Stability Indicating RP- HPLC - DAD Method for the Simultaneous Estimation of Hydrocortisone and Ketoconazole in Tablet Dosage form

[1]Ramavath Mohanbabu naik, [2]Syed Ahmed, [3]Dr.D.Ramakrishna, [4]Kambakam Venkatalakshmi

[1,2]Department of Pharmaceutical Analysis and Quality Assurance, Gland institute of pharmaceutical science,Kothapet(v), Near Narsapur,Medak(district),Telangana-502334.

[3]Principal of Shadan womens college of Pharmacy, Department of Pharmacognosy,Hyderabad,Telangana.

[4]Department of Pharmacognosy, Surabhi dayakar rao college of pharmacy,Gajwel (mandal),siddipet(district).

Word count:

Title	: 18
Abstract	:200
Manuscript	: 3327
References	: 10

***Abstract*: A new stability-indicating high performance liquid chromatographic method was developed and validated for the determination of Hydrocortisone and Ketoconazole in a tablet dosageform. The separation was achieved on a reverse phase C18 column (Agilent ODS) (250mm × 4.6 mm, 5 µ m) with mobile phase consisted of methanol:water (61:39 % *v/v* containing 0.2% HSA, pH 3.0 adjusted with Orthophosphoric acid) and the eluents were detected at 221nm. The retention time of Hydrocortisone and Ketoconazole was 6.0 min and 26.2 min respectively with the flow rate of 1mL.min^{-1}. Drugs were subjected to stress conditions of acidic, basic, oxidation, photolytic, neutral and thermal degradation and considerable degradants were detected in all stress conditions. A total of 17 degradation products were observed and well separated from analyte peaks, hence the method could effectively employed as stability-indicating one. The response of linear was in the range 5-50 µg/mL for Hydrocortisone and 10 to 70 µg/mL for Ketoconazole, respectively. The relative standard deviation values for intra- and interday precision studies were 1.06 % and 1.5 % for Hydrocortisone and 0.46 % and 1.1% for Ketoconazole.Recoveries ranged in between 98-102% for both Hydrocortisone and Ketoconazole..**

***Keywords*: RP-HPLC, Simultaneous, Hydrocortisone, Ketoconazole, stability-indicating**

Introduction

Hydrocortisone(HYDRO) is chemically (1S,2R10S,11S,14R,15S,17S)-14,17-dihyroxy-14-(2-hydroxyacetyl)-2,15-dimethyl tetracyclo[8.7.0.0^{2,7}heptadec-6-en-5-one. HYDRO belongs to Antiinflammato ry Agents. Structure of HYDRO was shown in figure 1[1].

Ketoconazole(KETO) is chemically 1-[4-(4-{2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl0piperazin-1-yl]ethan-1-one. It is used as Antifungal agents .Structure of KETO was shown in figure 2[2].

The review of literature revealed that various analytical methods involving spectrophotometry TLC, HPLC, HPTLC have been reported for HYDRO in single form and in combination with other drugs. Several analytical methods have been reported for KETO in single form and in combination with other drugs including spectrophotometry HPLC, HPTLC, LC – MS. To date, there have been no published reports about the simultaneous estimation of Hydrocortisone and Ketoconazole by HPLC in pharmaceutical

dosage forms. This present study reports for the first time simultaneous estimation of Hydrocortisone and Ketoconazole by HPLC in pharmaceutical dosage form. The proposed method is validated as per ICH guidelines.

Experimental

Materials and reagents

Analytically pure HYDRO was kindly provided by Hetero Laboratory, and KETO was provided by Mylan Laboratory, as gift samples. Analytical grade methanol was purchased from Merck & Co. Glasswares used in each procedure were soaked over night in a mixture of chromic acid and sulphuric acid rinsed thoroughly with double distilled water and dried in hot air oven. Water (HPLC grade) were purchased from Merck, India. Triple distilled water is used for all purpose.

Instrumentation

HPLC analysis was performed with Agilent-1200 binary pump plus manual sampler and Agilent photo diode-array detector (PDA). The output signal was monitored and processed using Ezchrome Elite software resident in a Pentium computer (Digital Equipment). Compounds were separated on a 250 mm-4.6 mm(id), 5µm particle size, Agilent C_{18} column with methanol and water (61:39 v/v) with 0.2 (%w/v) n-heptanesulfonicacid (HSA), 0.2%(v/v) and pH was adjusted to 3.0 with ortho phosphoric acid (OPA) as mobile phase. The pH meter equipped with a combined glass-calomel electrode, which was calibrated using standard buffer solutions of pH 4.0 and 7.0. The injection volume was 20µL, the mobile phase flow rate was 1.0 mL/min and the detection wavelength was 221nm.

Forced Degradation Studies

Stability testing is an important part of the process of drug product development. The purpose of stability testing is to provide evidence of how the quality of a drug Substance or drug product varies with time under a variety of environmental conditions, for example temperature, humidity, and light, and enables recommendation of storage conditions, retest periods, and shelf life to be established. The Stability-Indicating assay is a method that is employed for the analysis of stability samples in pharmaceutical industry. The ICH guidelines explicitly require conduct of forced degradation studies under a variety of conditions, like pH, light, oxidation ,dry heat, etc. and separation of drug from degradation products. The method is expected to allow analysis of individual degradation products. Forced degradations of HYDROand KETO drug substance were carried out under neutral, acidic, basic, oxidative, thermal and photolytic stress conditions. In stress study, aliquots of stress sample were diluted with mobile phase and achieved a concentration of 100 µg/mL of both drugs. The pH of stress sample was adjusted to 3-4 and injected in the optimized condition with appropriate blank. The samples from acid hydrolysis were neutralized with 1N NaOH and the samples from base hydrolysis were neutralized with 0.01N HCl. Blank solutions for each hydrolysis were prepared at the same time of preparation of stock solutions.

Preparation of Stock Solution for stress studies

The studies should be initiated at a concentration of 1 mg/mL. If solubility is a limitation, varying amounts of methanol may be used to get a clear solution or even the testing can be done on a Suspension**.** By using drug concentration of 1 mg/mL, it is usually possible to get even minor decomposition products in the range of detection. If several degradation products are formed in different conditions, the establishment of SIAM may involve a lot of development work. For this, repeat injections of reaction solutions might be required. Therefore, the volume of samples subjected to stress studies should be in sufficient quantity and also enough

sample volume should be drawn at each period. The withdrawn samples can be stored in cold cabinets to stop further reaction. The aliquots might be diluted or neutralized before injecting into HPLC.

An accurately weighed quantity of 10 mg of each drug substance was carefully transferred in to 10mL volumetric flask, dissolved completely in methanol and the volume was made up to the mark to get 1000 µg/mL. The same procedure was used to prepare stress solutions of acid hydrolysis, base hydrolysis and oxidation respectively with 1N HCl, 1N NaOH and 3%H_2O_2. Thermal degradation was carried out for solid state by means of heating the samples over a period in hot air oven, at 100ºC. Photo degradation was carried on solution sample as per outlined procedure in the following section. In all stress studies, stress was carried out for both HYDRO and KETO alone (control) as well as in combined form. Result of degradation studies for HYDRO and KETO alone was compared with degradation profile of combined studies.

Hydrolysis.

The hydrolytic stress studies were performed in acidic, basic and water (neutral) hydrolytic conditions. Generally the stress conditions used for the study of decomposition in acid, base revealed that HCl (0.1N), NaOH (0.1N) were most widely used respectively. Depending on the drug nature, the strength of the reagents may be increased to 1N, 2N, 2.5N and 5N etc.

The stock solutions of 1000 µg mL^{-1} were prepared in 1N NaOH (Basic), 1N HCl (Acidic) and water (neutral) at room temperature. 1mL volume of sample was withdrawn at different time points and made to 10 mL with mobile phase (100 µg/mL). The samples from acid hydrolysis were neutralized with 1N NaOH and the samples from base hydrolysis were neutralized with 0.01N HCl to protect the silica based column. Blank solutions for each hydrolysis were prepared at the same time of preparation of stock solutions.

Hydrogen Peroxide-induced Degradation

Evidently, H_2O_2 seems to be much more popular for the purpose of oxidation stress than any other oxidizing agent. The strength of the H_2O_2 used varies between 1% to 30%. 1 mL of sample was withdrawn from the stock solution (1000µg/mL) containing the required strength of the oxidizing agent (H_2O_2) in to 10 mL volumetric flask and made to 10mL with mobile phase to get the 100 µg/mL of each drug and then injected into the optimized conditions at various time intervals against blank.

Thermal Degradation

For, Solid state stability, it was performed on preheated sample as thin layer in the petridish at 100ºC. At various time intervals, 10 mg of the heated samples were weighed, dissolved in methanol and diluted with mobile phase to get a concentration of 100µg/ mL and injected into the HPLC system.

Photochemical Degradation

Photo degradation studies were conducted by exposing the solution sample in sunlight for a total period of 12 h. After degradation, sample was suitably diluted in mobile phase to concentration of 100 µg mL^{-1} and injected into the HPLC system.

Results and Discussion

Optimization of the Stability-Indicating HPLC Method

In this work we proposed a simple, fast, and accurate stability indicating RP-HPLC - DAD method for simultaneous determination of Hydrocortisone and Ketoconazole. For optimization of the chromatographic conditions and to obtain symmetrical peaks with better resolution and with peak purity, various conditions such as composition of mobile phase, mobile phases with different pH, stationary phases with different packing materials to Hydrocortisone and Ketoconazole combination.

The objectives of the present method were to separate both HYDRO and KETO from their possible degradants and to elute them as symmetrical peak. In this communication, Agilent Qualisil BDS column C_{18} (250mm×4.6mm, 5µm) was used as stationery phase. The flow rate was 1mL min^{-1} and the photo diode array (PDA) detection wavelength was 221nm. Various trials with methanol and water as mobile phase (70:30 v/v to 50:50 v/v) were performed, and HYDRO was not retained and KETO was observed in between 11 - 28 min with acceptable tailing. To retain the HYDRO, 0.2% HSA is added to the aqueous phase and the pH is adjusted to 3.0±0.2 with orthophosphoric (OPA) acid. But the HSA was selectively retained HYDRO,not shown effect on KETO. Hence the suitable retention time was obtained by optimizing % aqueous for stability assay and the retention times of HYDRO and KETO were 5.92 ± 0. 1min and 26.8 ± 0.4min, respectively [Figure 2]. The method has proven specificity by separating the degradants in various stress conditions.

Acid-Induced Degradation

Initial degradation study was performed in 0.1N HCl, observed that the drug was stable or showed negligible degradation. So, increasing in the strength of the acid is needed to get the sufficient degradation from the drug substances. Upon treatment with 1N HCl at RT for 12 h, sufficient degradation was 9.5% for HYDRO and 29% for KETO observed and it was considered as optimized for specificity. There were four degradant peaks, 9.6 min (D8) 11.2 min (D11), 13.41 min (D12), 46.7 min (D17) were formed. [Figure 3].

Base-Induced Degradation

When the drug was exposed to 0.1N NaOH, sufficient degradation was not observed hence a severe condition of 1N NaOH was chosen as stress condition. The drug degradation was 8.35% for HYDRO and 11.2% for KETO in 48 h with two impurities i.e. 2.86 min (D1), 3.16 min. (D2) ([Figure 4].

Neutral Hydrolysis

The degradation of 11% degradation was observed for HYDRO after 5 days at room temperature, but no degradation was observed for KETO. A total two impurities i.e. 4.02 min (D2), 4.80 min (D4) [Figure 8].

Hydrogen Peroxide-Induced Degradation

Both HYDRO and KETO showed negligible / no degradation in 0.3%.H_2O_2 for 5 days and hence severe stress condition of 3% H_2O_2 was used. The HYDRO showed 11% and KETO showed 9% degradation at 3days. Three degradants were formed i.e. 2.7 min (D1), 3.90 min (D2), 20.360 min (D15) [Figure 5].

Photochemical Degradation

Drugs were exposed to photolytic degradation in sunlight for 12 h, 7.5% and 30.2% degradation was observed for HYDRO and KETO respectively. Seven degradants were formed at 8.193 min (D7), 10.2 min (D9), 10.9 min (D10), 13.253 min (D12), 14.7 min (D13), 18.673 min (D14) and 21.06 min (D16). [Figure 6].

Thermal-Induced degradation

When drugs were exposed to dry heat in oven at 100°C for 2h, 6 degradation products were formed 4.147 min (D3), 4.947 min (D4), 5.553 min (D5), 6.80 min (D6), 10.153 min (D9) and 10.860 min (D10) with significant change in peak area of the parent drug. The TRA showed 11% and DIC showed 13% of degradation. [Figure 7] .

Specificity

Forced degradation studies were performed on HYDRO and KETO to support the specificity of the stability- indicating method. The study was employed on degradation of HYDRO and KETO by exposing to sun light (for 12 h), heat (100^0C for 2h), acid hydrolysis (1 N HCl, kept at RT for 12 h), base hydrolysis (1 N NaOH, kept at RT for 48 h), water hydrolysis (kept at RT for 5 days) and oxidation (3% H2O2, kept at RT for 3 days). All degradants adequately separated from HYDRO and KETO, thus the specificity of the method was proven.

Linearity and Range

The linearity of detector response to different concentrations of HYDRO and KETO was studied in the range from 5-50 µg/mL and 10-70 µg/mL, respectively [Table 2]. Samples were analyzed in triplicate at seven different concentrations. The correlation coefficient (R^2 value) obtained was 0.9990 for HYDRO and 0.9999 for KETO [Table 2].

Precision

Data for intraday and interday precision studies were obtained from three different concentrations (5, 30 50 µg/mL for HYDRO and 10, 50, 70 µg/mL for KETO) in the linearity range. The % RSD values for intraday and interday precision were below 1.5 %, indicating that the method was sufficiently precise [Table 3].

Recovery Studies

Accuracy was performed by recovery studies using standard addition method. Standard drugs in the range of 80, 100 and 120% of the sample concentration were added into the sample solution. Each concentration was analyzed in triplicate. Results of recovery studies were found to be in between 98 to 102 % for both HYDRO and KETO [Table 4].

Robustness

The robustness of the developed method was determined by analyzing the samples under a variety of conditions of the method parameters, such as change in flow rate (± 0.1 mL/min), pH (±0.2) of the buffer and Organic phase (±2 %). The method was robust for all the parameters tested. The % TEA and % HSA should be between 0.15 and 2.0 %.

Limit of Detection and Limit of Quantification

LOD and LOQ were determined based on signal to noise ratio. The S/N ratio of 3:1 was taken as LOD and S/N of 10:1 was taken as LOQ. LOD was found to be 0.42 µg/mL, 0.33 µg/ mL while LOQ was 1.46 µg mL^{-1} , 0.99 µg/mL for HYDRO and KETO, respectively [Table 2].

Analysis of Formulation

The assay of commercial tablets was established with present chromatographic condition developed and it was found to be more accurate and reliable. To determine the content of HYDRO and KETO in conventional tablet (50mg HYDRO/75mg KETO) twenty tablets were weighed; their mean weight was determined and was finely powdered. Tablet powder equivalent to 20 mg HYDRO with relevant quantities of KETO was weighed and transferred to a 100 mL volumetric flask, extracted for 30mins with methanol and volume was made up to 100 mL with diluent. 1 mL of above solution was taken in 10mL volumetric flask and volume was made up to 10 mL with mobile phase, and final solution (20µg/mL HYDRO, 30µg /Ml KETO) was filtered through 0.45 µ syringe filter and it was analyzed by HPLC system [Table 1].

Conclusion

A simple, fast and accurate stability indicating RP-HPLC-DAD method is described for simultaneous determination of Hydrocortisone and Ketoconazole in pharmaceutical formulations. The developed method was validated by testing its linearity, accuracy, precision, limits of detection and quantitation and specificity. The method is good enough to separate the peaks of active pharmaceutical ingredients (APIs) from the degradation products (produced during forced degradation studies) and concluded that the method can be successfully used for detect any kind of degradants during stability studies of tablet dosage forms.

References

[1] Simoncic, Z., Roskar, R., Gartner, A., Kogej, K., and Kmetec, V.; *International Journal of Pharmaceutics*, (2008); 356, 200.
[2] Medenica, M., Ivanovic, D., Maskovic, M., Jancic, B., and Malenovic A.; *Journal of Pharmaceutical* and *Biomedical Analysis*, (2007); 44, 1087.
[3] Validation of Analytical Procedures: Text and Methodology (Q2B), ICH Harmonised Tripartite Guideline.
[4] General Chapter 1225, Validation of compendial methods, United States Pharmacopeia 30, National Formulary 25, Rockville, Md., USA, The United States Pharmacopoeial Convention,Inc., (2007).
[5] Stability Testing of New Drug Substances and Products (Q1A (R2)), ICH Harmonised Tripartite Guideline.
[6] Nash, R.A., Wachter, A.H.; Pharmaceutical process validation, 3rd volume 129, pp.507–523. Grace S. N. Lau, J. A. J. H. Critchley,Ankit patel, Jaimin patel, Amit shah.; Development and validation of spectrophotometric method for Simltaneous estimation of Hydrocortisone and Ketoconazole in Tablet dosage form, International Journal of Pharmacy and Pharmaceutical Sciences,(2012); 56, 198.
[7] Anantha Kumar D.; Sujan D.P.; Vijayasree V.; Seshagiri rao J.V.L.N. Development and validation of dual wavelength spectrophotometric method for simultaneous estimation of Hydrocrtisone and Ketoconazole in their combined dosage form, *E.J.Chem.* 2009; 6: 541-544.
[8] ICH-Q2A. Text on Validation of Analytical Procedures, March 1995.
[9] Z.Zaheer, Manisha.M.Devdikar, A.G.Nikalje.; Stability indicating HPLC method for determination of Ketoconazole tablet dosage form, Inventi impact: Pharm Analysis & Quality Assurance, (2011), 34, 541-589.
[10] Hisham, E.; *Journal of. Pharmaceutical and Biomedical Analysis,* (1998); 17, 1267.

FIGURES & TABLES

Figure 1: Hydrocortisone

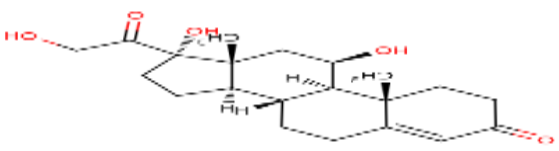
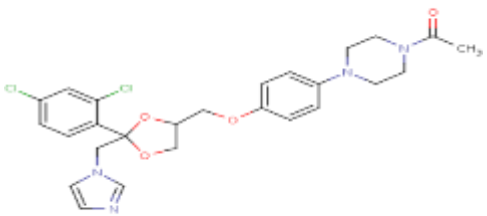


Figure 2: Ketocconazole



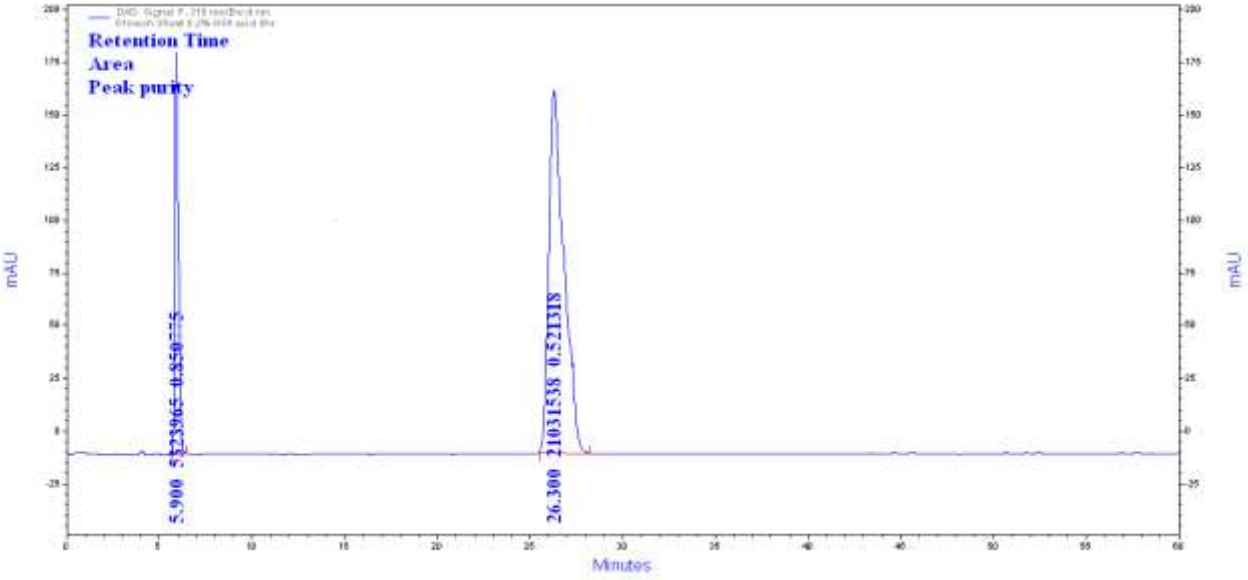


Figure 2. Optimized RP-HPLC Chromatogram of HYDRO (Rt : 5.9) and KETO (Rt: 26.30)

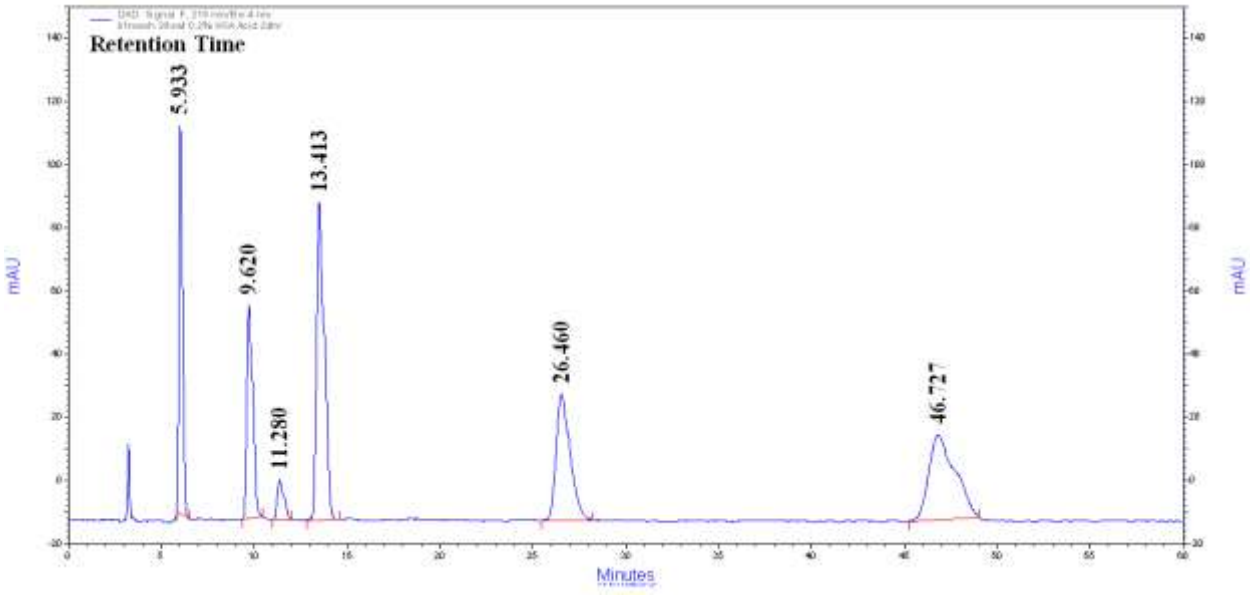


Figure 3. Acid degradation (1N HCl for 12 h) chromatogram of HYDRO and KETO

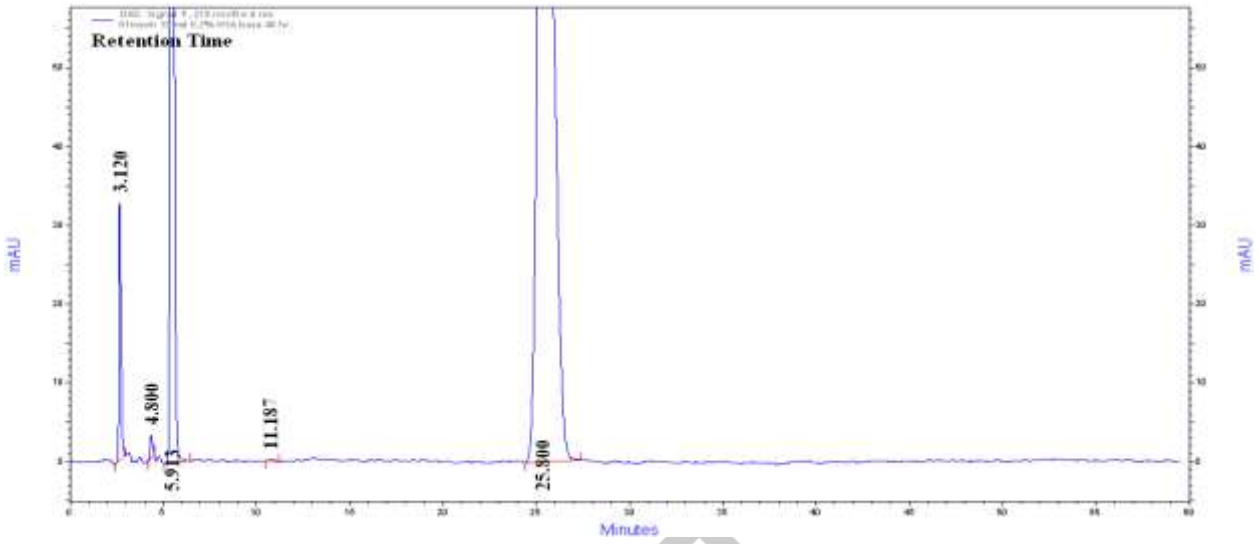


Figure 4. Base degradation (1N NaOH for 48 h) chromatogram of HYDRO and KETO

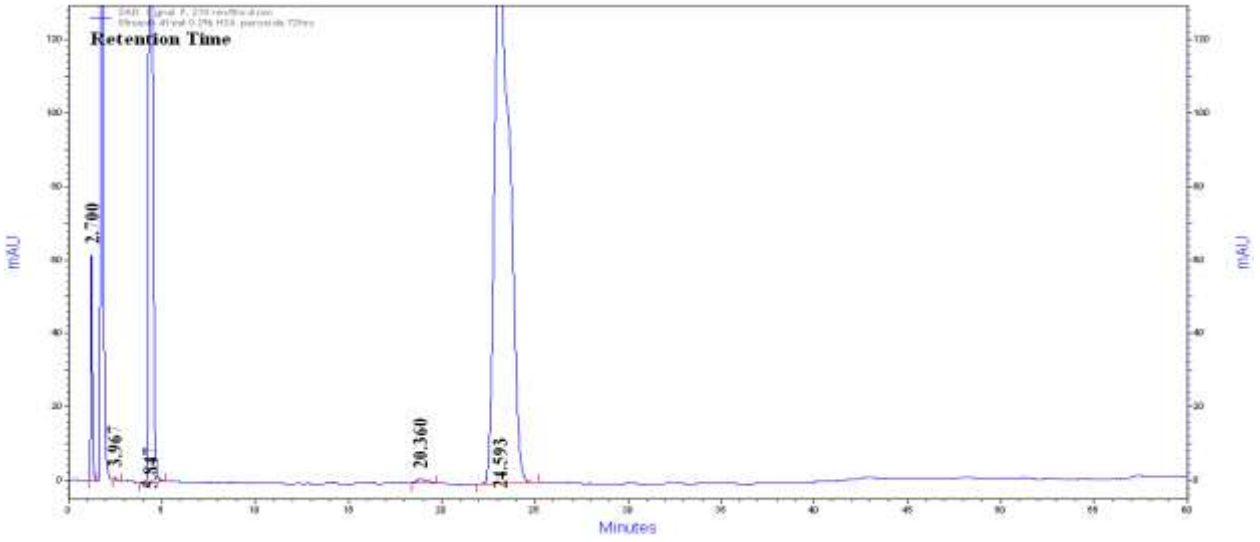


Figure 5. Oxidative degradation (3 % H_2O_2 for 3 days) Chromatogram of HYDRO and KETO

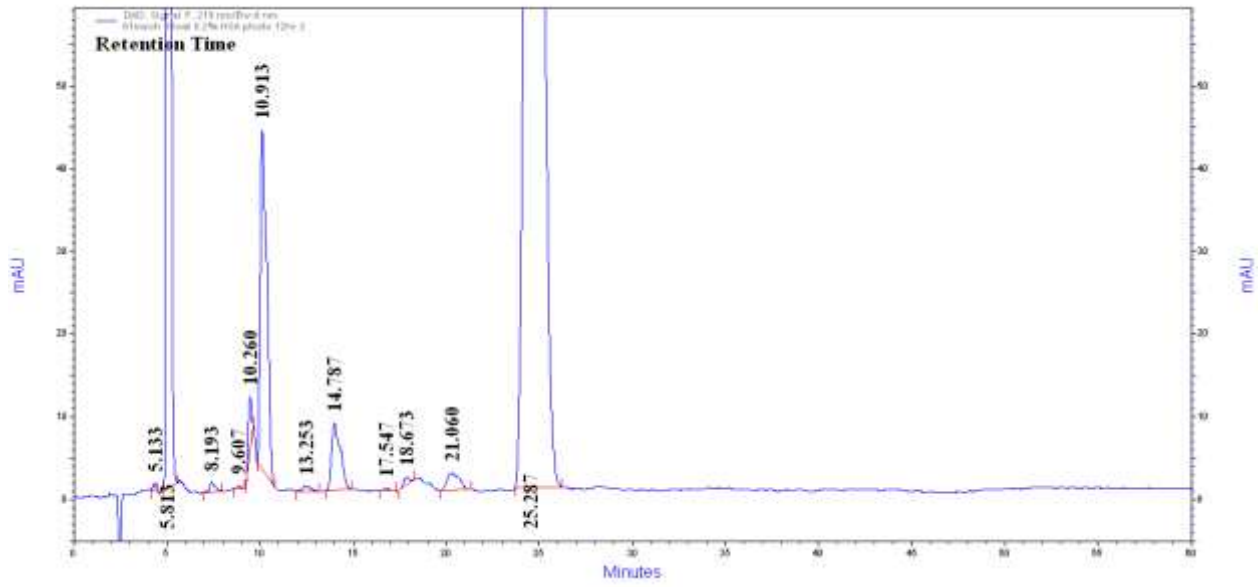


Figure 6. Photo degradation (sunlight for 12 h) chromatogram of HYDRO and KETO

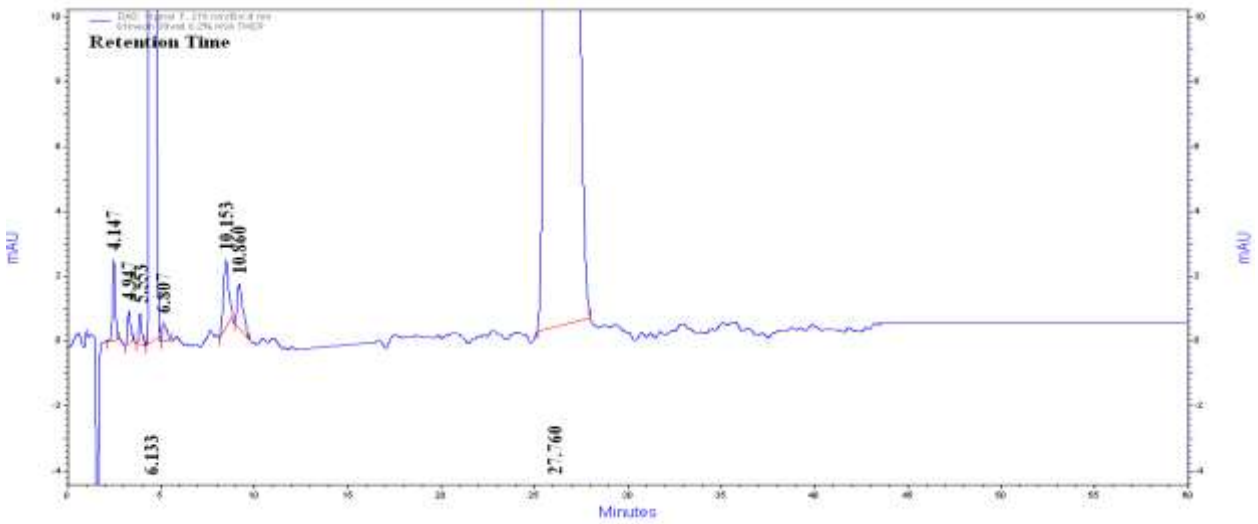


Figure 7. Thermal degradation (100º C for 6 h) chromatogram of HYDRO and KETO

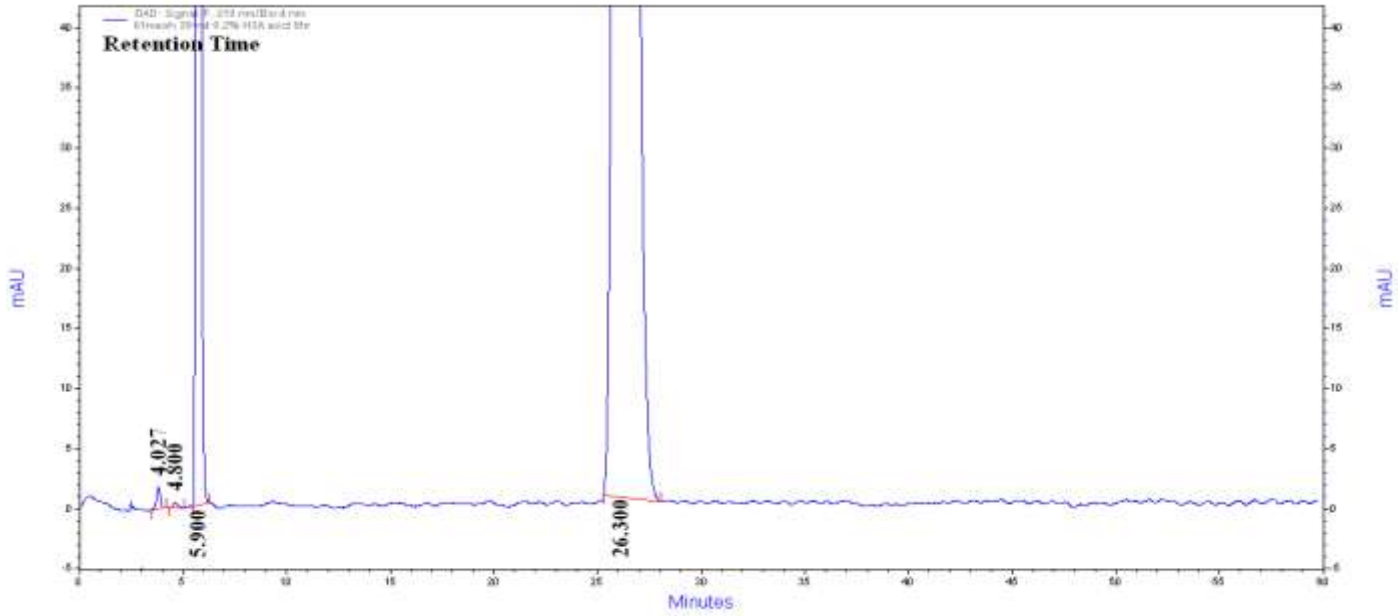


Figure 8. Neutral degradation (in Water for 5 days) chromatogram of HYDRO and KETO

Table 1. Analysis of Commercial Formulation

	HYDRO			KETO		
Label claim(mg/tablet)	Amount Found±SD	Label claim%	% RSD	Amount Found±SD	Label claim%	% RSD
TRA-50 DIC-75	49.1±0.66	98.2	1.3	74.1±0.65	98.8	0.8

Table 2. Regression parameters, LOD and LOQ

Validation parameters	HYDRO(221nm)	KETO(221nm)
Range(µg/mL)	5-50	10-20
Regression equation	y=79589x-58844	y= 94108x+988975
Correlation coefficient(r^2)	0.9990	0.9999
Limit of Quantification (µg/mL)	1.26	0.99
Limit of Detection (µg/mL)	0.42	0.33

Table 3. Precision Studies

Precision	HYDRO con. (µg/mL)			KETO con. (µg/mL)		
	5	30	50	10	50	70
Intra day						
Mean(n=3)	5.16	29.43	50.12	9.96	49.65	70.23
RSD (%)	1.1	0.8	1.3	0.3	0.2	0.9
Inter day						
Mean (n=3)	4.87	31.22	49.45	9.21	51.55	69.55
RSD (%)	1.4	1.3	1.8	1.1	0.9	1.3

Table 4. Recovery studies

Recovery Level	HYDRO con. (20 µg/mL)			KETO con. (30 µg/mL)		
	Amount Added (µg/mL)	Amount Found (µg/mL)	% Recovery	Amount Added (µg/mL)	Amount Found (µg/mL)	% Recovery
80 %	16	36.26	101.86	54	53.21	98.72
100 %	20	40.16	99.56	60	61.18	99.65
120 %	24	43.16	98.99	66	65.76	100.56

Table 5. Comparative study of degradation data of HYDRO and KETO: Control Vs Combination

Stress Conditions	% degradation				Impurities (D_X) x = 1, 2, 3…			
	Control		Combination		Control		Combination	
	HYDRO	KETO	HYDRO	KETO	HYDRO	KETO	HYDRO	KETO
Neutral (5 days)	10.1	--	11.2	0	D1, D2	--	D2, D4	--
1N HCl (12 h)	10.1	35.3	9.8	29	D1, D2	D7, D8, D11, D12, D17	D1, D2	D7, D8, D11, D12, D17
1N NaOH (48 h)	14.4	7.4	12.3	7.9	D1, D2, D3, D4	D5, D6	D1, D2, D3, D4	D5, D6
Peroxide (72 h)	9.8	8.7	14.5	9.6	D1, D3	D6, D13, D15	D1, D3	D6, D13, D15
Thermal 100ºC; 2 h	17.5	5.2	18.6	10.3	D1, D2, D3, D4, D9, D10	D5, D6	D1, D3, D4, D 9, D10	D5, D6, D8
Sunlight 12h	11.3	23.5	15.2	30.4	D1, D9, D10	D7, D11, D12, D13, D14, D15	D1, D9, D10	D7, D11, D12, D13, D14, D15, D16

R_t (min) of impurities: D1: 3.1, D2: 3.9, D3: 4.2, D4: 4.8, D5: 5.5, D6: 6.8, D7: 8.1, D8: 9.6, D9: 10.2, D10: 10.9, D11: 11.2, D12: 13.4, D13: 14.7, D14: 18.6, D15: 20.3, D16: 21.03, D17: 46.7.