GCMS given in Table-2, figure-2 and Figure-3.

Table-2: GCMS analysis details of benzoxazine-2-ones, mixed standard

S.#	R	Retention Time (Minute)	Area (%)	RRT	Retention Index	Mass by GCMS
1	-C_6H_5	12.698	12.85	1.00	2219	225.2
2	-4-$CH_3C_6H_4$	13.419	17.05	1.06	2339	239.2
3	-4-ClC_6H_4	14.067	16.73	1.11	2345	259.6
4	-4-BrC_6H_4	15.061	18.73	1.19	2553	304.1
5	1-Naphthyl	17.203	14.62	1.35	2741	275.3
6	-2-Aminopyridyl	12.403	8.24	0.98	2167	226.2
7	-3-Aminopyridyl	13.155	11.78	1.04	2298	226.2

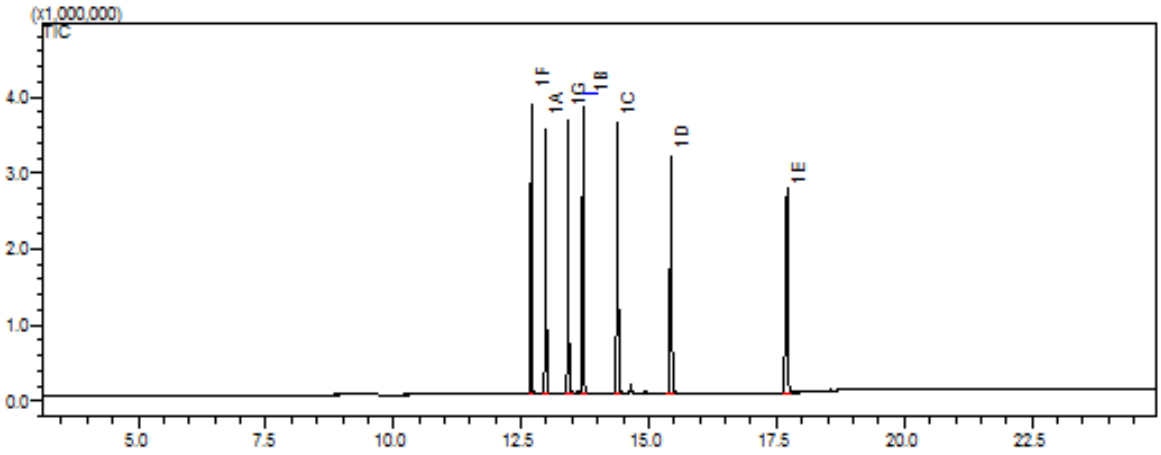
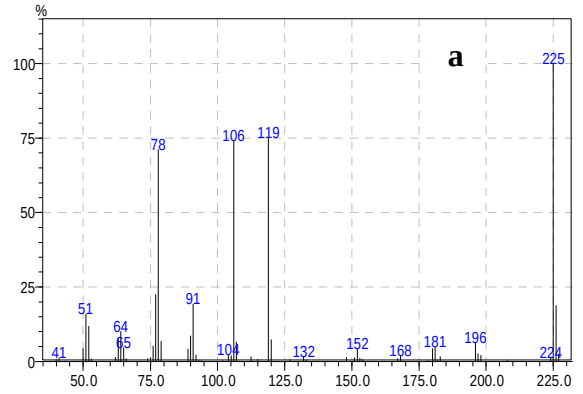
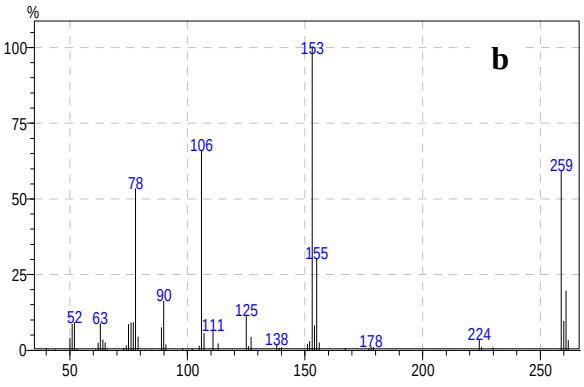
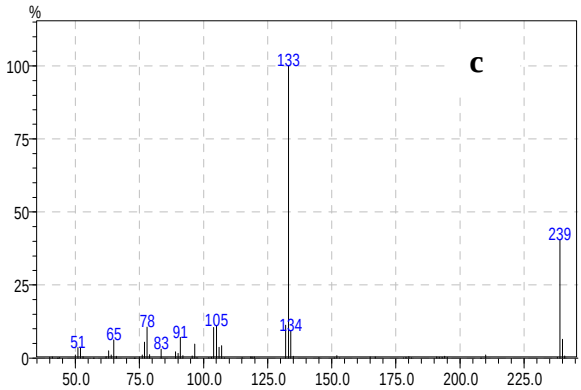


Fig-2: GC-MS Chromatogram, mixed standard of 3-aryl-3,4-dihydro-2H-benz[e]-1,3-oxazin-2-ones







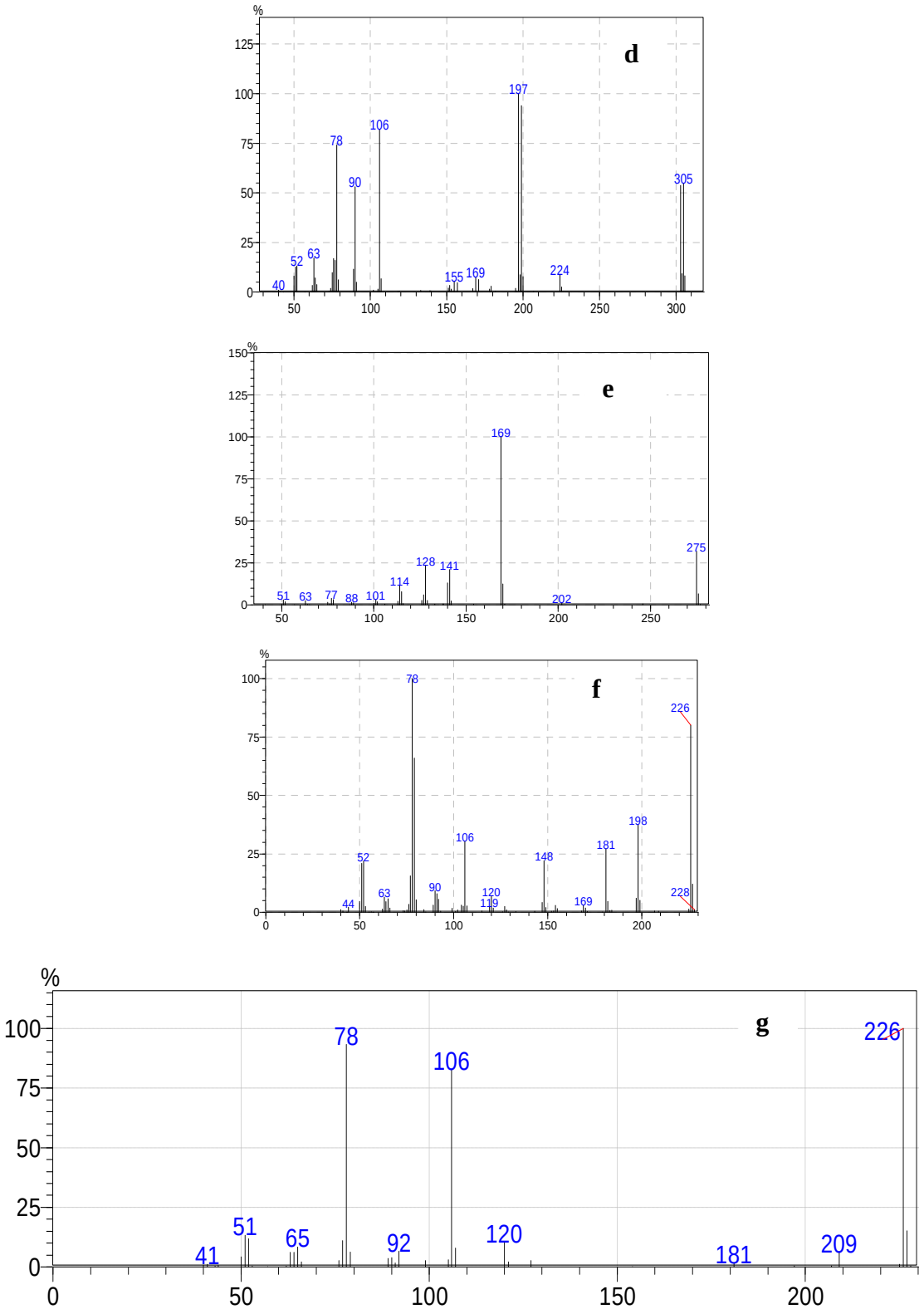


Fig-3: GCMS-EI Mass spectrums of peaks of 3-aryl-3,4-dihydro-2H-benz[e]-1,3-oxazin-2-ones

Standard preparation:

Stock solutions of all benz-1,3-oxazine derivatives were prepared 1 µg mL−1 by weighing 10mg of each standards in different 100 ml volumetric and making up volume with 100% HPLC grade methanol.

System Suitability solution
System suitability solution for separation of series of benz-1,3-oxazine-2-ones by single method has been prepared by weighing 10mg of each standard in same 100 ml volumetric flask and making up volume with 100% HPLC grade methanol.

Test preparation
Test samples have been prepared from the material obtained after the reaction without further purification to check the presence of unreacted materials as well as unknown impurities and intermediatesformed during reaction.

Method validation
System suitability
The system deemed suitable if the following acceptance criteria were satisfied. The relative standard deviation (% RSD) of the peak area responses for analytes from six standard solution injections should not more than 2.0%. The tailing factor for the benz-1,3-oxazine derivative peaks in the resolution solution should not more than 2.0. The minimum resolution between peaks should not less than 2.0 and Theoretical plate counts in standard solution should not less than 2000.

Specificity
Specificity demonstrated that the, process impurities and degradants peaks are not interfering with the analyte peak and suitability of analytical method for stability of benz-1,3-oxazine derivatives. To evaluate the interference from degradants force degradation experiment was carried out to ensure that the method used for determination of related substance of benz-1,3-oxazine-2-one derivatives[21].

Limit of detection (LOD) and Limit of quantitation (LOQ)
The limits of detection (LOD) and limits of quantitation (LOQ) have been evaluated by serial dilutions of known low concentrations of analytes by comparing measured signals from samples with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected and quantified. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit and 10:1 for quantitation limit[22].

Linearity
Linearity of the method was determined by plotting calibration curves of 3-aryl-3, 4-dihydro-2H- benz[e]-1,3-oxazin-2-ones for concentration of mixed stock solution of standardsof 1mg mL-1 was used for preparation linearity. Different concentration ranging from 1µg mL-1 to 150µg mL-1 prepared from stock solution of mixed standard. From each of these calibration standards 1µL was injected into the GC with the help of auto injector. The calibration curve obtained was subjected to regression analysis by the least square method to calculate the calibration equation and thecorrelation coefficient (r) by Lab Solution software. The response of the compound was found to be linear in the investigation concentration range with correlation coefficient 0.9990 or more [22-23].

Precision:
The precision of the method was determined in terms ofrepeatability or reproducibility and intermediateprecision studies. Repeatability was determined byevaluating five replicates of the three differentconcentrations standard solution of mixed standard on the same day (intraunder the mentioned chromatographic conditions. [24].

Intermediate Precision (Ruggedness)
Test samples of 3-aryl-3, 4-dihydro-2H- benz[e]-1,3-oxazin-2-one derivatives representing single batch was analysed by two different analysts on two different columns of the same specification, and on two different days. The ruggedness of the test method is calculated by difference between test results of six measurements and % RSD of standard solution [22].

Accuracy
A known concentration of standardsubstance (analyte) was added to blankpreparation of sample matrix and recovery ofanalyte is calculated on the basis of area obtainedin the chromatogram The result shows that bestrecoveries(99-101 %) of the spiked standardsare obtained at each added concentration, indicating that the method is accurate[23].

Robustness
The result of robustness study of the present assay method has been established by varying injector, detector temperature, initial column over temperature, carries gas from nitrogen to helium and instrument [24].The result shows that during all variance conditions, assay value of the test preparation solution was not affected and it was in

accordance with that of actual. System suitability parameters are also found under ICH criteria; hence the analytical method can be concluded as robust.

Solution stability

The results were obtained for the solution stability study at different time intervals for test preparation. It can be concluded that the test preparation solution was found stable up to 72 hours at 2-8 ˚C and ambient temperature, as during this time the result does not decrease below the minimum percentage [21].

RESULTS AND DISCUSSION

System suitability

In optimized chromatographic conditions, minimum resolution between peak were 4.617 (NLT- 2.0), maximum relative standard deviation of peaks for retention times and area were 0.029 and 0.810 (NLT–2.0), maximum tailing factor 1.319 (NLT -2.0) and minimum theoretical plate counts in standards solution 374463, which meet the ICH requirement (NLT 2000). These results conclude that method confirm system suitability criteria mentioned in the ICH and reported literature values Results of system suitability reported in Table-3.

Table-3:System suitability results

S.#	Parameter	Results	Acceptance criteria
1	Minimum Resolution R between benz-1,3-oxazine-2-one peaks 1B and 1G	4.733	NLT 2.0
2	Maximum relative standard deviation (RSD)	0.812	NMT 2.0
3	Maximum tailing factor for analyte peaks	1.198	NMT 2.0
4	Minimum Theoretical plat counts in standard solution (1C)	739696	NLT 2000

Specificity

Selectivity and peak purity on peaks were analysed by the comparison of retention times and mass spectra with reference compounds. Mass spectra were analysed at three levels (beginning, middle and end) of each peak investigated and found to be comparable. Minimum resolution between peaks is 3.395, maximum tailing factor is 1.26 and theoretical plates are more than 2000 for all peaks.Specificity results reported in Table-4.

Table-4:Specificity Results

S.#	Sample Id	Retention Time(min)	Resolution R between benz-1,3-oxazine-2one
1	Unk-1	4.294	First Peak
2	Phenol	4.599	4.578
3	Unk-2	11.809	140.673
4	Unk-3	12.308	9.891
5	1F	12.615	6.632
6	1A	12.909	5.851
7	1G	13.377	7.544
8	1B	13.638	4.079
9	1C	14.307	9.924
10	Unk-4	14.511	3.131
11	1D	15.34	11.358
12	1E	17.565	20.795
Acceptance criteria			**NLT 2.0**

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

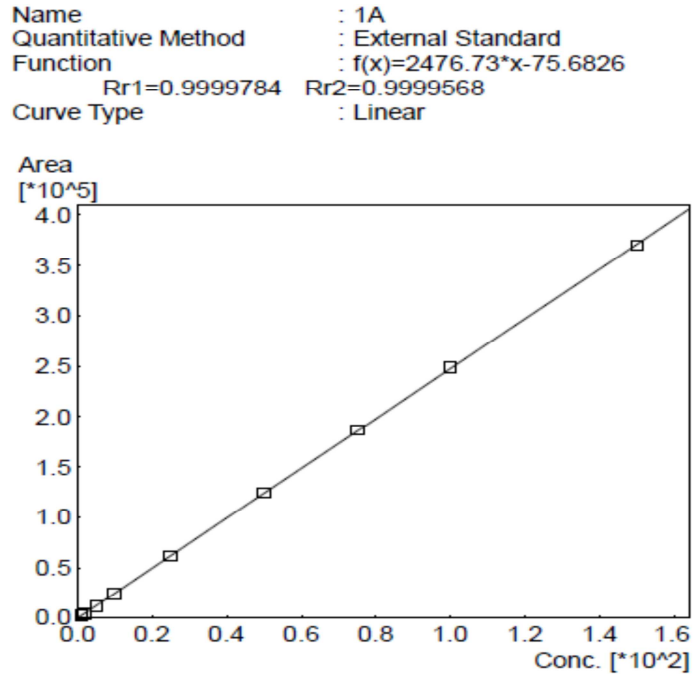
The value of LOD and LOQ for 3-aryl-3,4-dihydro-2*H*-benz[e]-1,3-oxazin-2-ones less than 1µg/ml with split injection, these values are better than reported values for similar compounds. %RSD was in the range of 1.0 -1.47 % (NMT 5.0%) for LOQ respectively. These results conclude that method confirms LOQ precision criteria mentioned in the ICH and reported literature values.The LOD value for -aryl-3,4-dihydro-2H-benz[e]-1,3-oxazin-2-ones,calculated by rms in Lab Solution software are given in table-5.

Table-5. LOD and LOQ results

S.#	Description	Limit of Detection (ppm)	Limit of Quantitation (ppm)
1	1A	0.22	0.67
2	1B	0.17	0.51
3	1C	0.22	0.67
4	1D	0.24	0.73
5	1E	0.26	0.78
6	1F	0.24	0.72
7	1G	0.34	1.00

Linearity

Linearity of peak area response versus concentration was studied over the calibration range 1µg mL-1 to 150µg mL-1 for all 3-aryl-3,4-dihydro-2*H*-benz[e]-1,3-oxazin-2-ones .The correlation co-efficient obtained was 0.999-0.9999 (NLT-0.990).The results show 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. (Figure-4).



#	Conc.	Area
1	1	2416
2	2	4829
3	5	12259
4	10	24620
5	25	61629
6	50	124461
7	75	186790
8	100	248892
9	150	370465

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

Precision:

The % RSD of the area for each benz-1,3-oxazin-2-one derivatives were calculated .The % RSD of six measurement of test sample was 0.16-1.38%. These results conclude that method confirm method precision criteria mentioned in the ICH values. (Table-6).

Table-6. System Suitability results of method precision

Parameter	Results	Acceptance criteria
Minimum Resolution R between analytes peaks	5.42	NLT 2.0
Maximum Relative standard deviation	0.66	NMT 2.0
Maximum tailing factor for benz-1,3-oxazine-2-ones	1.40	NMT 2.0
Theoretical plate count	648834	NLT 2000

Intermediate Precision (Ruggedness)

The intermediate precision of the method was evaluated using different analyst and different instrument in the same laboratory. The maximum % RSD of six measurement of test sample of analyst -1 and analyst-2 was 0.58 and 0.65 respectively (Table-7).

Table-7 : System Suitability results of IntermediatePrecision (Ruggedness)

S.#	Parameter	Results		Acceptance Criteria
		Exp-1	Exp-2	
1	Minimum Resolution R between analytes peaks	4.045	4.106	NLT 2.0
2	Maximum Relative standard deviation (%RSD)	0.58	0.65	NMT 2.0
3	Maximum tailing factor for benz-1,3-oxazine-2-ones	1.06	1.21	NMT 2.0
4	Theoretical plate count	331724	326781	NLT 2000

Accuracy

The result shows that best recoveries (99.05-100.33%) of the spiked samples were obtained at each added concentration, indicating that the method was accurate. (Table-8)

Table-8 : Accuracy results of analyte 1A

S.#	Accu. Level (ppm)	Area of Standard	Area of Spiked	Amount Recovered ppm	% Recovery	Average Recovery (%)
1	1	2282	2238	0.981	98.072	98.29
2		2270	2234	0.984	98.414	
3		2296	2259	0.984	98.389	
1	5	12236	12273	1.003	103.5	100.01
2		12308	12284	0.998	103.2	
3		12393	12385	0.999	103.8	
1	10	24195	24310	1.01	100.48	
2		24235	24332	1	100.4	100.53
3		24142	24316	1.01	100.72	
Average of average %recovery						**99.61%**

Robustness

The method was found to be robust with respect to column flow, column oven temperature and linear velocity without any changes in system suitability parameters such as resolution, tailing factor and theoretical plate. Resolution is 4.16-5.22, tailing factor is 1.009-1.168 and theoretical plate is 325892-766122 which is under acceptance criteria. These results conclude that method confirm robustness criteria mentioned in the ICH and reported literature. (Table-9)

Table-9: Robustness results

S.#	Description	Minimum Resolution	Maximum Tailing factor	Minimum Theoretical plate count
1	Condition 1.1	4.164	1.038	329167
2	Condition 1.2	4.285	1.011	386478
3	Condition 2.1	4.293	1.168	408667
4	Condition 2.2	4.344	1.006	438708
5	Condition 3.1	4.575	1.155	766122
6	Condition 3.2	4.279	1.049	464696
7	Condition 4.1	4.192	1.014	369122
8	Condition 4.2	4.141	1.048	325892
9	Condition 5.1	4.228	1.009	388197
10	Condition 5.2	5.252	1.165	649332
11	Condition 6.1	4.233	1.027	392474
12	Condition 6.2	4.556	1.114	473884
Average		**3.56**	**1.79**	**2701**
Acceptance criteria		**NLT 2.0**	**NMT 2.0**	**NLT 2000**

Solution stability

There were no significant changes in the amount of the analytes during solution stability experiment performed using the Assay method. The results from the studies indicated, the sample solution was stable at room temperature for at least 48 hour. These results conclude that method confirm specificity criteria as mentioned in the ICH and reported literature values. (Table-10)

Table-10. Solution stability results

S.#	Hours	Area (1A)	Area (1B)	Area (1C)	Area (1D)	Area (1E)	Area (1F)	Area (1G)
1	0	254640	286589	295766	316408	363966	279875	319030
2	6	254465	286528	295258	316451	363192	279810	319138
3	12	254352	285415	294043	316402	362233	279143	318071
4	24	254377	285427	294725	316212	363274	279625	318565
5	48	253786	285299	294043	315218	359623	278778	316831
6	72	253205	283025	292434	314921	355601	277953	312357
Average		254137	285381	294378	315935	361315	279197	317332
Std. Dev.		539.47	1291.27	1168.11	682.1	3185.9	740.96	2577.44
%RSD		0.21	0.45	0.4	0.22	0.88	0.27	0.81
Acceptance criteria		**NMT 2.0% (RSD)**						

CONCLUSION

The proposed GC method has been evaluated in terms of linearity, accuracy, precision, specificity, system suitability, and robustness and wasfound to be applicable in the analysisof a series of 3-aryl-3,4-dihydro-2H-benz[e]-1,3-oxazin-2-ones.The novelty of this method includes short analysistime even in the presence of unreacted materials as well as process impurities. To conclude,we consider our method to be a great tool in working with such compounds with good biological activities and class of efavirenz, a non-nucleoside reverse transcriptase inhibitor

(NNRTI).This method is recommended to the industry use for quality control of drug content in pharmaceutical preparations.

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