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Stability indicating RP-HPLC method development and validation for determination of potential degradation impurities of tretinoin in tretinoin topical pharmaceutical formulation

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ABSTRACT

A simple, specific, accurate and stability-indicating RP-HPLC method was developed and validated for determination of related substances in Tretinoin topical pharmaceutical formulation. Chromatographic separation was achieved on Hypersil BDS C18 250 × 4.6mm, 5µ column as stationary phase while mobile phase A was buffer, (Buffer=Water : Glacial acetic acid, 90:2 v/v) and mobile phase B as Methanol. Method was developed in gradient mode with 40 minutes, at flow rate of 1.2 mL/min. Effluents were monitored at 356 nm. The method was validated for specificity, linearity, accuracy, precision, limit of quantification, limit of detection, robustness and solution stability. The RRF (relative response factor) values of Isotretinoin impurity determined from linearity study were 0.91. Limit of quantification of Tretinoin and Isotretinoin was found to be 0.02 µg/mL and 0.02 µg/mL respectively. Recovery was found to be in the range of 95.0-105.0%. The method was proved to be robust with respect to changes in flow rate, buffer pH and column temperature. The proposed method was successfully applied for the quantitative determination of related substances in Tretinoin topical pharmaceutical formulation.

Keywords: Topical formulation , RP-HPLC, Related substances, Forced degradation, Trretinoin

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INTRODUCTION

Tretinoin (TRET), 3,7-Dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8-nonatetraenoic acid, is the all-*trans* stereoisomer of retinoic acid, is a first generation topical retinoid used as a topical keratolytic in the treatment of acne vulgaris in which comedones, pustules, and papules predominate; it prevents comedo formation and for topical use.). It is also used to treat acute promyelocytic leukemia [1-5].

Oral Tretinoin has been shown to be teratogenic in rats when given in doses 1000 times the topical human dose. Oral Tretinoin has been shown to be fetotoxic in rats when given in doses 500 times the topical human dose. Topical Tretinoin has not been shown to be teratogenic in rats and rabbits when given in doses of 100 and 320 times the topical human dose, respectively (assuming a 50 kg adult applies 250 mg of 0.1% cream topically). However, at these topical doses, delayed ossification of a number of bones occurred in both species [6-8]. Topical drug delivery systems have been used for centuries for the treatment of local skin disorders. Topical applications of the drug offer the potential advantages of delivering the drug directly to the site of action and delivering the drug for extended period of time at the effected site that mainly acts at the related regions [9].

A detailed literature survey for TRET revealed that stability of Tretinoin in tretinoin-minoxidil solution by HPLC reported by Zarghi A et al. [10]. A rapid simultaneous determination of Tretinoin and isotretinoin by HPLC reported by Tashtoush BM et al.[11]. Simultaneous determination of Tretinoin and Clindamycin phosphate and their degradation products in topical formulations by RP-HPLC reported Rose YY et al.[12]. Determination of tretinoin in creams by HPLC reported by Kril MB et al.[13]. Separation and Determination of Clindamycin Phosphate and

Tretinoin in Acne Gel by HPLC reported by Ya-zhong MA et al.[14]. HPLC method for simultaneous determination of tretinoin and isotretinoin in dermatological formulations reported by Bassam M et al.[15]. Simultaneous Determination of 13-Cis- and All-Trans-Retinoic Acids and Retinol in Human Serum by HPLC reported by Takeda N et al.[16]. Simultaneous Determination of All-Trans-, 13-Cis-, 9-Cis-Reti-noic Acid and Their 4-Oxo-Metabolites in Plasma by HPLC reported by Disdier B et al. [17]. Densitometric thin layer chromatographic analysis of tretinoin and erythromycin in lotions for topical use in acne treatment reported by Gabriels M et al.[18]. HPTLC Method for Simultaneous Estimation of Isotretinoin and Erythromycin in Bulk Drug and Topical Gel Form reported by Rathore AS et al.[19]. spectrophotometric determination of minoxidil and tretinoin reported by Bordbar M et al.[20]. Residue Determination of Clindamycin phosphate and Tretinoin on the Surface of Manufacturing Equipment by RP-HPLC also reported by Roy C et al.[21]. Assay by HPLC also reported in United State of Pharmacopeia [22].

To the best of our knowledge, none of the method is available for determination of potential known impurtiy of TRET from TRET and its deradation impurities from topical pharmaceutical formulation. The aim of the present work was to develop and validate a method for determination of degradation impurity in TRET topical formulation.

The drug product stability guideline Q1A (R2) issued by the International Conference on Harmonization (ICH) [23] suggests that stress studies should be carried out on a drug to establish its inherent stability characteristics, leading to identification of degradation products and, hence, supporting the suitability of the proposed analytical procedures. It also requires that analytical procedures for testing the stability of samples should be stability-indicating and should be fully validated. Chemical structures Tretnoin and Isotretinoin of all compounds are presented in Figure 1.

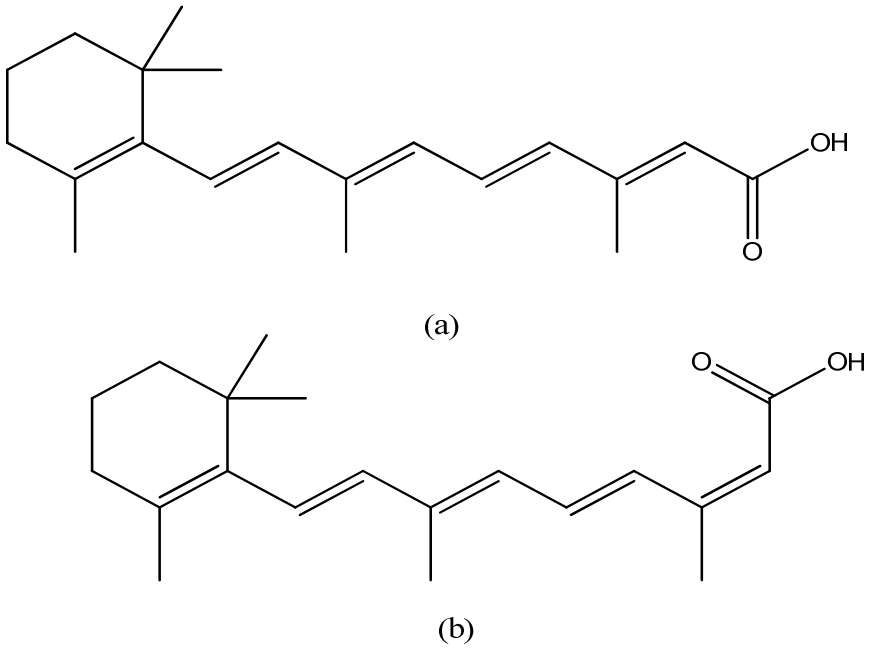


Figure 1: Chemical Structure of (a) Tretinoin and (b) Isotretinoin

MATERIALS AND METHODS

2.1 Reagents and chemicals

TRET gel, placebo, TRET working standards and Isotretinoin impurities (IUPAC name: (13cis)-retinoic acid) standards were provided by Dr. Reddy's laboratories Ltd., Hyderabad, India. HPLC grade methanol and glacial acetic acid were used of Rankem, India. 0.45 µm membrane filter, 0.22 µm PTFE syringe filter and 0.22 µm nylon syringe filter were used of Millipore, India. Water for HPLC was generated using Milli-Q Plus water purification system (Millipore, Milford, MA, USA).

HPLC (Alliance Waters, separation module 2695, photo diode array detector 2996, UV detector 2487 with Empower2 software. Photo-stability chamber (Sanyo, Leicestershire, UK). Dry air oven (Cintex, Mumbai, India).

2.2 HPLC instrumentation and chromatographic conditions

The LC system of Waters Alliance HPLC with PDA detector was used for this study and chromatographic separation was achieved on Hypersil BDS C18 (250 mm × 4.6 mm, 5 µm) column as stationary phase with binary gradient mode. Gradient program was used as time (min)/mobile phase-A (%)/mobile phase-B (%); 0.0/20/80,

30/10/90, 35/20/80, 40/20/80. Where, mobile phase-A was water : Glacial acetic acid, 90:02 v/v and mobile phase-B, Methanol. Buffer was filtered through 0.45µm membrane filter. Column oven temperature was maintained at 30°C and injection volume for each study preparation was 20 µL. Eluent with flow rate of 1.2 mL/min was monitored at 356 nm with PDA detector. Methanol was used as a final diluent.

The stress degraded samples and the solution stability samples were analyzed using a PDA detector covering the range of 200-400nm.

2.3 Diluted standard solution preparation

Standard solution was prepared by dissolving standard substance in diluent to obtain solution containing 0.5 µg/mL of TRET.

2.4 Sample solution preparation

An accurately weighed sample equivalent to 1 mg of TRET was taken into 10 mL volumetric flask. About 7 mL of diluent was added to this volumetric flask and sonicated in an ultrasonic bath for 15 min with intermittent shaking, diluted to the volume with diluent, mixed well, centrifuged at 7500 rpm for 15min. Supernatant solution was filtered through 0.22 µm Nylon syringe filter.

RESULTS AND DISCUSSION

3.1 Method development and optimization

Prime objective of an RP-HPLC method development for determination of related impurities in TRET topical dosage form was: the method should be able to determine all TRET related impurity in single run and should be accurate, reproducible, robust and stability indicating. All degradation products from stress conditions sholud be well separated from each other and method should be simple to user in analytical research and quality control laboratory for routine use.

The spiked solution of related compounds (Isotretinoin) and TRET were used for method development to optimize chromatographic conditions and separation by RP-HPLC. Isotretinoin impurity was spiked in TRET in such a way to achieve 1 µg/mL for each impurities and 100 µg/mL for TRET. Further more primary developed method was challanged by forced degradation as a pre-validation.

3.2 Mobile phase and gradient optimization

The optimization of stationary phase and mobile phase were done simultaneously. Stationary phases such as Waters symmetry C18, Luna C8 and inertsil ODS 3V were tried with mobile phase such as Ammonium phosphate buffer (pH 4.5), triethyl amine buffer (pH 2.5) and their composition with Methanol, acetonitrile and tetrahydrofuran were tried but problems such as co-elution of Isotretinoin impurity peak and principal peaks, peak broadening of Principal, placebo peak interferences, late elution of principal peak etc were observed. Good chromatography was observed using Hypersil BDS C18 250 mm × 4.6 mm, 5 µm column as stationary phase. Mobile phase A was buffer (water : Glacial acetic acid, 90:02 v/v), while mobile phase B was methanol. For gradient optimization first programme was time (min)/mobile phase-A (%)/mobile phase-B (%); 0.0/20/80, 30/10/90, 45/10/90, 46/20/80, 55/20/80, inorder to achieve shorter run time gradient programme changed to time (min)/mobile phase-A (%)/mobile phase-B (%); 0.0/20/80, 30/10/90, 35/20/80, 40/20/80 Column oven temperature was maintained at 30°C and injection volume for each study preparation was 20 µL. Eluent with flow rate of 1.2 mL/min was monitored at 356 nm with PDA detector. Methanol was used as the diluent for all preparations . So with this gradient elution it was found good separation of all impurities with main peak and tailing factor less than 2.0 and resolution > 2.0. Finally the praposed method was subjected to method validation according to the International conference on Harmonization guidelines, with consideration of sample concentration to achieve an LOQ below reporting threshold of the impurities.

3.3 Diluent selection

The extraction of the drug from formulation tried with different solvents such as acetonitrile, methanol, methanol with water, methanol with tetrahydrofuran. The complete extraction of drug was achieved with methanol. Tretinoin and Isotretinoin has solubility in methanol.

3.4 Method validation

After satisfactory development of method it was subjected to method validation as per ICH guideline [24]. The method was validated to demonstrate that it is suitable for its intended purpose by the standard procedure. Analytical method validation was carried out by means of system suitability, accuracy, precision, linearity, robustness, solution stability, filter compatibility, specificity and forced degradation study.

3.4.1 System suitability

The system suitability was performed by diluted standard solution and impurities spiked solution (1 µg/mL Isotretinoin impurity of TRET). The system suitability parameters were evaluated and found to be within the limits [Table 1].

Table No.1: System suitability results (Precision, Intermediate precision and Robustness)

Parameter	*Area ratio	USP tailing of TRET	USP theoretical plates of TRET	Relative retention time (RRT) Isotretinoin
Precision	1.00	1.0	27574	0.83
Intermediate precision	1.02	0.9	24577	0.84
At 1.4 mL/min flow rate	1.01	1.1	15071	0.84
At 1.0 mL/min flow rate	1.02	1.1	20240	0.85
At 35°C column temp.	0.99	1.1	14542	0.85
At 25°C column temp.	1.00	1.1	9909	0.84

**... Area ratio of TRET peak from two replicate diluted standard injections; USP...United state pharmocopeia*

3.4.2 Method Precision

The method was found to be precise with six sample preparations by spiking the impurities at 0.3% level [Table 2]. The % RSD of Isotretinoin in six sample preparation was found to be less than 3.5%.

Table No.2: Method precision of TRET

Sample No.	% Isotretinoin	% Total Impurities
Average #	1.00	1.15
%RSD*	3.40	3.30

#... Average of six determinations;
**… Determined on six values*

3.4.3 Forced degradation study

Forced degradation was performed on TRET topical formulation to achieve desired degradation and placebo as well as TRET drug substance were treated with similar conditions as mentioned below, based on development trials optimized forced degradation conditions were established. Final sample concentration was achieved 100 µg/mL of TRET with diluent as proposed sample concentration.

3.4.3.1 Oxidative degradation

TRET sample equivalent to 1mg of TRET was taken into 10 mL volumetric flask, added 5 mL Methanol and sonicated for 15minutes with intermittent swirling. Then subjected to oxidative stress condition by 0.5 mL of 1% v/v H_2O_2 solution in 10 mL volumetric flask and kept on room temperature for 45 min, diluted to volume with Methanol. Further a portion of sample centrifuged at 7500 rpm for 15min. Supernatant solution was filtered through 0.22 µm nylon syringe filter. Degradation in oxidative condition was achieved by 1.66 %.

3.4.3.2 Acid degradation

TRET sample equivalent to 1mg of TRET was taken into 50 mL volumetric flask, added 5 mL Methanol and sonicated for 15minutes with intermittent swirling. Then subjected to acid hydrolysis condition by 1 mL of 1N HCl solution in 10 mL volumetric flask and kept on room temperature for 60 min, sample was neutralised with 1 mL of 1N NaOH and diluted to volume with Methanol. Further a portion of sample centrifuged at 7500 rpm for 15min. Supernatant solution was filtered through 0.22 µm nylon syringe filter. Degradation in acid hydrolysis condition was achieved by 1.53 %.

3.4.3.3 Base degradation

TRET sample equivalent to 1mg of TRET was taken into 50 mL volumetric flask, added 5 mL Methanol and sonicated for 15minutes with intermittent swirling. Then subjected to acid hydrolysis condition by 1 mL of 5N NaOH solution in 10 mL volumetric flask and kept on water bath at 60°C for 15 min, sample was neutralised with 1 mL of 5N HCl and diluted to volume with Methanol. Further a portion of sample centrifuged at 7500 rpm for 15min. Supernatant solution was filtered through 0.22 µm nylon syringe filter. Degradation in base hydrolysis condition was achieved by 2.23 %.

3.4.3.4 Thermal degradation

TRET sample equivalent to 1mg of TRET was taken into 10 mL volumetric flask and exposed at 105°C for 2 hours. Added 5 mL Methanol and sonicated for 15minutes with intermittent swirling. Diluted to volume with Methanol. Further a portion of sample centrifuged at 7500 rpm for 15min. Supernatant solution was filtered through 0.22 µm nylon syringe filter. Degradation in thermal exposed condition was achieved by 3.46%.

3.4.3.5 Photolytic degradation:
TRET sample equivalent to 1mg of TRET was taken into 10 mL volumetric flask and kept in photo stability chamber to expose it for 1.2 million lux hours. Added 5 mL Methanol and sonicated for 15minutes with intermittent swirling. Diluted to volume with Methanol. Further a portion of sample centrifuged at 7500 rpm for 15min. Supernatant solution was filtered through 0.22 µm nylon syringe filter. Degradation in photolytic exposed condition was achieved by 3.04%.

3.4.4 Specificity
The forced degradation of TRET topical formulation was carried out as per ICH guideline [24]. All stress conditions and obtained results are shown in Table 2. The purity angle was less than purity threshold for all the stress conditions. Interference was not observed from blank and placebo preparations. Blank, placebo, control sample and impurity spiked sample chromatograms are presented in figure 4. Stress study samples are presented in Figure 5.

Table No.3: Data of forced degradation study of TRET

Degradation condition	Isotretinoin %	Unk. Max. Imp. %	Total Imps %	PA#	PTH#	PA*	PTH*	Drug assay %
Control sample	0.74	0.05	0.92	0.055	0.275	1.208	2.751	99.3
Acid degradation	1.13	0.17	1.53	0.103	1.361	1.091	2.554	98.9
Base degradation	1.66	0.21	2.23	0.085	1.221	1.553	2.501	98.3
Peroxide degradation	1.40	0.12	1.66	0.048	0.276	1.430	2.566	98.6
Photo degradation	2.79	0.08	3.04	0.051	0.283	0.886	1.509	97.2
Thermal degradation	3.10	0.11	3.46	0.054	0.267	0.533	1.008	97.0

*Unk. Max Imp.... Unknown maximum impurity; PA Purity angle; PTH Purity threshold; # For Tretinoin ; * For Isotretinoin*

3.4.5 LOD, LOQ and RRF establishment
LOD and LOQ were establised by linearity method. Linearity was performed with a range of 0.1µg/mL to 1.0 µg/mL concentration. The correlation co-efficient value was found to be more than 0.999 [Table 3]. Relative response factor (RRF) was calculated for each known impurities by taking the ratio of impurity slope to TRET slope.

Table No.3: LOD, LOQ and RRF results of TRET

Parameter	Isotretinoin	TRET
LOD (µg mL^{-1})	0.007	0.007
LOQ (µg mL^{-1})	0.02	0.02
Intercept (a)	-1614.83	-2078.15
Slope (b)	130363.5	143797.2
RRF	0.91	1.00

3.4.6 Linearity
Linearity was performed for Isotretinoin and TRET. The correlation coefficient value was found to be more than 0.999 [Table 4]. Bias was less then ±0.5% for each case.

Table No.4: Linearity of Isotrtinoin and TRET

Parameter	Isotretinoin	TRET
Linearity range (µg mL^{-1})	0.02-2.03	0.02-9.90
Correlation coefficient	0.99967	0.99993
Intercept (a)	294.7	-247.4
Slope (b)	121842.0	146067.6
Bias at 100% response	0.05	-0.2

3.4.7 Method Precision at LOQ
The precision of the test method at LOQ was conducted by injecting six placebo solution preparations spiked with each of known impurity (Isotretinoin) at LOQ concentration, calculated % of individual Impurity, % RSD from the six preparations are summarized in Table 5.

Table No.5:Method precision data at LOQ of TRET

Sample No.	Isotretinoin %
Average #	0.017
%RSD*	5.88

*#... Average of six determinations; *… Determined on six values*

3.4.8 Accuracy

Accuracy was established by recovery methodology by spiking of Isotretinoin impurity, at six different levels, in triplicate preparations, starting from LOQ to 150% of impurity specification of drug product [Table 6]. The recovery results for Isotretinoin impurity was found to be between 95.0-105%.

Table No.6 Accuracy data of Isotretinoin of TRET

Component		LOQ	50%	75%	100%	125%	150%
Isotretinoin	Concentration µg/mL	0.02	2.5	3.7	5.0	6.25	7.5
	#Mean Accuracy %	98.0	97.7	96.8	97.4	94.8	95.1
#... Average of three determinations							

3.4.9 Solution stability

The solution stability of the standard and spiked sample preparation in methanol was studied for 72 hrs. at bench top. The solution under study was compared with freshly prepared standard solution. The samples solution stability was established upto 72 hrs and found satisfactory [Table 7].

Table No.7 Solution stability results of TRET

Duration	% Isotretinoin	% Total Impurities
Initial	0.99	1.13
24 hrs.	0.99	1.16
72 hrs.	1.01	1.15

3.4.10 Filter compatibility

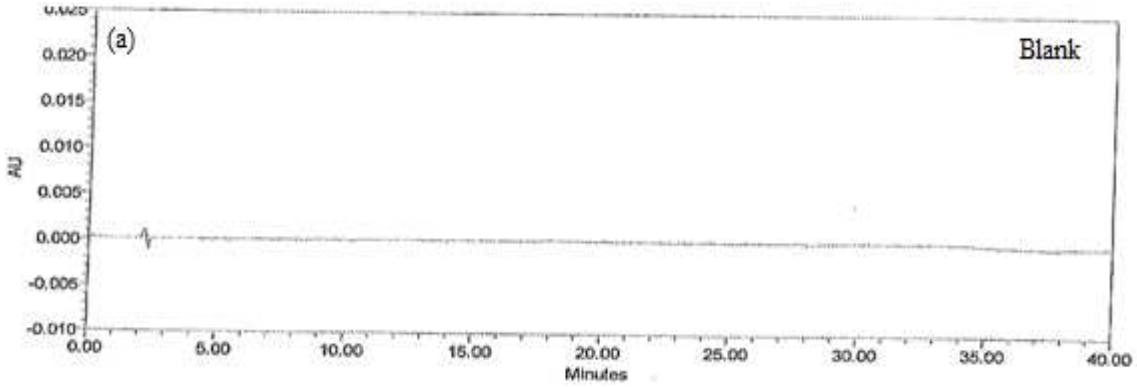
Filter compatibility was performed for PTFE 0.22 µm syringe filter (Millipore) and nylon 0.22 µm nylon syringe filter (Millipore). To confirm the filter compatibility in proposed analytical method, filtration recovery experiment was carried out by sample filtration technique. Sample was filtered through both syringe filters. Related substances were determined (in µg/mL) and compared against centrifuged sample. The sample solution was not showing any significant changes in related substances with respect to centrifuged sample. Filter compatibility results are presented in Table 9, which indicates that both syringe filters are compatible with sample solution.

3.4.11 Robustness

The robustnes was demonstrated by varying; flow rate and column oven temperature [Table 1]. The method was found to be robust with respect to flow rate and column temperature without any significant changes in system suitability parameters and relative retention time of impurities in sample.

Table No.9 Filter compatibility results of TRET

Components	Centrifuged	PTFE filter 0.22µm	Nylon filter 0.22µm
% Isotretinoin	1.17	1.17	1.16
% Total impurities	1.34	1.34	1.34



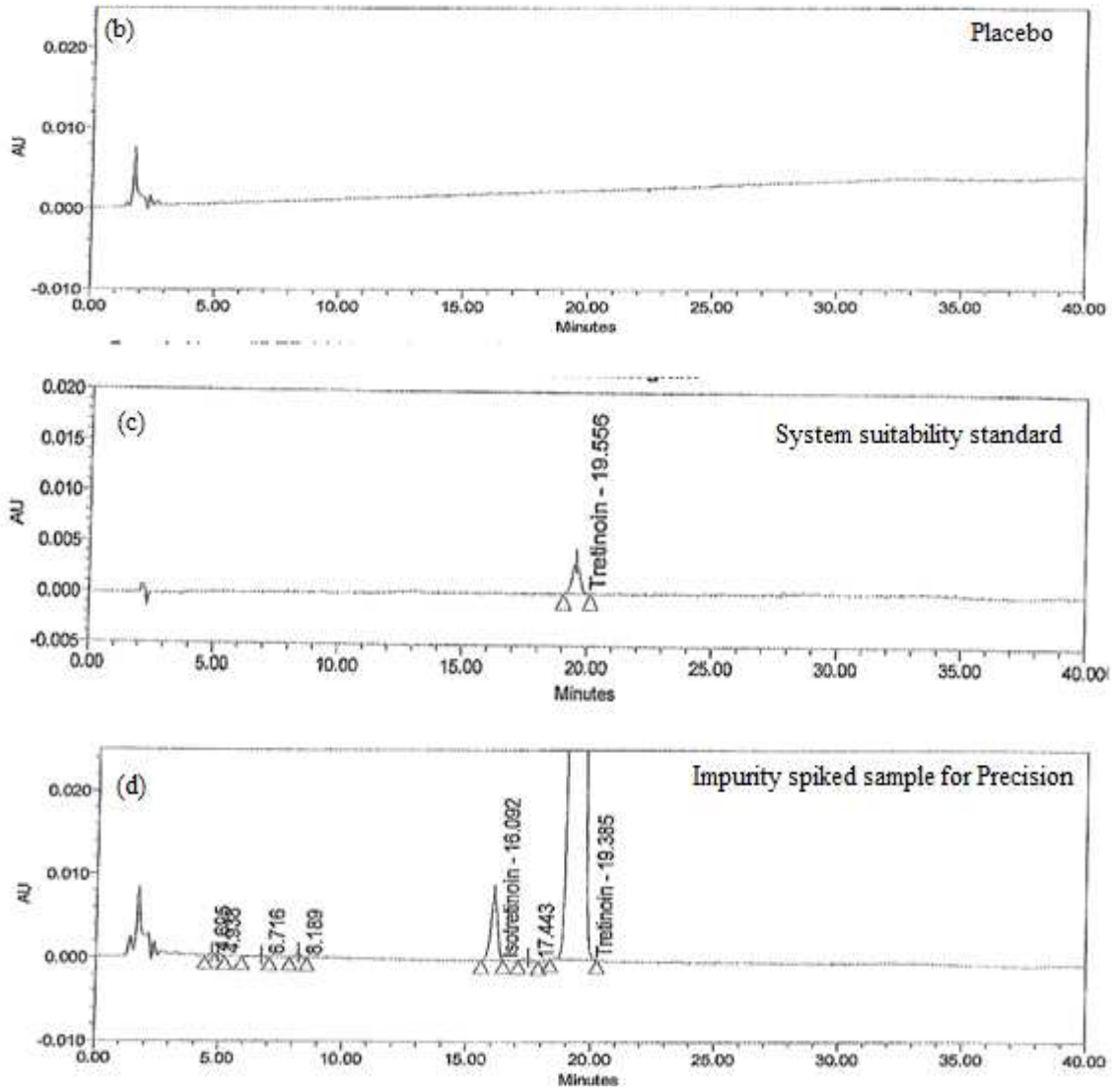
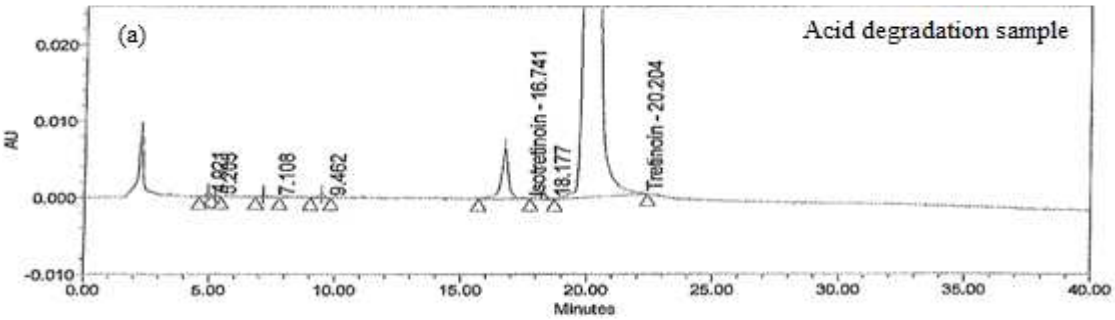


Figure No.2 The chromatogram of (a) blank (b) placebo (c) System suitability standard and (d) Impurity spiked sample for precision



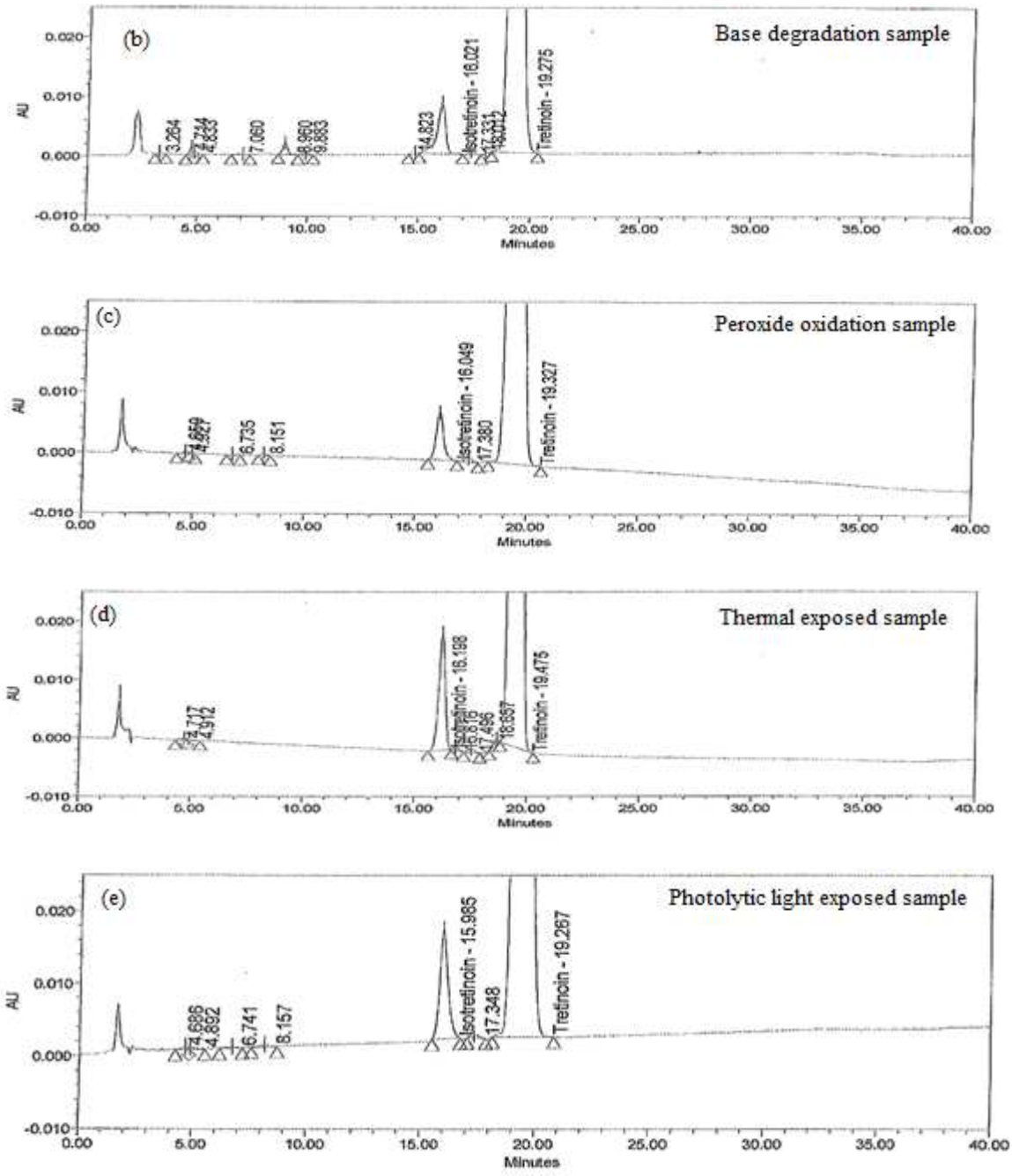


Figure No.3 The chromatogram of (a) acid degradation sample (b) base degradation sample (c) peroxide degradation sample (d) thermal degradation sample and (e) photolytic light exposed sample

CONCLUSION

A stability indicating RP-HPLC method was successfully developed for determination of related impurities in TRET gel and validated according to ICH guidelines. Established RRT and RRF values for known impurities can be useful to determine the related impurity in TRET gel using TRET standard only. Low cost, environment friendly and satisfactory LOD, LOQ, precision and accuracy are the main features of this method. The method was critically validated and statistically generated high quality data proves that the method is linear, sensitive, selective, specific and robust. This method can be applied for analytical development laboratory during product development, product stability testing and in quality control laboratory.

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REFERENCES

[1] http://www.drugs.com/cons/clindamycin-and-tretinoin-topical-application.htmL.
[2] http://medical-dictionary.thefreedictionary.com/tretinoin.
[3] http://www.rxlist.com/ziana-gel-drug.
[4] http://www.mims.com/USA/drug/info/Tretinoin%20Gel/Tretinoin%20Cream.
[5] Amichai B, Shemer A, Grunwald M, *Journal of the American Academy of Dermatology*, 2006, 54(4), 644-646.
[6] http://www.drugs.com/pro/tretinoin.html.
[7] Roy C, Patel HB, Chakrabarty J, *Indo Amer J. Pharm Res*., **2012**, 3(1), 1400-1413.
[8] Roy C, Chakrabarty J, *Sci Pharm.*, **2013**, doi:10.3797/scipharm.1303-22.
[9] Jain A, Gupta SP, Gupta Y, Khambete H, Jain S, Der Pharmacia Sinica, **2010**, 1(3), 221-231.
[10] Zarghi A, Jenabi M, Ebrahiman AJ, *Pharmaceutica Acta Helvetiae*, **1998**, 73(3), 163-165.
[11] Tashtoush BM, Jacobson EL, Jacobson MK, *J. Pharm. Biomed. Anal*., **2007** , 43(3),859-64. Doi: 10.1016/j.jpba.2006.08.027.
[12] Rose YY, Bektic E, Richard, Buchta, Houlden R, Hunt B, *Journal of Separation Science*, **2004**, 27(2), 71-77.
[13] Kril MB, Burke KA, DiNunzio JE, Gadde RR, *J. Chromatogr. A*, **1990**, 522(28), 227-234.
[14] Ya-zhong MA, Yan WU, Li-ping L, Xiang-yang L, **2011**, 30 (10), 1340-1343, Doi: 10.3870/yydb.2011.10.029.
[15] Bassam M. Tashtoush, Elaine L. Jacobson, Myron K. Jacobson, *J. Pharm. Biomed. Anal*., **2007**, 43(3), 859–864, Doi: 10.1016/j.jpba.2006.08.027.
[16] Takeda N, Yamamoto A, *J. Chromatogr. B*, **1994**, 657(1), 53-59.
[17] Disdier B, Bun H, Catalin J, Durand A, *J. Chromatogr. B*, **1996**, 683(2), 143-154.
[18] Gabriels M, Brisaert M, Vercammen JP, *European J, Pharm. Biopharm*, **1999**, 48(1), 53–58, Doi.: 10.1016/S0939-6411(99)00021-1.
[19] Rathore AS, Sathiyanarayanan L, Mahadik KR, *Am. J. Anal. Chem*., **2010**, 1, 144-149, Doi:10.4236/ajac.2010.13018.
[20] Bordbar M, Faal AY, Ghasemi J, Mostafavi MM, Sarlak N, Baharifard MT, *Chemical Papers*, **2009**, 63(3), 336-344, Doi: 10.2478/s11696-009-0003-0.
[21] Roy C, Chakrabarty J, Rao S, Modi PB, Vairale A., *J. Pharm.Rese.*, **2012**,5(7), 3665-3660.
[22] *The United States Pharmacopoeia* 29th NF 24, **2007**, Tretinoin, Tretinoin gel, Tretinoin cream, Tretinoin topical solution.
[23] International Conference on Harmonization .Stability Testing of New Drug Substances and Products. Q1A (R2), IFPMA, Geneva, Switzerland, **2003**.
[24] International Conference on Harmonization .Validation of Analytical Procedure, Text and Methodology Q2(R1), IFPMA, Geneva, Switzerland, **2005.**