Development of a stability-indicating UPLC method for determination of Isotretinoin in bulk drug

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Development of a stability-indicating UPLC method for determination of isotretinoin in bulk drug

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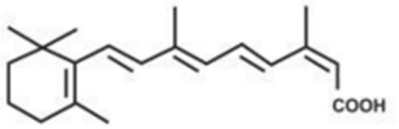
Abstract

A highly sensitive and rapid stability indicating Ultra Performance Liquid Chromatographic (UPLC) method was developed for the quantification and identification of isotretinoin in bulk. Chromatographic separation was developed using a gradient elution in a reversed-phase system at flow rate of 0.5 ml/min with 12 minutes run time. The mobile phase was a gradient mixture of mobile phase A (contained a 30:70:0.5 mixture solution of methanol / Purified water / Glacial Acetic Acid) and mobile phase B (contained a 70:25:4.5:0.5 mixture solution of methanol /Acetonitrile/Purified water / Glacial Acetic Acid). Eluents were monitored at 355 nm. The analytical method was validated for accuracy, precision, robustness, linearity and forced degradation in accordance with ICH topic Q2 (R1) Validation of Analytical Procedures: Text and Methodology. The method was linear over a concentration range of (1-7 µg/mL) with correlation coefficient of (r^2 > 0.9999). The accuracy was confirmed by calculating the % recovery which was found to be 100.1 – 101.6%. The RSD values obtained for repeatability and intermediate precision experiments were less than 2%. The limit of detection (LOD) was (0.12 µg/mL) while the limit of quantification (LOQ) was (0.38µg/mL). The drug samples were exposed to different stressed conditions and the results showed that all degradation products were satisfactorily separated from each other and from the peak of the drug using the developed method. The proposed method can be used for the quantitative determination of isotretinoin with confidence.

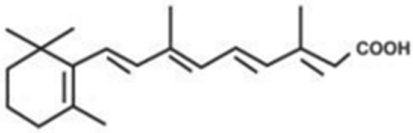
Keywords: Isotretinoin; Method development, UPLC, Forced degradation studies, ICH guidelines.

1. Introduction

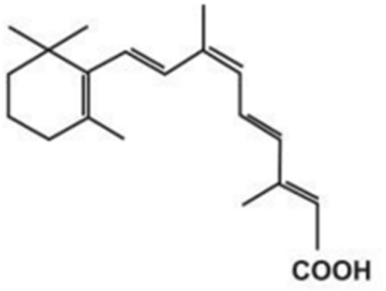
Isotretinoin is an active pharmaceutical ingredient (API) used as an oral (systemic) treatment of severe form of acne which is also known to be one of the most useful vitamin A derivatives that affects all the major factors involved in the pathogenesis of acne [1]. It is a cis-vitamin A acid (synthestic vitamin A acid derivative) and a stereoisomer of Tretinoin and isomer of Alitreitnoin. It is chemically described as (2Z,4E,6E,8E)-3,7-dimethyl-9-(2,6,6-trimehtyl-1-cyclohexenyl)nona-2,4,6,8-tetraenoic acid (also known as 13-cis-retinoic acid) [2]. Isotretinoin has a small molecular weight (300.44) and is pratically insluble in water [3]. The structural formula of isotretinoin and its isomoers, tretinoin and alitretinoin are as shown in schemes 1.a-c [4]:



Scheme 1.a. Chemical structure of Isotretinoin (13-cis-retinoic acid) (4)



Scheme 1.b. Chemical structure of Tretinoin (all-trans-retinoic acid) (4)



Scheme 1.c. Chemical structure of Alitretinoin (9-cis-retinoinc acid) (4)

Several analytical methods utilizing High Performance Liquid Chromatography (HPLC) have been developed for quantitation of isotretinoin in pharmaceutical dosage forms including both capsule and topical gel [2]. An isocratic reversed phase HPLC was developed by Mahajan et al. (2016) [2]. Normal phase HPLC method was employed in the United States Pharmacopoeia (2007) and another method was reported to determine both isotretinoin and tretinoin simultaneously in the dermatological formulations by Tashtoush et al. (2007)[, a reversed phase method equipped with fluorescence detector has also been described by Gatti et al. (2000)[6]. However, most of these methods were associated with large consumption of solvents, long run time, the use of undesirable reagents such as Tetrahydrofuran (TFA), poor sensitivity and poor resolution from retinoic isomers [2, 5].

In contrast to HPLC, Ultra-performance liquid chromatography (UPLC) is functionally advanced in its rapidness, sensitivity and resolution [7]. It can withstand much higher back pressure with smaller packed column, lower injection volume and shorter run time [7, 8]. UPLC Technology is able to provide numerous benefits to routine testing of commercialised drug products, with increased sample throughput and decreased solvent consumption [9, 10]. This indicates that an analytical testing method using UPLC may offer significant cost effectiveness by analysing more samples per system in lesser time. The overall operating expense in both development and quality control thus can be reduced with increased productivity. All these advantages over conventional HPLC has allowed UPLC to gain its place and popularity in the industry for both routine and research purpose nowadays [11]. It is thus highly desirable to develop and validate an UPLC method for isotretinoin with satisfactory resolution, sensitivity and relatively short analysis run time in line with routine analysis for quality control purposes. To the best of our knowledge, there is no published work involves development of an analytical method for quantification of isotretinoin using UPLC. Therefore, aim of the present work was to develop and validate a stability indicating method for isotretinoin using UPLC method.

2. Materials and Analytical Testing Method

2.1 Chemicals and reagents

Isotretinoin Standard (USP) and Tretinoin (USP) were purchased from United States Pharmacopeial Convention, USA. Alitretinoin was purchased form Olon S.P.A, Italy. Tetrahydrofuran, Methanol and Acetonitrile were procured from EMD Millipore

Corporation, Germany. Glacial Acetic Acid (GAA) was purchased from VWR International S.A.S, France. All the organic solvents were gradient grade. Purified water was obtained by MllieQ water purifier system, Auckland, New Zealand, Isotretinoin Active Pharmaceutical Ingredient (API), BASF, New Zealand.

2.2 Laboratory Preventive Measure

Isotretinoin is sensitive to light, heat and air, hence exposure of isotretinoin to strong light is to be avoided [2]. Therefore, all experiments were performed under the recommended conditions of protection from actinic light, i.e. performing the test in a room fitted with the appropriate light filters.

2.3 Chromatographic System and Condition

The UPLC analysis was performed on UPLC System gradient with UV Detector (Dionex UltiMate or equivalent) controlled through Chromeleom Software. Analytical Column used for this method was C18, SB, 1.8 µm, 2.1mm x 150 mm (Waters Acquity HSS C18 SB, or equivalent). The mobile phase was a gradient mixture of mobile phase A (contained a 30:70:0.5 mixture solution of methanol / Purified water / Glacial Acetic Acid) and mobile phase B (contained a 70:25:4.5:0.5 mixture solution of methanol /Acetonitrile/Purified water / Glacial Acetic Acid). Both mobile phase A and B were filtered and degassed through 0.22 µm membrane filter. The final gradient program [(min)/%B] was 0/62, 7/62, 8.5/100, 9.5/100, 9.5/62 and 12/62 (Table 1). The flow rate was set at 0.5 ml/min. The column temperature was 55^{o}C. The sample compartment was 4^{o}C. The injection volume of sample was 2 µl. The eluent was analyzed by UV Detector at wavelength of 355nm. An ultrasonic bath from Bandelin Sonorex (Germany) was used for standard and sample sonication.

2.4 Preparation of solutions

2.4.1 Standard solution preparation

Isotretinoin stock standard solution (250µg/mL) was prepared by weighing appropriate amount isotretinoin USP standard into 100 mL volumetric flask. 20 mL THF (Tetrahydrofuran) was added to completely dissolve the standard with at least 5 minutes of sonication and made up to volume with methanol. 5 µg/mL working solution was prepared by further dilution from the stock solution and made up to volume with methanol.

2.4.2 Sample solution preparation

Isotretinoin sample stock solution was prepared by weighing appropriate amount isotretinoin into volumetric flask, dissolve with 20 mL THF with at least 5 minutes of sonication and made up to volume with methanol. 5 µg/mL working solution was prepared by further dilution from the stock solution and made up to volume with methanol.

2.5 Method validation

The developed UPLC method was validated in accordance to ICH topic Q2 (R1) validation of Analytical Procedures: Text and Methodology [11] for the following.

2.5.1 Accuracy

Accuracy of the developed method was determined by calculating the percentage recovery of the analyzed spiked samples [11]. Known amount of isotretinoin were spiked in triplicates at different concentration levels of 60%, 100% and 140%.

2.5.2 Precision

Six individual samples of 100% were prepared to assess the closeness of agreement between series of measurement [12]. The %RSD value was calculated.

2.5.3 Intermediate precision

Reproducibility of testing method was examined as follows: Six samples at 100% level were prepared and analyzed on different days, instrument, column, mobile phase [12]. The % RSD values was calculated for all the replicates (combining from two analysis)

2.5.4 Linearity and range

Linearity was required to demonstrate that the detector response is directly proportional to the analyte concentration over a specific range [12]. A minimum of five solutions at the concentration from 1 to 7 µg/mL were prepared for linearity study. Duplicate injections of each solution were performed and 10 injections were performed on 100% working solution for instrument repeatability. The calibration curve was constructed and linear regression equation was obtained. The %CV (RSD) between the peak area and the actual concentration should be not less than 0.999.

2.5.5 Robustness

Robustness was assessed by altering chromatographic condition [2,12]. Chromatographic system variation was evaluated by using flow rates of ± 0.05 mL/min and column temperature of ± 2°C from the nominal conditions of 0.5 mL/min and 55°C. Combinations of variation are presented in Table 2, which shows four different variables. The recovery was studied and compared with nominal condition. The suitability of variation was thus determined.

2.5.6 LOD and LOQ

Detection and Quantitation limits are the lowest detectable and quantifiable concentration that a method can achieve. As per ICH guideline, the LOD and LOQ were determined based on the standard deviation of the response and the slope using the formulae provided below.
The detection limit (DL) is expressed as [12]:

$LOD=3.3*\sigma/S$
where σ = the standard deviation of the residue
S = the slope of the calibration curve

The quantitation limit (QL) is expressed as:
$LOQ=10*\sigma/S$
where σ = the standard deviation of the residue
S = the slope of the calibration curve

2.5.7 Forced degradation studies

Forced degradation studies were conducted to ensure that the analytical method was stability indicating and to demonstrate the ability of the method to separate isotretinoin from its degradation end products. Isotretinoin active ingredients were weighed and stressed with stressors that are recommended in the regulatory guideline [12] and analysed for isotretinoin contents. Acid degradation was carried out by incubating isotretinoin with 2M Hydrochloric acid (HCl) for 1 day. Alkaline degradation was carried out by stressing isotretinoin for 7 days with 1M Sodium Hydroxide (NaOH). The oxidative study was carried out by stressing isotretinoin with 20 mL of 30% H_2O_2 for 8 hours. Thermal degradation was done by exposing sample at 95°C for 3 days. Hydrolysis was done by adding 20 mL of purified water into sample for 3 days. Light degradation studies on isotretinoin were carried out by exposing the sample to visible light which was not less than 1200000 lx.h and UVA radiation of 220

W.h/m^2. Samples were withdrawn at specified time points. The stressed samples were neutralized (acid and base only), prepared to assay concentration and analyzed according to the chromatographic conditions with UV detection at 355nm.The stressed conditions are listed in Table 4.

3. Results and Discussion

3.1 Method development and optimization

Originally, the UPLC method we developed for the analysis of isotretinoin was based on an isocratic HPLC method as detailed by [2]. The method developed using a RP-C18 column (250 x 4.6mm, 5 µm particle size) with 10 minutes run time at flow rate of 0.8ml/min. Wavelength set at 355 nm with injection volume of 20 µl. The Mobile phase was constituted of 50:45:5 (Acetonitrile/Methanol/water), pH adjusted to 4.5 using (Glacial acetic acid) GAA. A wavelength of 355 nm was used as it has identified as a suitable wavelength for quantifying isotretinoin. A trial injection was made for isotretinoin standard sample spiked with alitretinoin and tretinoin. The results showed that the reference method was able to separate isotretinoin from alitretinoin and tretinoin (Figure 1.a). However, (Figure 1.b) shows that both tretinoin and alitretinoin may have co-eluted as one single peak.

When an UV degraded isotretinoin standard solution was injected, a shoulder peak that was not separated from isotretinoin peak was also observed which suggested that there was co-elution underneath isotretinoin peak (Figure 1.c). These findings suggested that the current column or mobile phase composition was inefficient to separate the impurities or isomers that were closely related to isotretinoin. Adjustments in flow rate, run time or temperature under an isocratic elution program were thought to be uneconomical, as a prolonged run time would be required to achieve satisfactory resolution.

Due to the observed deficiencies, we s decided to modify our original UPLC method in order to capture any possible degradants as well as to ensure that they were well separated from isotretinoin peak with reasonably short run time. Several modifications based on the current isocratic method were considered for the development of UPLC method. The UV degraded standard solution (isotretinoin) and spiked tretinoin and alitretinoin solutions were used to modify the method and to optimize the chromatographic conditions. To achieve satisfactory

resolution and good chromatography, the RP-C18 column (250 x 4.6mm, 5 µm particle size), used in our original method which was a long column packed with larger particles and showed lack of resolution, was replaced with a shorter column packed with smaller particles eg. C18, SB, 1.8 µm, 2.1mm x 150 mm. The injection volume was lowered to less than 2 µL due to smaller column volume.

The composition of mobile phase with 0.5% glacial acetic acid (GAA), methanol and acetonitrile were re-adjusted and optimized through multiple trials by achieving the separation of isotretinoin, light degraded impurities, tretinioin and alitretinoin using the UV degraded standard solution (isotretinoin). Introduction of gradient elution program with high organic portion near the end of each injection may have served as a strong wash phase to elute out the impurities, isomers, unwanted waste or residue to avoid blockage of column or carry over. The details of the gradient elution programme and the modified mobile phase can be seen in (section 2.3 and table 1). Moreover, acetonitrile was used as a solvent of isotretinoin to prepare the samples in the original method. Since the proportion of methanol in mobile phase was relatively higher than acetonitrile, methanol was proposed to replace acetonitrile in the modified method. Standard or bulk active pharmaceutical ingredient (API) would therefore be prepared in methanol. Figure 2 shows the UV light degradants (degradant 2 and degradant 3) were separated from isotretinoin with a resolution of 1.5 and 2.0, while the resolution between tretinoin and alitretinoin was 3.3. That was a great improvement in terms of resolution from the original isocratic HPLC method with the run time of 12 minutes only.

3.4 Method Validation

Ovearll, a Gradient reverse-phase UPLC method has been followed to develop an assay for determination of isotretioin cocentration in isotreitnoin or related products. Quantitation of isotreitnoin was carried out via comparison with an external isotretinoin standard. The validation would be in accordance with ICH topic Q2 (R1) validation of Analytical Procedures: Text and Methodology (2005).

3.4.1 Accuracy

Accuracy was required to demonstrate that the analytical procedure would provide results that reflect the true value for the material across the specified range of the analysis. The mean percentage recovery at each level should be within 98.0-102.0 with %RSD less than 2.0%. The recovery was calculated using linear regression equation (Table 3.a) that was established within the range of 60% - 140% (3µg/ml - 7µg/ml). Table 3.b shows the results of accuracy

studies. As the recovery values were in the range of 100.1 – 101.6% and the %RSD of all three levels was 0.79, the relative response factors and recovery data reported in Table 3.b indicate that the method is accurate. The retention time, theoretical plate and tailing factor were also reported to demonstrate that the chromatographic condition of isotretinoin peak was successfully obtained.

3.4.2 Precision, Intermediate Precision, Injection Repeatability

The repeatability and intermediate precision were determined as described in Section 2.5.2 and 2.5.3. As Per ICH guidelines (2005), the %RSD value between six individual samples should be less than 2.0%. Our results which are listed in Tables 4.a and b reveal that the %RSD value for precision study of six 100% solutions was 1.5 %, which was meeting criteria of 2.0%. The %RSD value of twelve individual results from two days of study was 1.5% which was also less than 2.0% for intermediate precision. This suggested that the method was accurate and precise as acceptable recovery and precision has been achieved from 60%-140%. %RSD value for Instrumental repeatability of ten injections on the 100% sample solution was 0.76 indicating the injection repeatability was also precise (Table 4.b).

3.4.3 Linearity and Range

A series of solutions containing isotretinoin at concentration of 20% to 140% (1µg/ml - 7µg/ml) of the nominal concentration were analysed to determine the linearity of the proposed method. The peak area versus concentration data were treated by least-squares linear regression analysis. The result from Linearity and range assessment suggested that the method is linear with R^2 value equal to 0.9999, the linear regression equation obtained was 22410x+213.33 (Table 5).

3.4.4 Limit of detection (LOD) and limit of quantitation (LOQ)

The analytical method showed good linearity within the specified range. The LOD and LOQ were calculated based on standard deviation of residue (843.2795) and slope (22410) as per formula provided in ICH guideline (11) and they were 0.12µg/ml and 0.38 µg/mL respectively.

3.4.5 Robustness - Chromatographic Parameter Variations

Robustness was assessed by altering the chromatographic conditions including flow rate and column temperature listed in Table 6. The number of theoretical plates and the tailing factors

were within the acceptance criteria of greater than 20000 and 0.8-1.5 respectively. The %RSD value of results between nominal and combination of changes was only 0.54 demonstrating that the chromatographic condition was robust and reliable results can be obtained within the prescribed degree.

3.4.6 Forced degradation studies

Stress study should be carried out to establish the inherent stability characteristics of drug as according to drug stability guideline Q1A (R2) issued by ICH [13]. Therefore, it is essential to have a stability indicative testing method for pharmaceutical development. The method is used to detect how the stability of the drug substances or products that can be changed with time under specified stress conditions. It should be able to measure the changes in active ingredient level accurately without interference from degradants, impurities or formulation matrix [14]. It is also useful to predict the degradation of drug compound at early stage of development when the availability of information is limited. Several methods using HPLC for isotretinoin quantitation were recently developed and validated by Mahajan et al., 2016 Guimaraes et al., 2010; and Dalvi et al., 2016 [2, 15, 16], however, only accuracy, linearity, precision and robustness were covered by the validation [14, 15] . Forced degradation was not being conducted for all the HPLC methods. Tashtoush et al (2007) [5] has conducted photostability study on tretinoin and isotretinoin, however, the authors claimed that the method was not qualified as a stability indicating method as the separation parameters between retinoic acids and its degradant required more attention in order to achieve better resolution. Hence, the proper degradation profile of isotretinoin remains unknown**.** In our study, the degradation experiments were therefore carried out on isotretinoin using the newly developed UPLC method to determine the stability-indicating property of the method.

Recovery of isotretinoin in each stressed sample was calculated relative to an unstressed sample using data acquired at 355 nm. As the concentration of working solution was only 5µg/ml, degradation under mild stress conditions might be insufficient to produce desired amount of degradants to be detected on the chromatograms. Our aim was therefore to achieve degradation with relatively harsh conditions to generate desired amount of degradation products that can be used to assess the analytical methods performance suggested by Singh et al (2013) [17]. The results of the percentage recovery of the stressed samples and the number of degradants produced are summarized in Table 7. The percentage recovery of the light

stressed sample was 90.9%, 87.9% for heat, 81.0% for oxidation, 93.7% for hydrolysis, 73.7% for acid stressed sample, and 97.3% for base stressed samples. Among all of the stressed samples studied, only based stressed samples showed a degradation of less than 5% even after 7 days incubation with 1M NaOH. This suggests that isotretinoin is relatively stable in basic conditions. On the other hand, extensive degradation was observed for acid stressed sample with production of seven degradants. Other stressed samples including heat, hydrolysis, light and oxidation have all produced various degrees of unknown degradants at 355nm. Calculation of mass balance (using % area) was attempted but was not precise as 100% recovery cannot be obtained for some of the stress conditions e.g. acid. This might be due to the degradants not having the same UV spectra of isotretinoin or the absorptivity coefficient may have different values according to Cione et al. (2011) [18].

The ICH guideline (2003) has also commented that mass balance is not achievable in all circumstances with due consideration of the margin of analytical precision. The focus of the forced degradation may instead be on assuring the specificity of the assay and completeness of the investigation of the degradation route (Riley and Rosanske 1996) [19]. It was therefore important to demonstrate the ability of the method to resolve the degradants from its principle peak which was confirmed by analysing the resolutions of the nearest degradants. Figures 3.a-f of light, heat water, and acid stressed samples show unknown degradant peaks eluted before isotretinoin peak. The resolution between isotretinoin peak and the peaks of nearest impurities eluted before isotretinoin were 1.6, 1.7, 1.6 and 10.5 respectively. Apart from base stress sample which showed no degradant before and after isotretinoin peak at wavelength 355nm, a resolution of more than 1.5 was achieved for all nearest degradants that eluted after isotretinoin for all the stressed samples investigated. The method was therefore proven to be stability-indicative as it was not only able to reflect on the recovery of isotretinoin under various stressed conditions, but resolution between the degradants and isotretinoin peak was also achievable with satisfaction at 355nm.

According to ICH guidelines, the information generated from the forced degradation studies is important to determine the intrinsic stability of the drug molecule and to select proper formulation, packaging and appropriate storage conditions to maintain the drug in stable form for long period of time.

3.4.7 Specificity

USP defines specificity as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components. Our method was considered a specific as there was no interference from other spiked components including tretinoin and alitretinoin with the isotretinoin peak at wavelength 355 nm (Figure 4). Table 8 shows that the resolution between isotretinoin and alitretinoin was 6.981. Even alitretinoin and tretinoin were also well separated from each other with resolution of 3.461, which allowed identification of individual isomers that might be appearing during processing or storing. This improvement in separation between retinoic related substances eg tretinoin, isotretinoin and alitretinoin has addressed the poor resolution issue that was previously raised by Tashtoush et al (2007) [5]. Moreover, the principle peak in the stressed sample has demonstrated to be resolved from other forced degradants.

4. Conclusion

A simple, rapid and economical UPLC method has been developed for qualification and quantification of isotretinoin in bulk drug. The developed method was validated against the current ICH guideline and showed the ability of delivering accurate, precise, specific, linear and robust results within the validated range. The study has also concluded that isotretinoin is most labile to acid, oxidative stress and thermal degradation, and more stable against photolysis, alkaline and hydrolysis. Degradants and spiked retinoic acids were present without interfering isotretinoin to show the method is stability indicating. The analytical method therefore may be used for assay of isotretinoin and identification of isotretinoin and its isomers ie. tretinoin and alitretinoin for routine quality assessment and stability study.

Conflict of interest

The authors declare that there is no conflict of interest

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Table 1. Gradient elution program for isotretinoin assay.

Time (min)	Mobil Phase A %	Mobile Phase B %
0	38	62
7	38	62
8.5	0	100
9.5	0	100
9.5	38	62
12	38	62

Table 2. Chromatographic variation condition

Method	Column Temperature(C^0)	Flow Rate (mL/minute)
1	53	0.45
2	53	0.55
3	57	0.45
4	57	0.55

Table 3. Range for Accuracy data calculation. a

Parameter	Values
Linearity range (3-7µg/mL)	60-140%
Slope	5842.5x
Intercept	471.43
Correlation Coefficient	0.9999
Regression equation	y=5842.5x + 471.43

Accuracy data. b

Concentration (%)	Theoretical (mg)	Recovered (mg)	Accuracy (%)	Retention Time (min)	Theoretical plate	Tailing Factor
60	15	15.24	101.6	7.7	20550	1.1
100	25	25.1	100.4	7.7	20624	1.1
140	35	35.04	100.1	7.7	20626	1.1
Mean			100.7	7.7	20600	1.1
RSD (%)			0.79	0.0	0.2	0.0

Table 4.

Precision data and intermediate precision. a

Concentration (%)	Precision 1	Precision 2
100%	101.9	101.5
	100.5	97.6
	98.7	98.9
	98.8	98.0
	98.1	100.8
	98.3	100.4
Average (%)	99.4	99.5
%RSD	1.5	1.6
	Intermediate Precision (n=12)	
Average (%)	99.5	
%RSD	1.5	

Injection Repeatability. b

Injection Number (n)	Peak Area
1	114571
2	113440
3	114119
4	113394
5	112009
6	112073
7	113271
8	112508
9	113128
10	114058
Average (%)	113257
%RSD	0.76

Table 5. Linearity and Limits

Parameter	Values
Linearity range (1-7µg/mL)	20-140%
Slope	22410x
Intercept	213.33
Correlation Coefficient	0.9999
Regression equation	y=22410x + 213.33
LOD (µg/mL)	12µg/ml
LOQ (µg/mL)	0.38 µg/ml

Table 6. Robustness data

Method	Column Temperature(C^o)	Flow Rate (mL/minute)	Retention Time (min)	Tailing Factor	Theoretical plate	Resolution with nearest peak		Recovery (%)
						Before	After	
Nominal	55	0.50	7.8	1.2	23303	1.6	2.1	98.1
1	53	0.45	8.8	1.1	25562	1.6	2.1	98.3
2	53	0.55	8.3	1.2	24796	1.7	2.1	98.2
3	57	0.45	8.1	1.2	24221	1.6	2.1	98.2
4	57	0.55	7.2	1.2	23285	1.5	2.0	99.4
Mean								98.4
%RSD								0.54

Table 7. Forced degradation data

Stress Types	Stress conditions	Assay Recovery (%)	Mass Balance (%)	No. of Degradants/impurities at 355 nm		Resolution between nearest degradant	
				Known (Tretinoin)	Unknown	Before	After
Light	visible light not less than 1200000 lx.h followed by UVA ration at 220 $W.h/m^2$	90.9	98.5	1	1	1.6	12.6
Heat	95°C for 3 days	87.9	97.5	1	2	1.7	12.6
Oxidation	20 mL of 30% H_2O_2 for 8 hours	81.0	99.4	1	peak not found	-*	12.2
Hydrolysis	20 mL of	93.7	98.0	1	1	1.6	12.4

	purified water into sample for 3 days						
Acid	2M HCl for 1 day	73.7	88.7	1	6	10.5	12.6
Base	1M NaOH for 7 days	97.3	97.3	peak not found	peak not found	-*	-*

-*: No nearest peak was identified.

Table 8. Specificity study

Compound	Retention time	RSD (%)	Tailing Factor	RSD (%)	Resolution	RSD (%)
Isotretinoin	7.56	0.06	1.1	0.0		
					6.987	0.01
Alitretinoin	9.075	0.03	1.0	0.0		
					3.461	0.02
Tretinoin	9.657	0.0	1.1	0.0		

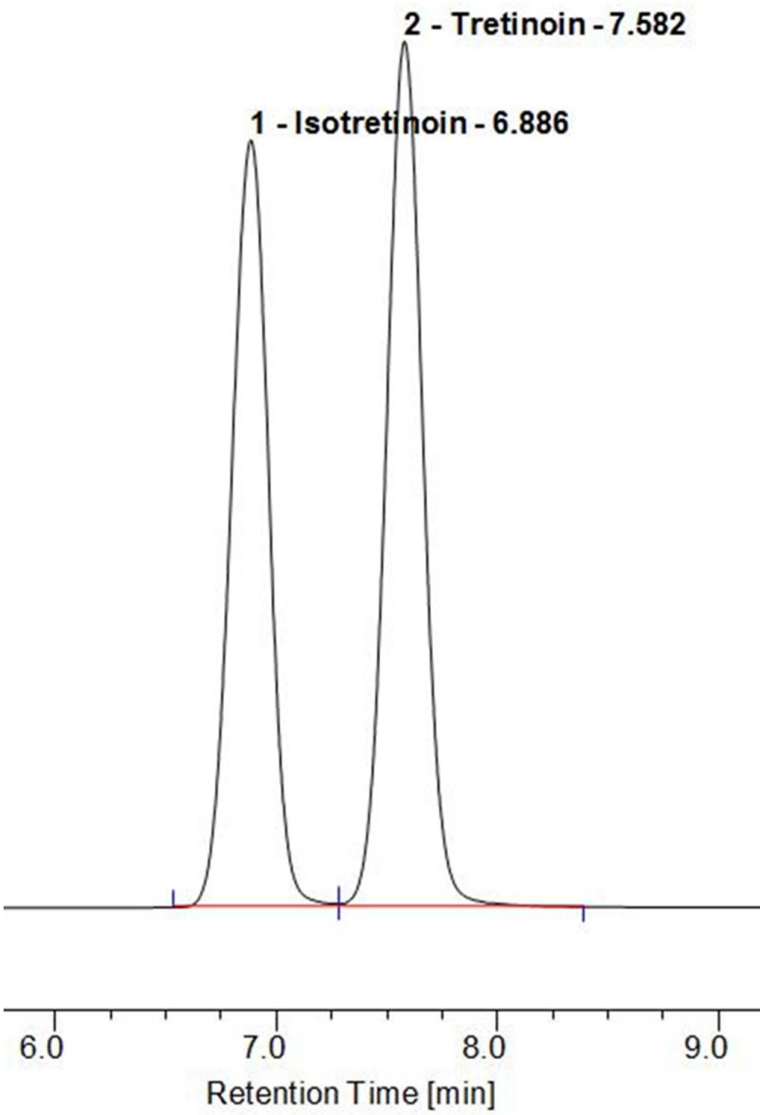


Figure 1.a. Standard solution spiked with isotretinoin and tretinoin

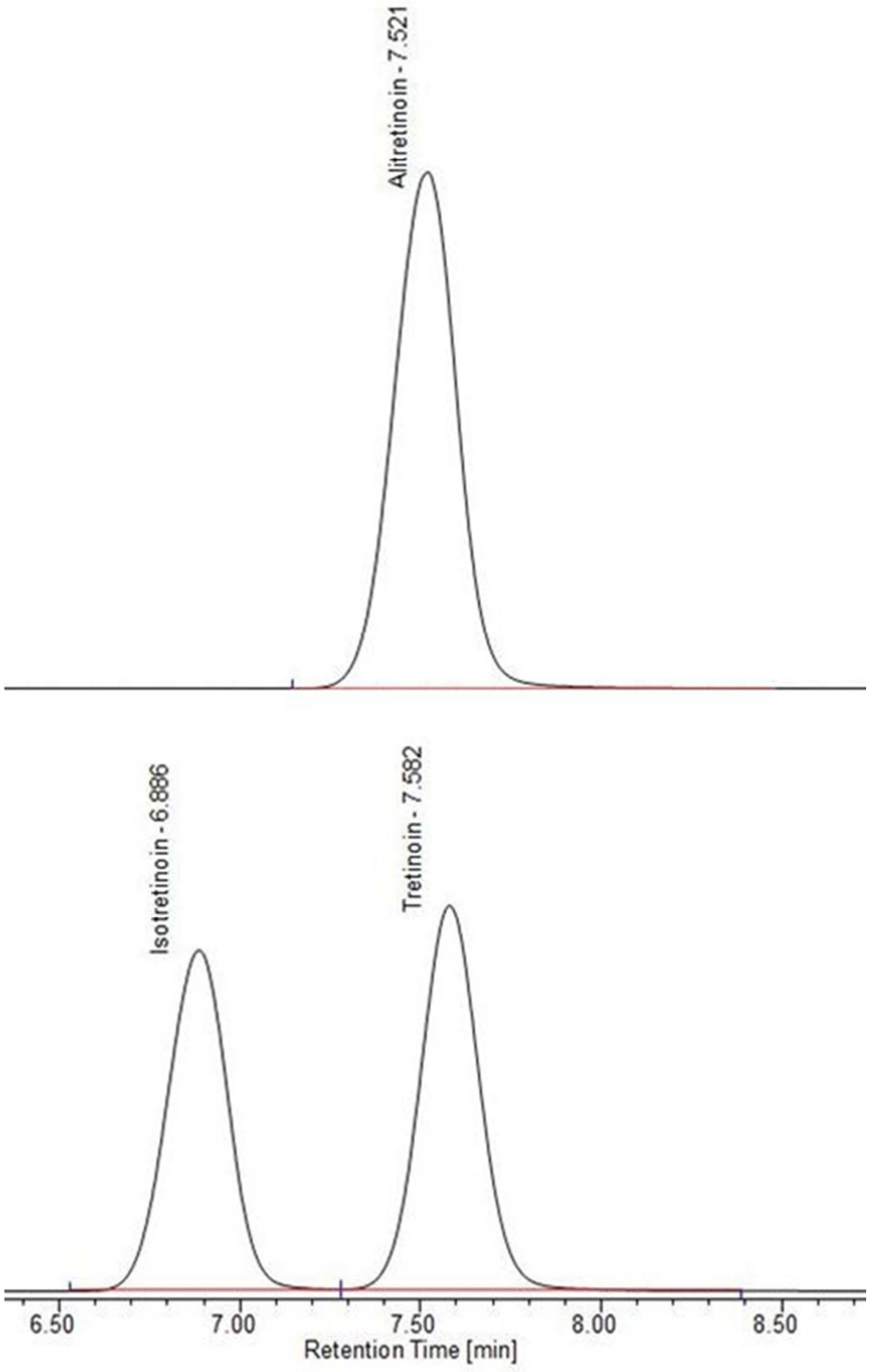


Figure 1.b. An overlay of alitretinoin (7.521min), isotretinoin(6.886 min) and tretinoin (7.582 min) solution. The alitretinoin peak elute about the same time as tretinoin.

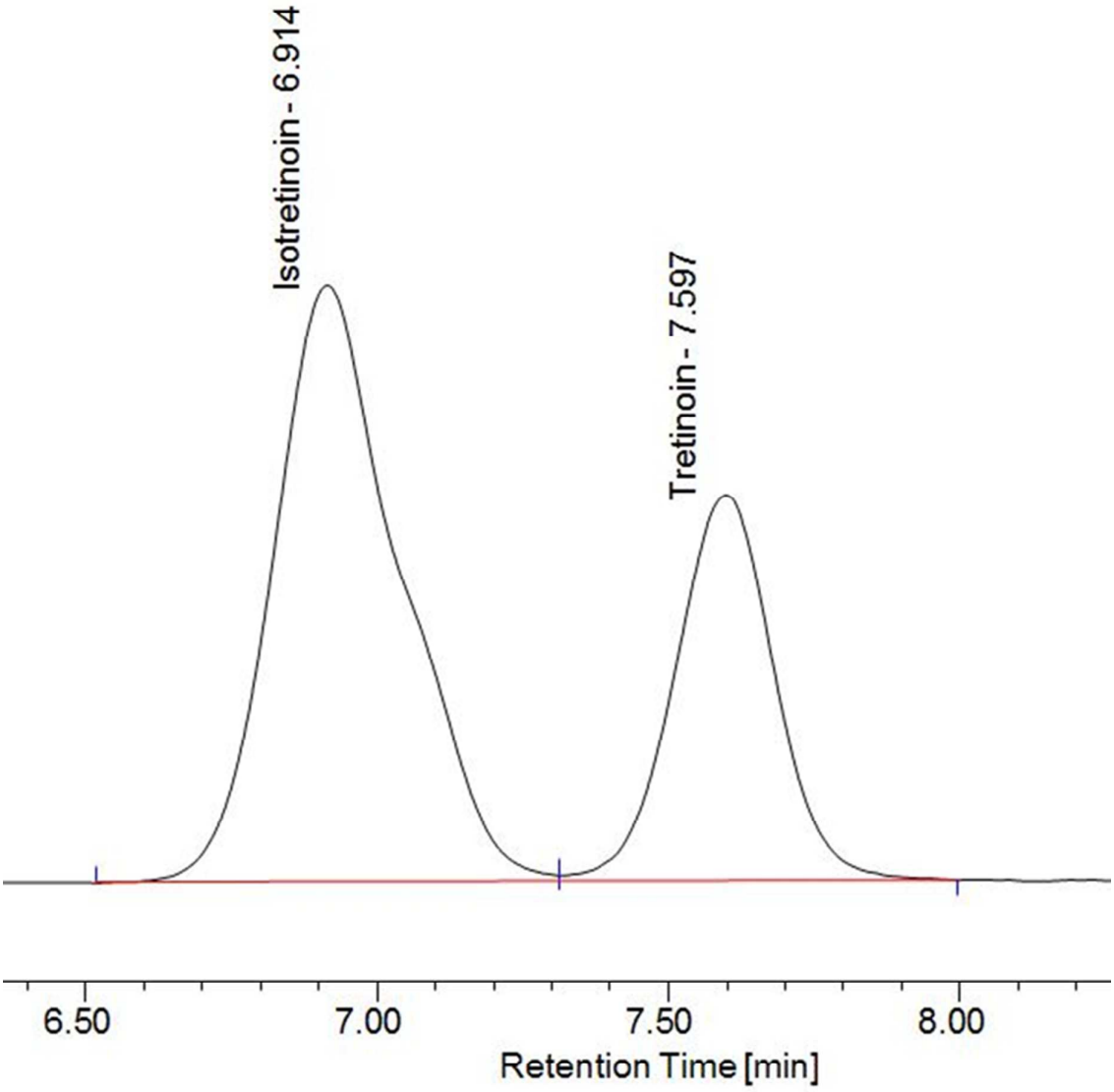


Figure 1.c. UV light degraded standard solution, a shoulder was noticed on the isotretinoin peak (isotretinoin at 6.914 min, tretinoin at 7.569min).

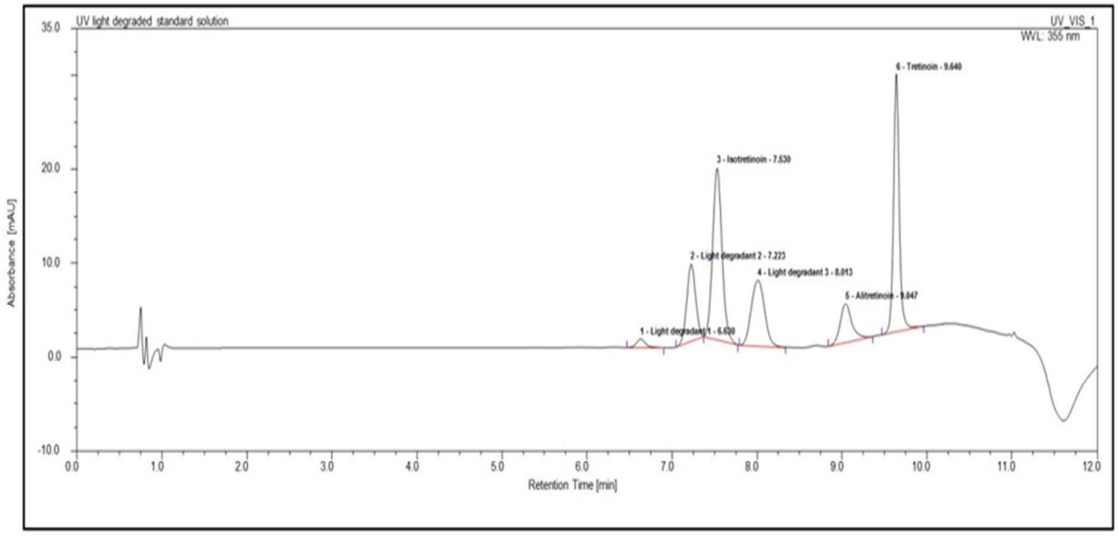
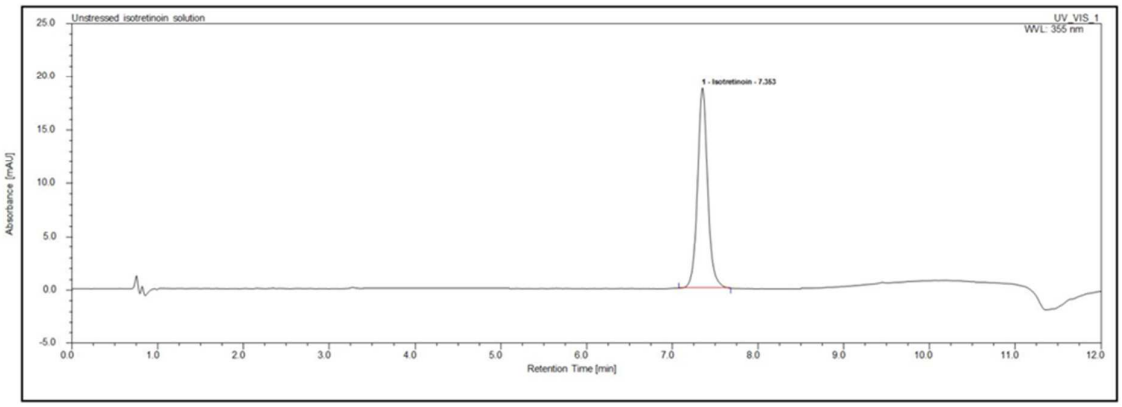
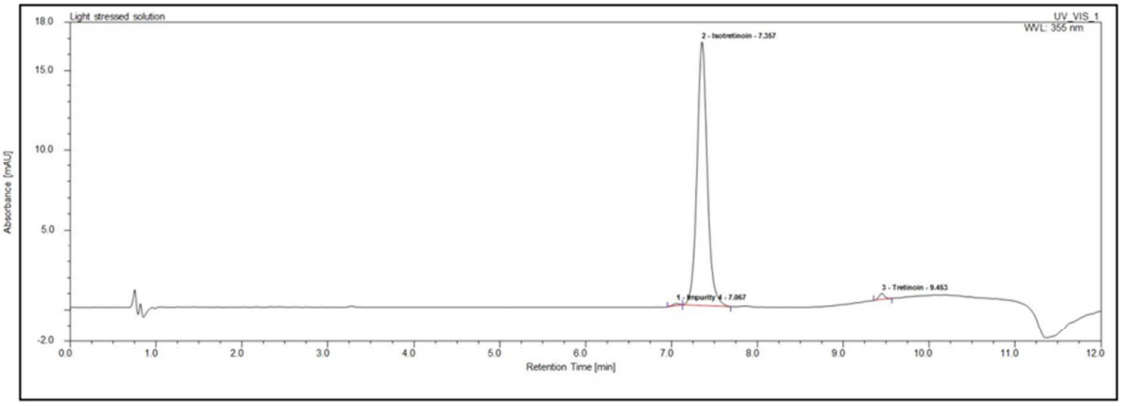


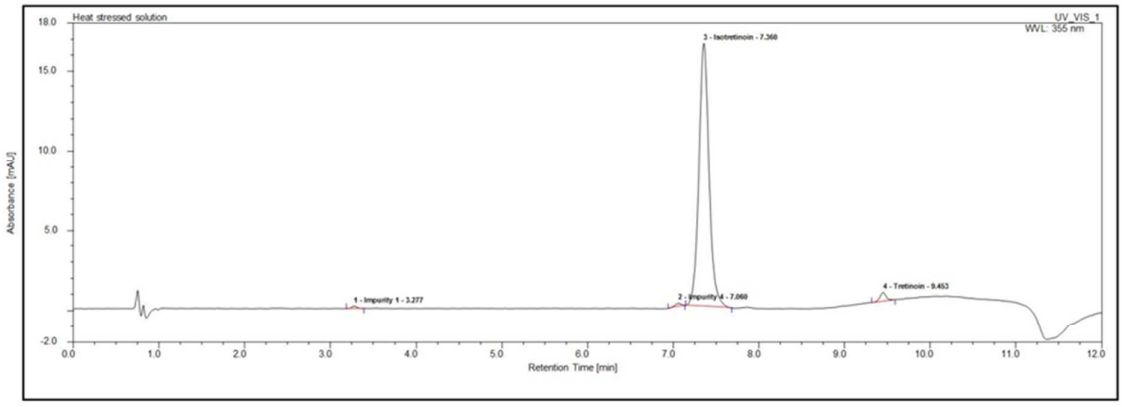
Figure 2. UV light degraded standard solution. Resolution between of isotretinoin and its light degradant 1 was 1.5 and 2.0 for light degradant 2. Resolution between alitretinoin and tretinoin was 3.3.



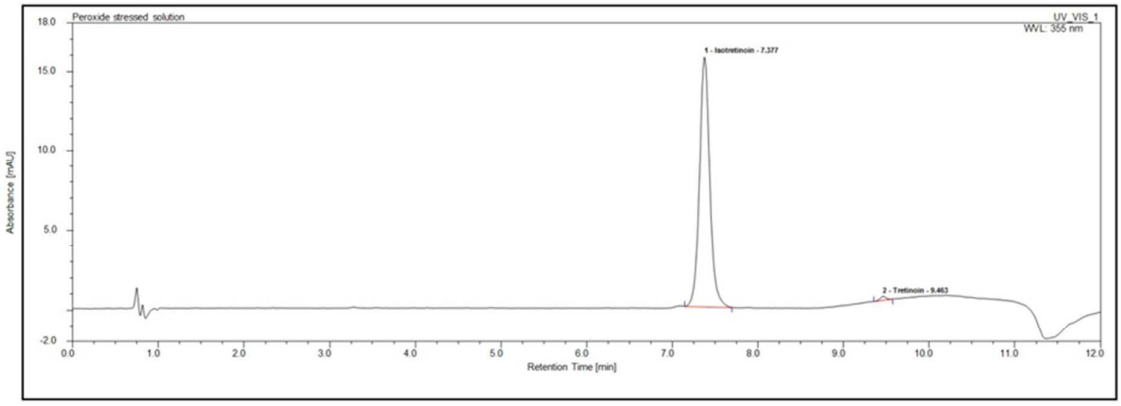
a. Unstressed isotretinoin solution.



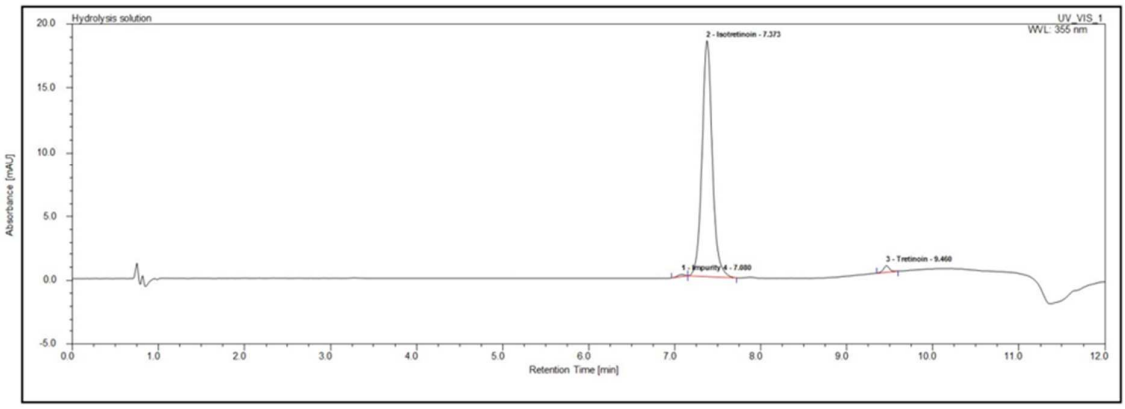
b. Light Stressed solution



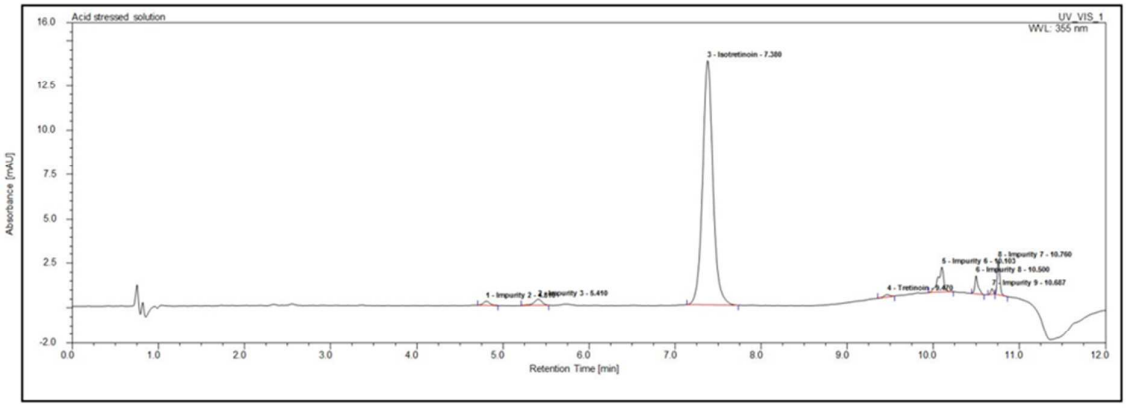
c. Heat stressed solution



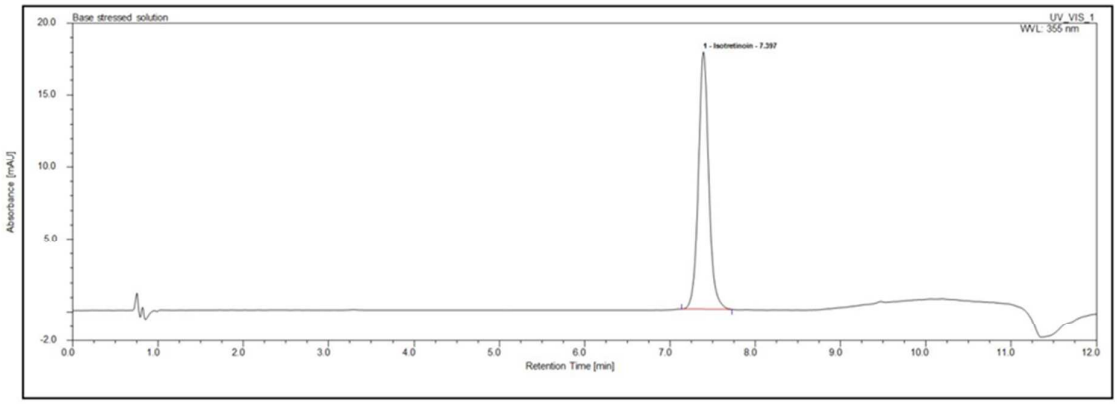
d. Peroxide stressed solution



e. Hydrolysis solution



f. Acid stressed solution



g. Base stressed solution

Figure 3. Chromatograms of isotretinion samples exposed to different stressed conditions

(a) Unstressed samples (b) light (c) Heat, 95C (d) H2O2 (e) hydrolysis by exposing to water (f) HCl (g) 1M NaOH.

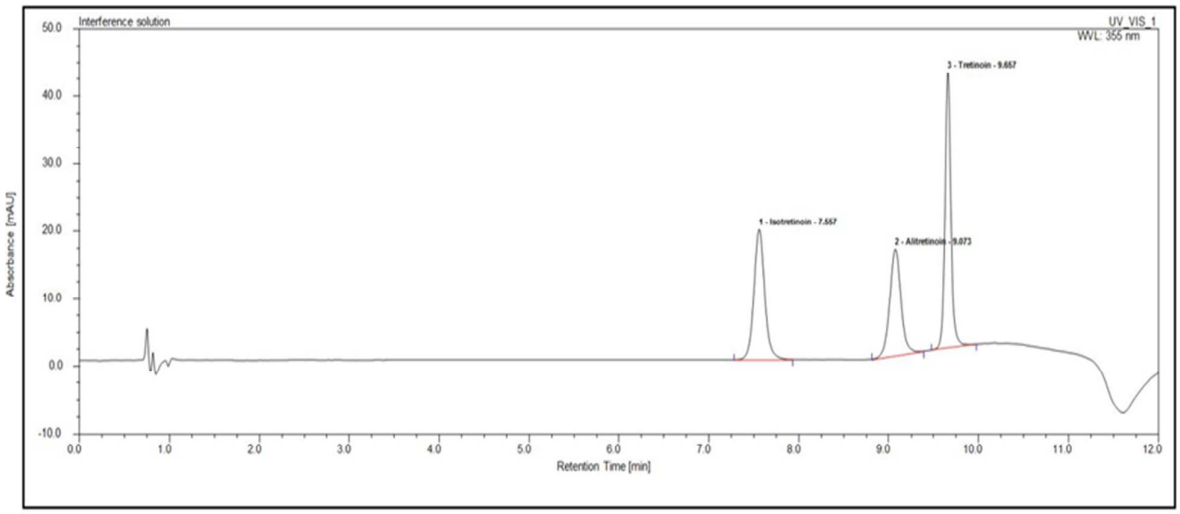


Figure 4. Spiked alitretinoin (3rd peak) and tretinoin (2nd peak) did not interfere with isotretinoin (1st peak). Alitretinoin and tretinoin were also well separated from each other.