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Research Article

DEVELOPMENT AND VALIDATION OF METHODS FOR ESTIMATION OF ADRENOCHROME MONOSEMICARBAZONE IN INJECTION

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ABSTRACT

Objective: The present research work aims to develop a simple, sensitive, accurate and reproducible method for the estimation of Adrenochrome monosemicarbazone by spectrophotometric and chromatographic methods.

Methods: In spectrophotometric method an absorbance maximum for Adrenochrome monosemicarbazone was found to be at 354 nm using water as a solvent. In RP-HPLC, chromatographic separation was carried out on Shimadzu LC-2010 CHT using KROMACIL C-18 (250 mm × 4.6 mm, 5.0µ) column as stationary phase and mobile phase containing Methanol:Water (50:50 v/v) at flow rate of 1 ml/min using UV detection at 354 nm. The retention time was found to be 2.7 min. The methods were successfully validated in accordance to ICH guidelines.

Results: Linearity for the spectrophotometric method was observed in concentration range of 1-7µg/ml. The RP-HPLC method was found to be linear in the range of 10-60 µg/ml. The drug was found to undergo substantial degradation when exposed to acidic, basic, oxidation, thermal and photo degradation conditions.

Conclusion: The developed method can be applied successfully to estimate Adrenochrome monosemicarbazone in injection without the interference of common excipients.

Keywords: Adrenochrome monosemicarbazone, UV Spectrophotometry, RP-HPLC, Forced degradation.

INTRODUCTION

Adrenochrome monosemicarbazone (AMC) also known as Carbazochrome a haemostatic suitable for all types of bleeding. Chemically it is Hydrazinecarboxamide, 2-(1,2,3,6-tetrahydro-3-hydroxy-1-methyl-6-oxo-5H-indol-5-ylidene), an indole derivative (Figure 1). It is an oxidation product of adrenaline[1]. Comprehensive literature survey reveals that several analytical methods have been reported for the estimation of Carbazochrome, Carbazochrome sodium sulfonate and Epinephrine which includes Potentiometric titration[1], UV Spectrophotometric[2] RP-HPLC[3-8], Spectrofluorimetric method[9] and Liquid chromatography coupled with atmospheric pressure chemical ionization tandem mass spectrometry (LC-APCI-MS-MS) in human plasma[10] and Stability studies[11,12]. However, the literature survey does not reveal any spectrophotometric, chromatographic and forced degradation method for the estimation of Adrenochrome monosemicarbazone. The present paper presents the development of a simple, sensitive and accurate UV spectrophotometric, RP-HPLC and forced degradation method for estimation of Adrenochrome monosemicarbazone in injection.

MATERIALS AND METHODS

Chemicals and Reagents

The pure drug AMC was obtained as a gift sample from Roselab Biosciences Ltd., Ahmedabad. Kerutin-C injection (Adrenochrome monosemicarbazone-1mg) was purchased from local market. HPLC grade Methanol (RANKEM), 3% H_2O_2 (RANKEM), water for injection, hydrochloric acid and sodium hydroxide of AR grade were used for the work..

Instrumentation

A UV–Visible double beam spectrophotometer (Shimadzu) model 2550 with spectral slit width of 2.0 nm was used for experiments. Chromatographic separation was carried out on Shimadzu LC-2010 CHT equipped with KROMACIL C-18 column (250 mm × 4.6 mm, 5.0µ). Vacuum oven and UV cabinet were used for forced degradation.

Experimental Work

UV-Spectrophotometry Method

Selection of solvent

The drug is soluble in water. Therefore water was selected as solvent.

Preparation of stock solution

The standard stock solution of AMC was prepared by dissolving 25 mg AMC in water and final volume was adjusted with same solvent in 100 ml of volumetric flask to get strength of 250 µg/ml. Further dilution was carried out with water to get 10 µg/ml of AMC.

Selection of analytical wavelength

1-7 µg/ml solutions of AMC were prepared in water and spectrum was recorded between 200-600 nm and 354 nm was obtained as the λmax.

Preparation of Sample Solution from Injection

Accurately measured 2.5 ml of AMC was transferred to 10 ml volumetric flask, 5 ml of water was added to the same flask, sonicated for 5 min and volumes were made up to mark with water to get strength of 250 µg/ml. Further dilution was carried out by diluting 4 ml of sample solution in 10 ml volumetric flask with water to get 100 µg/ml. Transfer 1.5 ml of solution into 50 ml volumetric flask and dilute to the mark with water to get a final concentration 3 µg/ml. The sample solution was scanned in the wavelength range of 200–600 nm and measured the absorbance at 354nm. The % drug found was calculated by y=mx+c equation.

Method Validation [13]

Linearity

A calibration curve was plotted over a concentration range of 1-7µg/ml. Accurately measured standard stock solution of AMC (1, 2, 3, 4, 5, 6 and 7 ml) were transferred to a separate series of 10 ml of volumetric flasks and diluted to the mark with water. The absorbance of each solution was measured at 354 nm. Calibration curves were constructed by plotting absorbance versus concentrations at 354 nm. Each reading was average of three determinations. (Fig. 2-3)

Precision

The intra-day and inter-day variation for determination of AMC was carried out three times in the same day and three consecutive days and % RSD were calculated. The method was found to be precise due to low values of the %RSD (Table 2).

Accuracy (% Recovery)

Accuracy of the method was determined in terms of % recovery of standard. Recovery studies were carried out by addition of standard drug solution at the level of 50%, 100% and 150% to the preanalyzed sample. In this method the known concentration of standard drug was added to the assay sample. The average percent recoveries for AMC are shown in Table 3.

LOD and LOQ

The LOD and LOQ of developed method were studied as per ICH guidelines. Several approaches for determining the LOD & LOQ are possible, depending on the procedure i.e, a non-instrumental or instrumental. Among them here employed method was,

LOD= 3.3σ/S and

LOQ= 10σ/S

Where σ = the standard deviation of response,

S = the slope of calibration curve.

The results obtained are shown in Table 1.

RP-HPLC Method

Preparation of Mobile phase

Mobile phase composition was Methanol: water (1:1). The mobile phase was sonicated for 15 min and then it was filtered through 0.45µm membrane filter paper.

Working Standard Stock Solution

An accurately weighed quantity of standard AMC (5 mg) powder was weighed and transferred to 50 ml volumetric flask and dissolved in 30 ml of mobile phase. The flasks were shaken and sonicated for 15min and volumes were made up to mark with mobile phase to get 100 µg/ml. Then 1, 2, 3, 4, 5, 6 ml of AMC were transferred to 10 ml volumetric flasks and made up to mark with mobile phase.

Sample Solution

Accurately measured 5 ml of Adrenochrome monosemicarbazone formulation (1 mg/ml) was transferred to 50 ml volumetric flask and dissolved in 30 ml of mobile phase. The flask was shaken and sonicated for 15 min and volumes were made up to mark with mobile phase to get 100 µg/ml. The sample solution was filtered through 0.45µm membrane filter paper. The sample solution was further diluted with mobile phase to obtain the final concentration (40 µg/ml).

Chromatographic condition

Chromatographic separation was carried out on Shimadzu LC-2010 CHT using KROMACIL C-18 (250 mm × 4.6 mm, 5.0µ) as stationary phase and mobile phase of Methanol:Water (50:50 v/v) at flow rate of 1 ml/min using UV detection at 354 nm.

Method Validation [13]

Linearity

The linearity of the response for AMC assay method was determined by preparing and injecting standard solutions with concentrations of 10-60µg/ml. The calibration curve (Fig. 5) indicates that the response is linear over the concentration range studied with correlation coefficient (R^2) value 0.9986.

Precision

The precision of the method was demonstrated by inter-day and intra-day variation studies. In the intra-day studies, three repeated injections of standard and sample solutions were made and the response factor of drug peaks and percentage RSD were calculated. In the inter-day variation studies, three repeated injections of standard and sample solutions were made for three consecutive days and response factor of drugs peaks and percentage RSD were calculated. From the data obtained, the developed RP-HPLC method was found to be precise (Table 7).

Accuracy

It was performed at three levels 50%, 100%, 150% by Standard addition method. Each concentration was analyzed 3 times and average recoveries were measured (Table 8).

Limit of Detection and Limit of Quantification

The limit of detection (LOD) and the limit of quantification (LOQ) of the drug were derived by calculating the signal-to-noise ratio (S/N, i.e., 3.3 for LOD and 10 for LOQ) using the following equations as per International Conference on Harmonization (ICH) guidelines.

LOD = 3.3 × σ/S

LOQ = 10 × σ/S

Where σ = the standard deviation of the response and S = Slope of calibration curve.

The results obtained are shown in Table 6.

Robustness

Robustness of the method was determined by making slight changes in the chromatographic conditions. It was observed that there were no marked changes in the chromatographic parameters (Table 9).

Analysis of AMC in Dosage Form

Pharmaceutical formulation of AMC was purchased from local pharmacy. The response of formulation was measured at 354 nm for quantification of AMC by using RP-HPLC. The amount of drug present in sample solution was determined by fitting the response into the regression equation for AMC. Result is given in Table 10.

Forced degradation studies [14-15]

Acid hydrolysis

About 2.5 mg of AMC was weighed accurately and transferred in to 25 ml volumetric flask. Add 2 ml of 0.1N HCl in flask for acid hydrolysis, Solutions were kept for 30 min at room temperature. Then 2 ml of 0.1N NaoH added in to flask for neutralization for acid hydrolysis and mobile phase was added to volume this yielded 40 µg/ml. Filtered through 0.45 µ filter paper and injected into HPLC system.

Base hydrolysis

About 2.5 mg of AMC was weighed accurately and transferred in to 25 ml volumetric flask. Add 2 ml of 0.1N NaoH in flask for base hydrolysis, Solutions were kept for 60 min at room temperature. Then 2 ml of 0.1N HCl added in to flask for neutralization for base hydrolysis and mobile phse was added to volume this yielded 40 µg/ml. Filtered through 0.45 µ filter paper and injected into HPLC system.

Oxidative condition

About 2.5 mg of AMC was weighed accurately and transferred in to 25 ml volumetric flask. Add 2 ml of 3% H_2O_2 in flask for oxidative hydrolysis, Solutions were kept for 30 min at room temperature and mobile phase was added to volume this yielded 40 µg/ml. Filtered through 0.45 µ filter paper and injected into HPLC system.

Thermal Degradation

For thermal Degradation, the standard was kept in Petri dish and placed in oven at 60°c for 24 hour. Then solution was prepared 40 µg/ml.

Photodegradation studies

For photodegradation studies, the standard was kept in Petri dish and placed in UV cabinet for 2 days. Then solution was prepared 40 µg/ml.

RESULTS AND DISCUSSION

In spectrophotometry linearity was observed for 1-7µg/ml (Figure 2) and correlation coefficient was found to be 0.999 with %RSD below 2% (Tables 1-4). In RP-HPLC method linearity was found in the range of 10-60µg/ml with correlation coefficient of 0.9986 and total run time of 5 minutes (Tables 5-10, Figures 4-5). The validation

for both the developed methods were performed as per the ICH guideline (Q_2R_1)[13]. The forced degradation studies indicate that appreciable changes were observed by treating the drug with acidic hydrolysis, basic hydrolysis, oxidative condition, thermal degradation and photodegradation (Table 11 and Figure 6). The developed methods were successfully applied to the estimation of the drug in commercially available adrenochrome monosemicarbazone injection. The results obtained indicate the additives present in the formulation do not interfere with analysis of the injection. The developed methods can be used in quality control laboratories for analysis of Adrenochrome monosemicarbazone in injection formulation.

CONCLUSION

The proposed methods are accurate, precise, simple, sensitive and rapid and hence can be applied for routine estimation of adrenochrome monosemicarbazone in injection without interference.

ACKNOWLEDGEMENTS

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Table 1: Regression characteristics and validation parameters of AMC

Parameters	Result
Solvent	water
λ_{max} (nm)	354
Beer's law limit (µg/ml)	1-7
Regression equation	y = 0.127x + 0.032
Slope	0.127
Intercept	0.032
Correlation coefficient (R^2)	0.999
Linearity Range (µg/ml)	1-7
LOD (µg/ml)	0.171
LOQ(µg/ml)	0.520

Table 2: Precision data

Concentration (µg/ml)	Intraday Mean ± S.D.*	% RSD	Interday Mean ± S.D.*	% RSD
1	0.161±0.0025	1.57	0.168±0.0030	1.79
2	0.271±0.0040	1.49	0.280±0.0035	1.26
3	0.409±0.0064	1.57	0.416±0.0079	1.91
4	0.551±0.0044	0.79	0.545±0.0059	1.08
5	0.683±0.0095	1.38	0.667±0.0057	0.85
6	0.831±0.0081	0.98	0.816±0.0157	1.93
7	0.924±0.0068	0.74	0.923±0.0091	0.98

***** Mean of three estimations

Table 3: Determination of Accuracy

% Level of spike	Amount Taken (µg/ml)	Amount Added (µg/ml)	Amount Found* (µg/ml)	%Recovery± SD
50%	2	1	3.003	100.11±0.0252
100%	2	2	3.957	98.92±0.0416
150%	2	3	4.963	99.27±0.0777

***** Mean of three estimations

Table 4: Analysis of Marketed formulation by UV-spectrophotometer

Formulation	Amount labeled (mg)	Amount found* (mg)	% Recovery ± S.D (n=3)
Kerutin-C	1	0.996	99.55 ± 0.021

***** Mean of three estimations

Table 5: Chromatographic conditions

Parameters	Optimized condition
Column	KROMACIL C-18 (250 mm × 4.6 mm, 5.0µ)
Mobile phase	Methanol:Water (50:50 v/v)
Flow rate	1 ml/min
Detection wavelength	354 nm
Injection volume	20 µl
Run time	5 min

Table 6: System Suitability Parameters for HPLC method

Parameters	AMC
Calibration range (µg/ml)	10-60
LOD (µg/ml)	0.059
LOQ(µg/ml)	0.179
Slope	126927
Intercept	331739
Correlation coefficient	0.9986
Intraday RSD, %	0.09-0.27
Interday RSD, %	0.05-0.20

Table 7: Precision Data

Concentration (µg/ml)	Intra-day precision		Inter-day precision	
	Mean ± S.D (n=3)	% RSD	Mean ± S.D (n=3)	% RSD
10	1599136 ± 3293.64	0.21	1600681 ± 3068.18	0.19
20	2949326 ± 5029.23	0.17	2953938 ± 5805.56	0.20
30	4041683 ± 11030.13	0.27	4053860 ± 2003.20	0.05
40	5422966 ± 14340.99	0.26	5446745±5058.54	0.09
50	6546079 ± 7855.19	0.12	6579252±10649.97	0.16
60	8017574 ± 7473.40	0.09	8045764 ± 8715.33	0.11

Table 8: Determination of Accuracy

Drug	Amount taken (µg/ml)	Amount added (µg/ml)	Amount found* (µg/ml)	% Recovery ± S.D (n=3)
AMC	10	5	14.82	98.80 ± 0.050
	10	10	19.79	98.95 ± 0.029
	10	15	24.86	99.44 ± 0.081

* Mean of three estimations

Table 9: Robustness (20 µg/ml)

Parameters	Conditions	Area
Mobile Phase	Methanol : Water(45 : 55)	2864997
	Methanol : Water(50 : 50)	2867820
	Methanol : Water(55 : 45)	2883411
	MEAN ± S.D	**2872076 ± 9917.36**
	% RSD	**0.35**
Wavelength (nm)	349	2781648
	354	2867820
	359	2829274
	MEAN ± S.D	**2826247 ± 43165.66**
	% RSD	**1.53**
Flow Rate (ml/min)	0.8	2891010
	1.0	2867820
	1.2	2797039
	MEAN ± S.D	**2851956 ± 48952.83**
	% RSD	**1.72**

Table 10: Assay of AMC in Dosage Form by RP-HPLC

Formulation	Amount labeled (mg)	Amount found* (mg)	% Recovery ± S.D (n=3)
Kerutin-C	1	1.003	100.28 ± 0.11

* Mean of three estimations

Table 11: Forced degradation study data

S. No.	Condition	% degradation*	
		Std	Test
1	Acid hydrolysis	15.97	15.54
2	Base hydrolysis	13.11	13.1
3	Oxidative condition	22.25	21.92
4	Thermal Degradation	20.64	20.26
5	Photodegradation studies	20.79	20.45

* Mean of three estimations

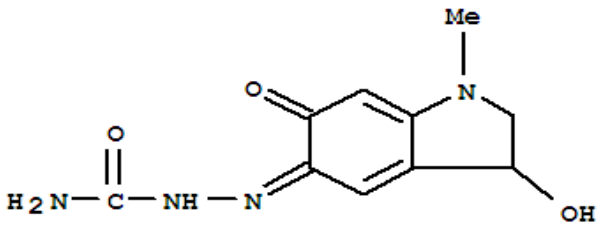

Fig. 1: Structure of Adrenochrome monosemicarbazone

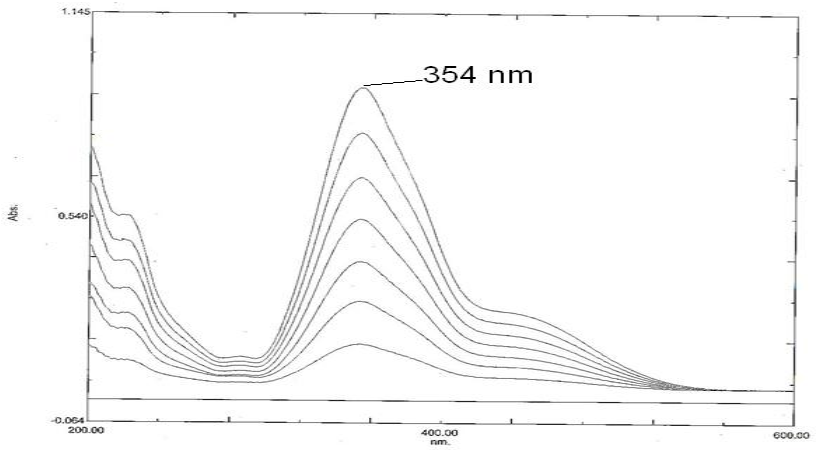

Fig. 2: Overlay spectra of AMC

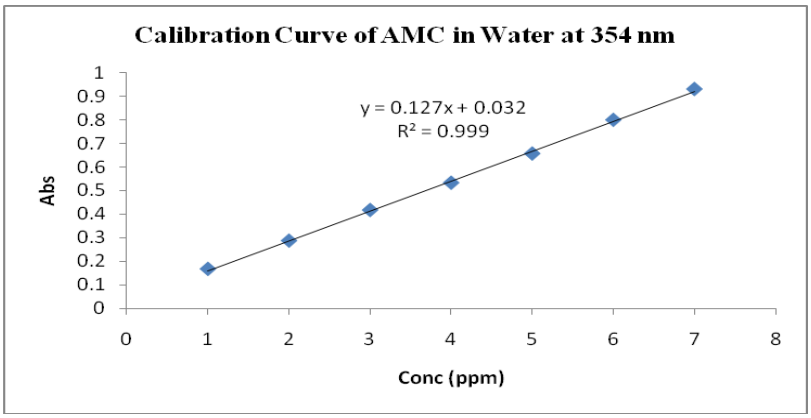

Fig. 3: Calibration curve of AMC at 354nm

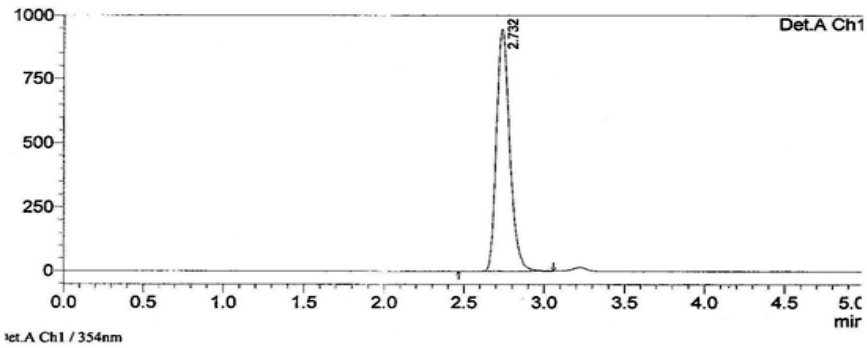

Fig. 4: RP-HPLC chromatogram of AMC(40 µg/ml)

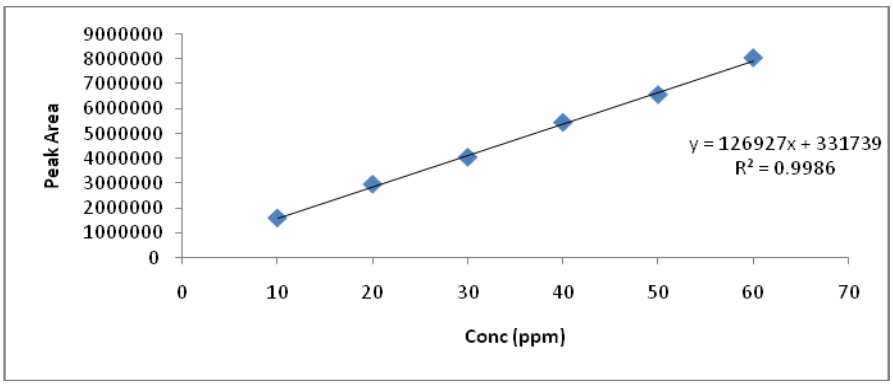

Fig. 5: Calibration curve of AMC for RP-HPLC method

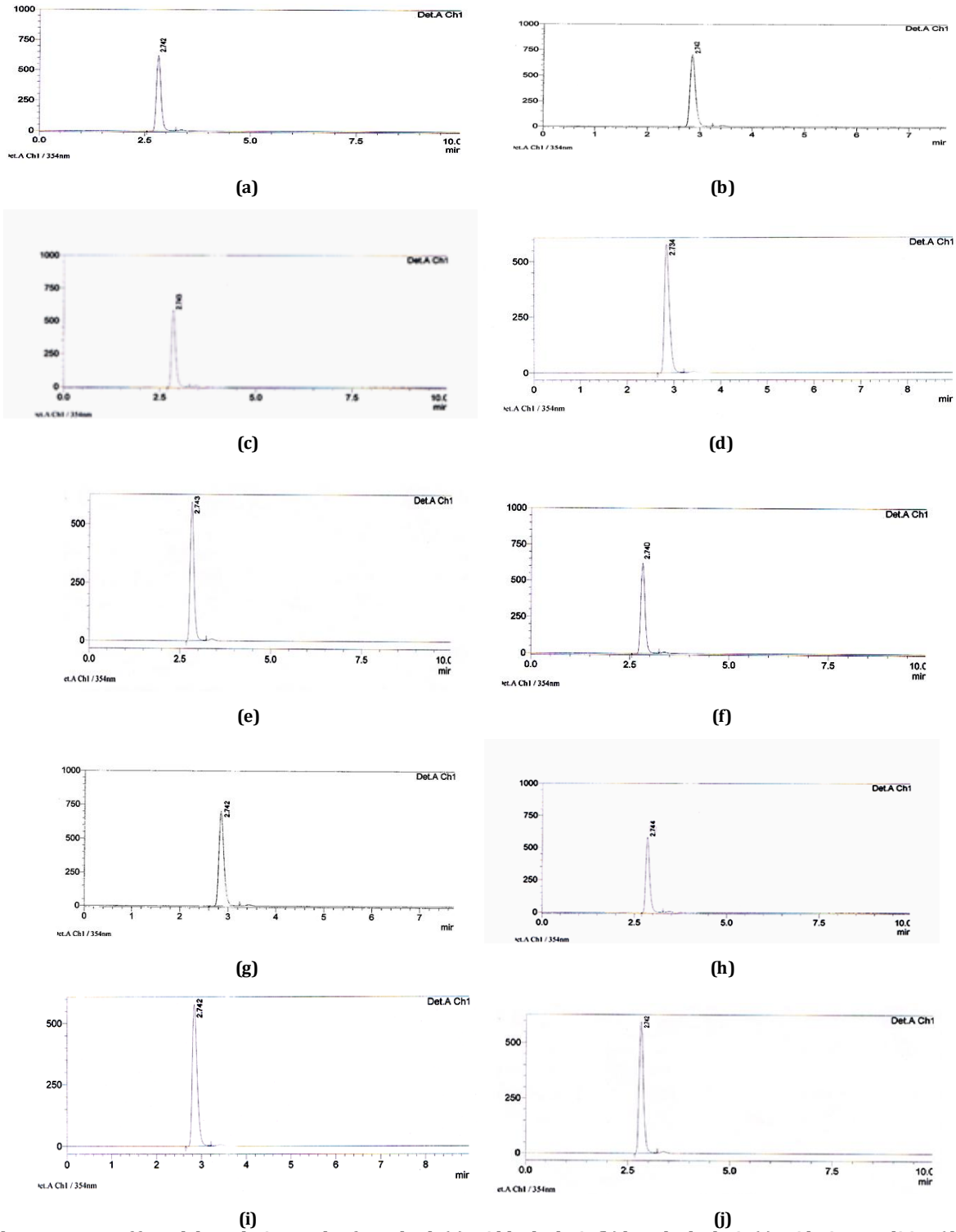

Fig. 6: Chromatogram of forced degradation study of standard: (a) acid hydrolysis (b) base hydrolysis (c) oxidative condition (d) thermal degradation (e) photodegradation and Chromatogram of forced degradation study of test : (f) acid hydrolysis (g) base hydrolysis (h) oxidative condition (i) thermal degradation (j) photodegradation.

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