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Research Article

DEVELOPMENT AND VALIDATION OF STABILITY INDICATING LIQUID CHROMATOGRAPHIC METHOD OF A SEMICARBAZONE DERIVATIVE OF HIGH MEDICINAL VALUE

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ABSTRACT

An isocratic reversed phase stability-indicating high-performance liquid chromatographic (HPLC) assay method was developed and validated for quantitative determination of Semicarbazone Derivative and the simultaneous stability indicating study was carried out by using gradient pump system the mobile phase methanol : water 70:30v/v was selected to achieve maximum detection, sensitivity at ambient temperature using Lichrospher C_{18} column (250 mm × 4.6 mm,5µl) flow rate 1.0 ml/min, at 285 nm. The proposed method was found to be rapid, accurate and consistent.

Keywords: HPLC method development, Stability indicating method, Semicarbazone Derivative.

INTRODUCTION

A semicarbazone is a derivative of imines formed by a condensation reaction between ketone or aldehyde and semicarbazide. Recently some researcher had reviewed bioactivity of semicarbazones as anti-convulsant, anti-tubercular, anti-oxidant, anti-microbial, analgesic, anti-pyretic anti-inflammatory antimicrobial, antioxidant, and Anticancer activities. Synthesis of N^1-(3-cloro-4-flurophenyl)-N^4-substituted semicarbazone as novel anticonvulsant agent and several 3-chloro-4-flurophenyl substituted semicarbazones have been systhsied in three steps involving aryl urea and aryl semicarbazides formation[1] Synthesis characterization and in vitro anticancer activity of semicarbazone and thiosemicarbazone derivatives of salicylaldehyde and their copper complexes against human Brest cancer was carried out.[2] Design and synthesis of some Novel-4-(4-substituted aryl) semicarbazone as anticonvulsant agent was reported.[3] So carry out instability indicating method of such a potent drug and to get acquainted with shelf life and storage condition is a major concern of the researcher.

High performance Liquid Chromatography (HPLC) is an analytical technique to study the separation, identification and quantification of each component in a sample.Optimization and validation of Rp-HPLC stability indicating method for determination of Olmesartan Medoxomil and its degraded products was established and stability testing of an active substance or finished product providing evidence as to the quality that it remains acceptable up to the stated period under storage condition as on label.[4] Analysis of Celecoxib(CXB) in bulb drugs and in microemulsions by HPLC technique was carried out.[5]

Forced degradation is a powerful tool used routinely is pharmaceutical development in order to develop stability indicating method that leads to quality stability data and to understand the degradation pathways of drugs substance and drug products in current [6] Stress degradation studies of Cetirizine Dihydrochloride had been carried out using HPLC and analytical method developed to optimally dissolved the drugs peak and degradation products[7] Forced degradation studies of expired and marketed tablets of Amlodipine by RP-HPLC had been carried out by some researchers[8] The development and validation of thiazole derivatives under various stress conditions was carried out by HPLC technique[9] A stability-indicating HPLC method for the determination of Riluzole hydrochloride in bulk and pharmaceutical dosage form has been reported.[10]A stability-indicating liquid chromatography method has been developed

and validated for the determination of Cinacalcet hydrochloride in a laboratory mixture as well as in a tablet formulation developed in-house.[11]

MATERIALS AND METHOD

High Performance Liquid Chromatography method had been developed on SHIMADZU LC 20 at dual pump system. The system was controlled and data analysis were performed with spinchrom CFR software. The assay were performed on LC system consisting of SHIMADZU LC-20 and SHIMADZU UV detector, samples were injected with Rheodyne injector system with 20µl sample loop. The detector was set at 285 nm and peak areas were integrated automatically by computer using the spinchrom CFR software program. Detection carried out at ambient temperature using LichrospherC_{18} column [250mm×4.5mm,5µ]. All the calculation concerning the quantitative analysis were performed by the measurement of peak areas. The sample solution and mobile phase were filtered through 0.25 and 0.45 membranes respectively and degassed through ultrasonic bath.

Chemical

(2Z)-2-[(2Z)-1-(2-hydroxyphenyl)-2-(4-methoxy -benzylidene)-3-Oxobutylidene] hydrazine carboxamide was synthesized in laboratory , and high purity was maintained.HPLC grade methanol and water (Qualigen fine chemicals) of analytical grade.

Preparation of Mobile Phase

The mobile phase was prepared by using the solution of methanol and water in the ratio 70:30 respectively and filtered through 0.45µ filter paper and degassed.

Preparation of Standard Solution

Standard stock solution of Semicarbazone had been prepared by dissolving 1mg/ml. This stock solution was used to prepare the ten solution of various concentration i.e.5-50µg/ml by dilution of suitable aliquots of mobile phase.

Preparation of Sample Solution

The ten samples solution of Semicarbazone Derivative were prepared by using stock solution and centrifuged at appropriate rpm then filtered through 0.25µ membrane filter paper and degassed. All solution were freshly prepared before the analysis.

RESULT AND DISCUSSION

Method Optimization

HPLC analysis of (2Z)-2-[(2Z)-1-(2-hydroxyphenyl)-2-(4-methoxy-benzylidene)-3-Oxobutylidene] hydrazinecarboxamide was performed using methanol and water in ratio70:30 as a mobile phase. The method had been developed on SHIMADZU LC-20 at dual pump system and the system was controlled and data analysis were performed with spinchrom CFR software . Assay were performed on LC system consisting of SIMADZU LC-20 shimadzu UV detector. Samples were injected with a Rheodyne injector system with a 20µl sample loop. The mobile phase methanol : water 30:70 was selected and to achieve maximum detection sensitivity ,was carried out at ambient temperature using Lichrospher c18 column (250 mm × 4.5 mm ,5 µl)
flow rate 1.0 ml/min, it was filtered through 0.45 µ & 0.25 µ filter & sonicate for 5min in ultrasonic bath. Samples were analyze at 285 nm at an injection volume 20µl . The chromatographic run time was 5 minutes and column void volume 1.870 minute.(fig-1). The developed chromatographic method was validated for precision, accuracy, LOD,LOQ, Linearity, Robustness, Solution Stability, Specificity as per ICH guidelines.

Optimized Chromatographic Condition

Parameter	Optimised Condition
Chromatograph	SHIMADZU-HPLC
Column	Lichrospher C_{18},250×4.6mm,5µl
Mobile phase	Methanol: water(70:30v/v)
Flow rate	1.0ml/min
Detection	285nm
Injection volume	20µl
Temperature	Ambient
Retention time – semicarbazone	1.870 min

Precision

Precision of the method was determined by repeatability and intermediate precision for three consecutive days. Four different concentrations of semicarbazone were analysed in five independent series during the same day (intraday precision) and over three consecutive days (inter day precision). Every sample was injected six times.The result shown Table-01

Accuracy

The accuracy of an analytical method is the closeness of the test results obtained by method or true value. The accuracy define in terms of % deviation of the calculated concentration from the actual concentration is listed Table-02

LOD and LOQ

The limit of detection (LOD) can be defined as the smallest level of analyse that gives a measurable response.The LOD is based on a certain signal to noise (S/N) ratio.Typically 2 or 3 are recommend that a (S/N) ratio of 3 be used as the limit of detection for HPLC. The limit of quantification (LOQ) can be "define as the smallest concentration of analyte which gives a response that can be accurately quantified".

LOQ was calculated to be 5.412 µg/ml which is mention in Table-03

Robustness

Robustness relates to the capacity of the method to remain unaffected by small but deliberate variation introduced into the method parameters. Several experimental parameters like mobile phase, methanol and water ratio (70:30) and flow rate (1.0ml/min) were varied around the value set in method to reflect changes likely to rise in different test environment.The determination of 10µg/ml under the various conditions was performed.Each mean value was compared with the mean value obtained in optimum conditions and no difference was found between the results. Therefore the method is robust to the small change in experimental condition listed in Table-04

Linearity

Linearity is the ability of assay to retain values that are directly proportional to the concentration of the target analyse in the sample.The linearity of a method is measure of how well of calibration plot of a response v/s concentration in fig.02, approximately a straight line. Linearity can be assume by performing single measurement at a several analyse concentration. The resulting plot slope intercept and correlation coefficient provide the desired information on linearity. The numerical value of the slope and intercept will depend on the response measured, Intercept greater than 2% are typically expected with well designed HPLC method for major compound analysis linearity correlation coefficient above 0.9880 is accepted for most method especially for major component in assay method.

Table-03 represent the equation of the regression line correlation coefficient(r^2) Relative Standard Deviation(RSD) value of the slope and intercept for compound. Excellent linearity was obtained for compound between the peak area and concentration of 5-50µg/ml with r^2= 0.9880 for semicarbazone.

Ruggedness

The ruggedness of the HPLC method evaluated by caring out analysis using a standard working solution, the same chromatographic system and the same column on different days. The standard solution was injected six times as a test sample.Small difference in area and good consistency in retention time were observed after 90hrs.an RSD of less than 0.4853% for area and for retention time were obtained on different days indicates that the method is capable of producing results with high precision on different days, method is fairly rugged which is mention in Table-05

STABILITY INDICATING METHOD

Degradation of molecule to an appropriate extent by means of various stressing agent like temperature ,light, chemical agent, mechanical stress. The final purposes is to mimic what could happened under storage condition and to confirmed that stability indicating analytical method are appropriate.

All degradation studies of (2Z)-2-[(2Z)-1-(2-hydroxyphenyl)-2-(4-methoxy-benzylidene)-3-Oxobutylidene] hydrazinecarboxamide were carried out by using concentration 50µg/ml. Natural degradations was studied using a solutions of semicarbazone derivative in methanol and refluxed for 24 h. For Acidic degradation 0.1 M HCL was used at room temperature and reflux conditions, Alkaline degradations was also studied using 0.1 M NaOH at room temperature and reflux condition and Oxidative stress degradation 3% and 5% H_2O_2 room temperature and reflux conditions, The disappearance of Semicarbazone derivatives was observed by injection of samples at different time intervals to HPLC system and comparing the peak area with a standard solution. The experiment was performed in six times at each temperature and time interval.

Alkaline Hydrolysis

At room temperature

1 mg/ml of Semicarbazone derivative solution,was prepared in 10ml volumetric flask and working solution treated with 0.1 M sodium hydroxide solutions .Equal volume of reactant & reagent was transferred to round bottom flask and kept 24 hrs. at room temperature, after 24 hrs. reaction mixture was neutralized by dilute HCl , reaction mixture was diluted with mobile phase ((70:30) methanol/water) filtered and sonicated , 20µl of the dilute reaction mixture was injected to HPLC system to analyse the degradations of the product in replication.

At high temperature

1ml working solution of Semicarbazone derivative was refluxed with 1 ml,0.1M NaOH solutions at 80^0C for 30-minuites in round bottom flask, After cooling, the reaction mixture diluted with appropriate mobile phase, filtered and sonicated, 20µl diluted solution was injected to HPLC systems six times to observe degradation at high temperature.

A standard solution of semicarbazone derivative was subjected to alkaline hydrolysis at room temperature (24 hrs.) and to reflux at 80^0C. The disappearance of signal from RT 1.903 and appear at 2.493 and 2.450 RT indicate that compound had been completely hydrolysed or degraded in to different product. Since the reactive functionalities in semicarbazone is –N=N-CO-NH_2 may be hydrolysed to -N=N-COOH in the products and so signal appears at different retention time as shown in Table-06

Acid Hydrolysis

At room temperature

1 mg/ml of Semicarbazone derivative solution was prepared in 10 ml volumetric flask and treated with 0.1M HCL solution. Equal volume of reactant & reagent was transferred to round bottom flask and kept 24 hrs at room temperature. After 24 hrs reaction mixture was neutralized by dilute NaOH, dilute the solution with mobile phase (70:30 methanol/water), filter and sonicate.20µl of the diluted reaction mixture was injected to HPLC system to analyse the degradation of the product in replicate.

At high temperature

1 mg/ml Semicarbazone derivative solution was refluxed with 1ml,0.1M HCL solution at 80^0C for 30 min in a round bottom flask, after cooling, the reaction mixture ,diluted with appropriate mobile phase, filtered and sonicated. 20µl diluted solution was injected to HPLC system in six times to observe degradation at high temperature .

A standard solution of semicarbazone derivative was subjected to acid hydrolysis at room temperature (24 hrs.) and to reflux at 80^0C. On method optimization its signal appear at 1.903RT on hydrolysis disappearance of this signal indicate that semicarbazone derivative was completely degraded into different products. So signal shifted at 2.530 and 2.413 respectively as in Table-07

Oxidative Stress Degradation

At room temperature

1 mg/ml of Semicarbazone derivative solution was transferred to 10ml volumetric flask and added in 3% v/v hydrogen peroxide solution immediately after making up the volume, the solution was transferred to round bottom flask and kept at room temperature. Sample was withdrawn at 24 hrs. and diluted to 10 ml with mobile phase filtered, sonicated and injected into HPLC system. Same procedure repeated with 5% v/v H_2O_2 Solution.

At high temperature

1 mg/ml of Semicarbazone derivative solution and 1 ml of 3% H_2O_2 solution was transferred in a round bottom flask and reflux for 30 minutes after cooling the solution transferred in volumetric flask filtered, sonicated and injected to HPLC system six times. Same procedure repeated with 5% v/v H_2O_2 Solution.

In these stress condition sample was subjected to mild oxidation by using 3% and 5% hydrogen peroxide solution. Appearance of the signals at 1.903 but with different peak area indicate that semicarbazone derivative was not completely get oxidised in these condition as shown in Table –08

CONCLUSION

A rapid RP-HPLC method was successfully developed for the determination of Semicarbazone derivative .the developed method is selective, precise,accurate,and linear, forced degradation data proved that the method is specific for the analysis and free from the interference of blank and unknown degradation products the result indicate the suitability of the method for acid, base,oxidation and sunlight degradation studies.The method is suitable for the analysis of stability of samples and the routine analysis of semicarbazone derivative in drugs.

Instability study of the (2Z)-2-[(2Z)-1-(2-hydroxyphenyl)-2-(4-methoxy- benzylidene)-3-oxobutylidene]hydrazinecarboxamide under various stress condition shown that the sample is get completely hydrolysed in acidic and alkaline condition at RT and on reflux condition as well. Whereas mild oxidising agent is not completely oxidised sample. This study acquainted us with the degradation pathway of drug in extreme chemical and environmental conditions and chemical behaviour of the molecule which helps in the development of formulation and packaging of potent drug to researchers and may be beneficial to society.

Table 01

Compound	Λmax	Peak area	RSD%
Semicarbazone Derivative	285nm	0.9500	3.8476

Table 02

Compound	Spiked concentration µg/ml	Measured concentration µg/ml	% RSD	%Deviation
Semicarbazone	15	14.15	1.8064	5.333
Semicarbazone	20	17.92	3.8476	10.00

Table 03

Compound	λmax	Equation	R^2	LOQ µg/ml	LOD µg/ml
Semicarbazone Derivative	285nm	Y=0.04434x	0.9880	5.412	1.786

Table 04

Time	0.9ml/min	1ml/min	1.1ml/min
Mean(RT)	2.643	1.870	1.713
S.D.	0.092	0.141	0.156
%RSD	1.423	3.092	3.745

Table 05: Day to Day variability according to area (n=6)

Date	12 April	13 April	14 April
Compound	Semicarbazone	Semicarbazone	Semicarbazone
Area	0.9500	0.9439	0.9444
S.D	0.0892	0.0156	0.0165
R.S.D%	3.8476	0.6744	0.7013

Table 06: Alkaline Hydrolysis

Sr. No.	Reaction condition	Retention time	Λmax	Peak area	Concentration	% Degradation
1	At rt24 hrs.	2.493	285	2.111	37.83	100%
2	At 80^0C	2.450	285	3.317	59.44	100%

Table 07: Acid Hydrolysis

Sr.No.	Reaction condition	Retention time	λmax	Peak area	Concentration µg/ml	% Degradation
1	rt24 hrs.	2.530	285	1.275	22.84	100%
2	At 80^0C	2.413	285	1.430	25.62	100%

Table 08: Oxidative Stress Degradation

Sr. No.	Reaction condition	Retention time	Λmax	Peak area	% Concentration	% Degradation
1	3% H_2O_2at rt	1.920	285	0.253	9.08	90.93
2	3% H_2O_2 at 80^0C	1.950	285	0.492	17.63	82.37
3	5% H_2O_2 at rt	2.167	285	0.490	17.56	82.44
4	5% H_2O_2 at 80^0C	1.953	285	0.937	33.58	66.42

Table 09: Quantification of Semicarbazone derivative under various stress conditions

Sr.No.	Reaction condition	% Recovery	% Degradation
1	**Acid hydrolysis**		
	rt-24hrs	0	100
	Hyd. At 80 degree	0	100
2	**Base hydrolysis**		
	rt-24hrs	0	100
	Hyd. at 80 degree	0	100
3	**Peroxide oxidation**		
	3% H_2O_2 at rt 24 hrs.	9.08	90.92
	3% H_2O_2 oxidation at 80^0C	17.63	82.37
4	5%H_2O_2 at rt 24 hrs	17.56	82.44
	5% H_2O_2 oxidation at 80^0C	33.58	66.42

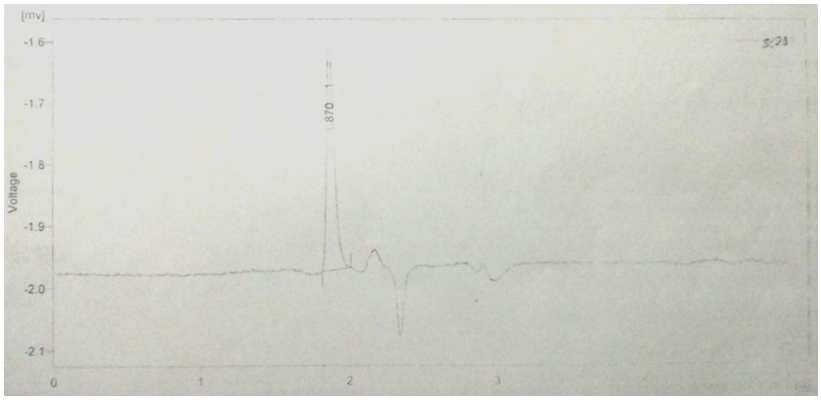

Fig. 01: Standard Chromatogram

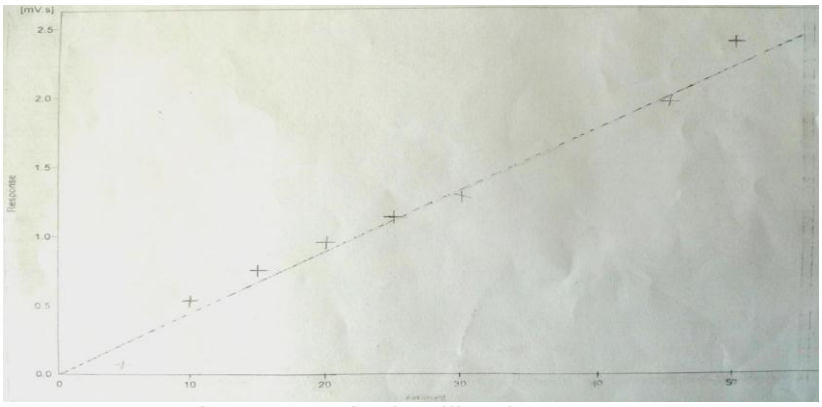

Fig. 02: Standard Calibratiom Curve

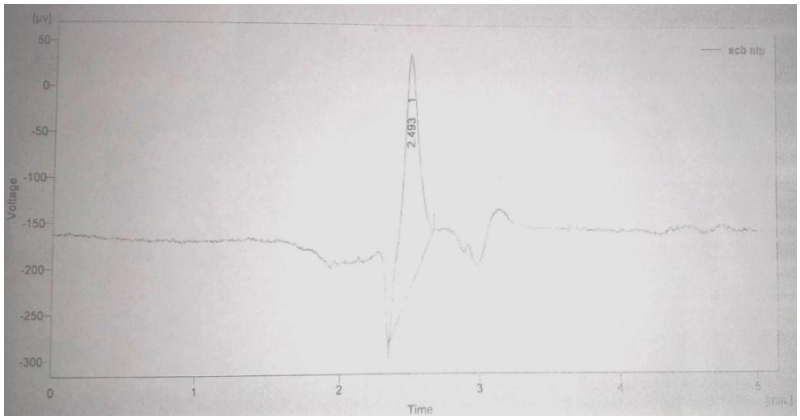

Fig. 03: Chromatogram: Effect of alkali at rt-24hrs

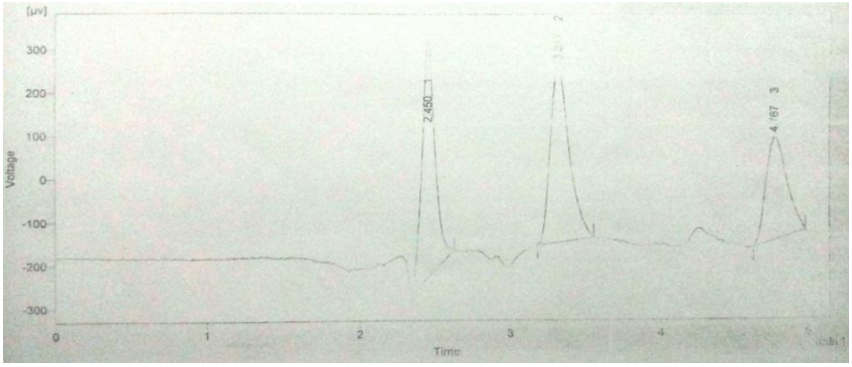

Fig. 04: Chromatogram: Effect of alkali at 80°C

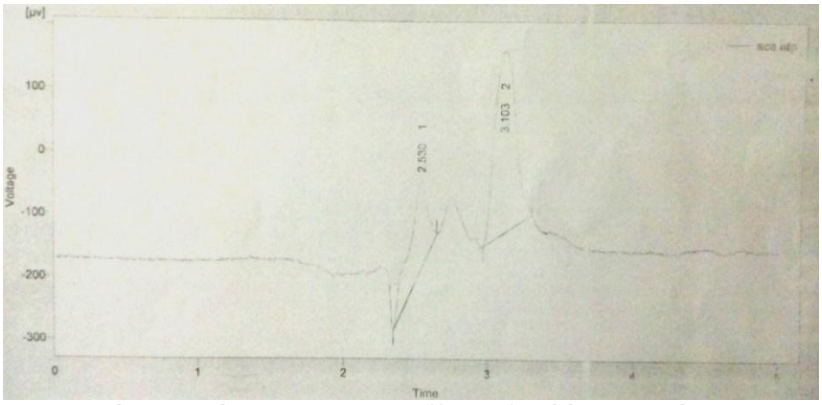

Fig. 05: Chromatogram: Effect of acid at rt-24 hrs

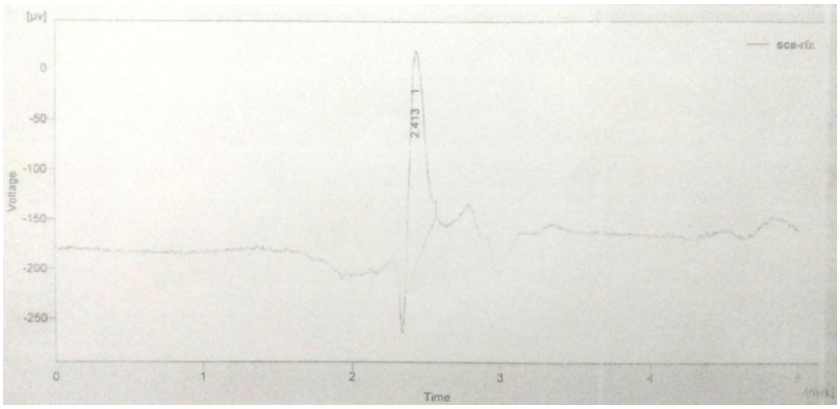

Fig. 06: Chromatogram: Effect of acid at 80°C

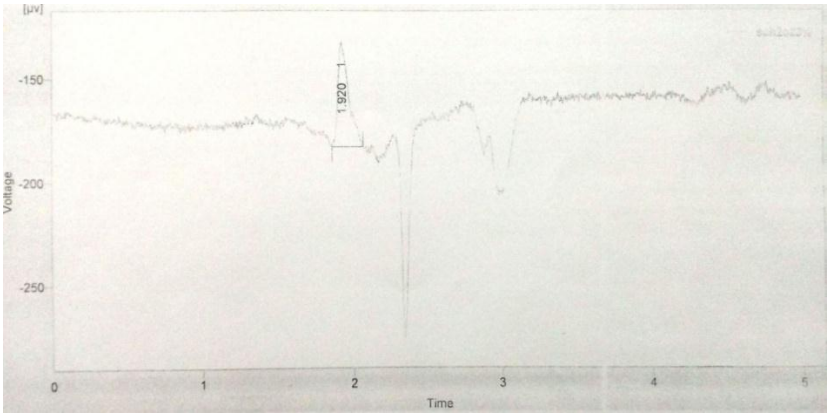

Fig. 07: Chromatograms: 3% H_2O_2 at rt

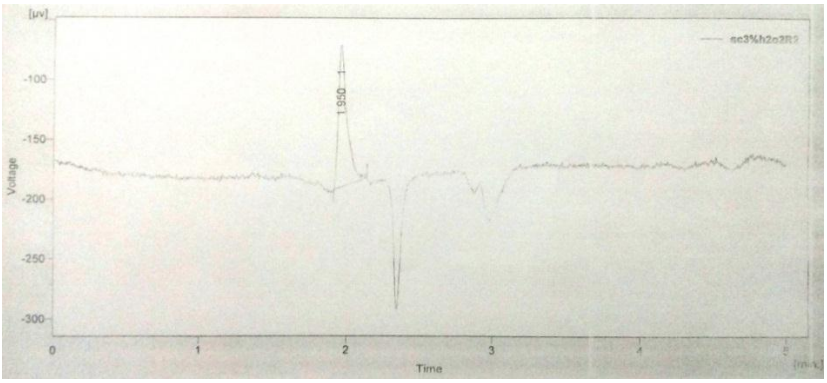

Fig. 08: Chromatogram:3% H_2O_2 80°C

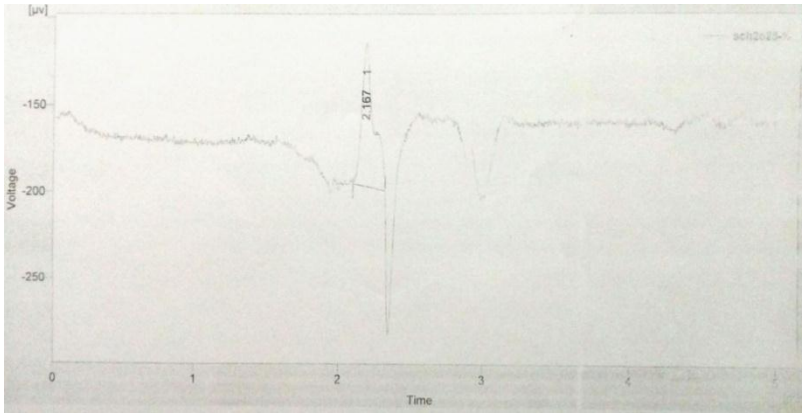

Fig. 09: Chromatogram: 5% H_2O_2 at rt

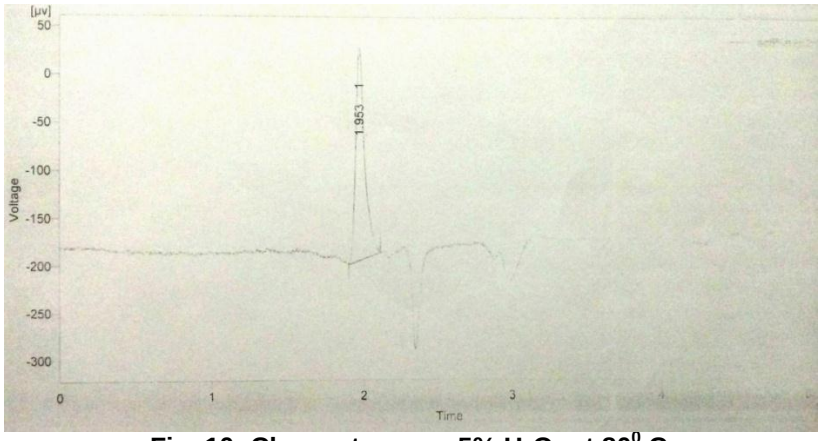

Fig. 10: Chromatogram: 5% H_2O_2 at 80^0 C

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