Research Article

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Development and Validation of a stability indicating method for the simultaneous determination of Atenolol and Hydrochlorothiazide by HPLC

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Abstract:

A simple, selective, rapid, precise and stability indicating high performance liquid chromatographic method was developed and validated for the simultaneous estimation of Atenolol and Hydrochlorothiazide in pharmaceutical Tablet dosage form. **Materials and Methods:** The method involves the use of easily available inexpensive laboratory reagents. The mobile phase consisted of 50:50 % (v/v) of Methanol & 0.1% v/v orthophosphoric acid operated on isocratic mode. The flow rate is 0.6 ml/min. Chromatographic separation of Atenolol and Hydrochlorothiazide was performed on Phenomenex Prodigy C_{18} column (150 X 4.6 mm id, ODS 2, 5µm). The wavelength of detection is 271 nm. The injection volume is 20µL. The retention time of Atenolol and Hydrochlorothiazide are 2.68 ± 0.10 minutes and 3.49 ± 0.10 respectively. The run time of analysis is 5 minutes. **Results:** A linear response was observed over the concentration range 10.00-100.00 µg/mL of Atenolol and the concentration range 1.00-10.00 µg/mL of Hydrochlorothiazide. Limit of detection and limit of quantitation for Atenolol were 0.1µg/mL and 1 µg/mL, and for Hydrochlorothiazide were 0.05µg/mL and 0.5µg/mL, respectively. The method was successfully validated in accordance to ICH guidelines acceptance criteria for linearity, accuracy, precision, specificity, robustness. The influence of acid, alkaline, oxidative Stress and photolytic stress conditions on both the drugs was studied. Results indicated complete degradation in alkaline medium for Atenolol and Hydrochlorothiazide **Conclusion:** The analysis concluded that the method was selective for simultaneous estimation of Atenolol and Hydrochlorothiazide can be potentially used for the estimation of these drugs in combined dosage form.

Keywords: Atenolol, Hydrochlorothiazide, HPLC, Antihypertensive agents, thiazide diuretic.

Introduction

Atenolol **(Fig-1a)** may be chemically described as a benzene acetamide, 4-[2-hydroxy-3-[(1-methyl et-[2-hydroxy-3-[(1-methyl ethyl) amino] propoxy [1]. Atenolol is a selective beta-1 adrenergic receptor antagonist [2]. It is used in the treatment of cardiovascular diseases such as angina, hypertension, cardiac arrhythmias and myocardial infarctions. Atenolol competitively blocks beta-adrenergic receptors in the heart and juxtoglomerular apparatus. It decreases the release of renin from the kidney, thus lowering the blood pressure [3]. It is official in IP [4], BP [5] and USP [6]. Few chromatographic methods such as HPLC [7], RP-HPLC [8] and Bio-analytical [9] methods have been reported for estimation of Atenolol.

Hydrochlorothiazide [1] **(Fig-1b)** is chemically 6-Chloro-3-4-dihydro-2H-1-2-4-benzothiadiazine-7-sulfonamide1,1-dioxide belongs to the thiazide class of diuretics [2]. It is used for the treatment of edema associated with heart (congestive heart failure), liver

Fig-1a: Structure of Atenolol

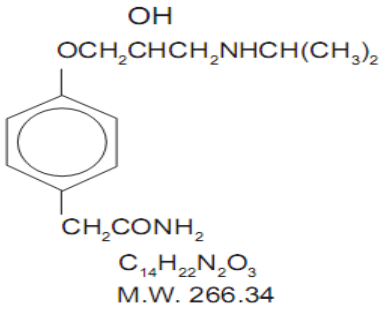
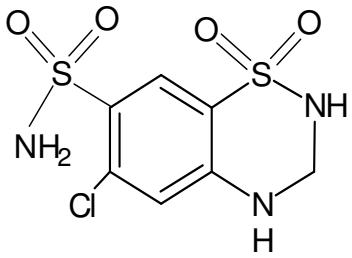


Fig-1b: Structure of Hydrochlorthiazide



(hepatic cirrhosis), renal (nephrotic syndrome, chronic renal failure and glomerulonephritis) diseases. Hydrochlorothiazide acts on the kidneys to reduce sodium (Na) reabsorption in the distal convulated tubule [10]. It is official in IP, BP and USP. Some Spectrophotometric methods [11,12] have been reported for the estimation of Hydrochlorothiazide.

The combination of Atenolol and Hydrochlorothiazide is useful in the treatment of hypertension and congestive heart failure (CHF). The combined drug therapy of Atenolol and Hydrochlorothiazide has greater antihypertensive activity literature review reveals that HPLC [13], RP-HPLC [14] and spectrophotometric [15] and bio-analytical methods for Atenolol alone or in combined dosage forms and various UV, HPLC, RP HPLC methods for Hydrochlorothiazide alone or in combined dosage forms. The aim of the present study was to develop accurate, precise and selective reverse phase HPLC methods for the simulated analysis of Atenolol and Hydrochlorothiazide.

2. EXPERIMENTAL

2.1. Reagents and chemicals

Orthophosphoric acid (AR Grade, Merck ltd), Methanol (HPLC grade, Merck ltd), Milli-Q water, Atenolol (99.8 % w/w is a gift sample from Zydus Cadila) and Hydrochlorothiazide [100 % w/w is a gift sample from Zydus (Biogen)], glacial acetic Acid (GR Grade, SD Fine Chem Ltd). All other chemicals are of the highest grade commercially available unless otherwise specified. ATEN-H tablets for evaluation of the assay content were purchased from a local pharmacy.

Atenolol (99.8 % w/w is a gift sample from Zydus Cadila) and Hydrochlorothiazide [100 % w/w is a gift sample from Zydus (Biogen)], glacial acetic Acid (GR Grade, SD Fine Chem Ltd). All other chemicals are of the highest grade commercially available unless otherwise specified. ATEN-H tablets for evaluation of the assay content were purchased from a local pharmacy.

2.2. Apparatus and chromatographic conditions

The Chromatographic system consisted of a Shimadzu Class VP Binary pump LC-10ATvp, SIL-10ADvp Auto sampler, CTO-10Avp Column Temperature Oven, SPD-10Avp UV-Visible Detector. All the components of the system are controlled using SCL-10Avp System Controller. Data acquisition was done using LC solutions software. The mobile phase consisted of 50:50 % (v/v) of Methanol and 20mM Orthophosphoric acid (pH adjusted to 3.0 with acetic acid) operated on isocratic mode. The flow rate is 0.6 ml/min. Chromatographic determination of Atenolol and Hydrochlorothiazide was performed on PHENOMENEX Prodigy C_{18} column (150 X 4.6 mm id, ODS 2, 5µm). The wavelength of detection is 271 nm. The injection volume is 20µL.

2.3. Preparation of standard solutions, Calibration Standards & Quality Control Samples

Stock solutions of Atenolol (1mg/mL), & Hydrochlorothiazide (1mg/mL) were prepared separately in a volumetric flask using methanol and labeled accordingly. Suitable dilutions were then prepared using 50:50 %v/v Methanol & Milli-Q water as Diluent Solution. A Linear Calibration curve containing 8 non-zero standards were prepared using Diluent solution in the concentration range of 1.00-10.00 µg/mL for Hydrochlorothiazide & 10.00-100.00 µg/mL for Atenolol. The calibration standard sample is then transferred into the auto sampler for analysis. Samples for Specificity (Sample with Hydrochlorothiazide alone, sample with Atenolol alone, Blank Sample and sample containing both the drugs) were also prepared accordingly.

For the preparation of quality control samples, a separate stock containing approximately the same concentration of the Hydrochlorothiazide and Atenolol were prepared and labeled as quality control stocks. From these stocks, quality control samples containing Hydrochlorothiazide and Atenolol were prepared at three concentration levels namely LQC, MQC and HQC so as to obtain low,

median and high concentration quality control samples. The performance of the linear calibration curve is then evaluated using quality control samples.

2.4. Assay

The assay of tablets containing Atenolol and Hydrochlorothiazide (Brand name: ATEN- H) is done using the procedure given in Indian Pharmacopoeia under tablets. The active ingredients in each of 10 dosage units is taken by random sampling and analyzed by the developed method.

For the current assay ten tablets were randomly taken and transferred separately into 100ml volumetric flasks and dissolved in 20 ml methanol. The solution was then ultrasonicated for 10min and then made up to volume. Required amount of solution is then taken and filtered through 0.45µ nylon membrane and diluted with diluent solution so that the resultant concentrations are within the calibration range of the developed method. The samples are then analyzed by using the validated method. The sample is then injected in triplicate.

2.5 Method Validation

2.5.1 System Suitability

A sample containing mixture of Atenolol (at concentration of 50.00µg/ml) and Hydrochlorothiazide (at concentration of 5µg/ml) was used as system suitability sample. System suitability was assessed by six replicate analysis. A percent coefficient of variation (% CV) less than 1 % for retention times for the drugs is taken as the acceptance criterion.

2.5.2 Detection and Quantitation Limits (Sensitivity)

Limits of detection (LOD) and quantification (LOQ) (**Fig-2**) were estimated from both linearity calibration curve method and signal to noise ratio method. The detection limit was defined as the lowest concentration level resulting in a peak area of three times the baseline noise. The quantification limit was defined as the lowest concentration level that provided a peak area with signal to noise ratio higher than 5, with precision (%CV) and accuracy with (±) 20%.

2.5.3 Linearity (Calibration Curve)

The calibration curve was constructed with eight non-zero standards ranging from 1.00 to 10.00 µg/mL for Hydrochlorothiazide and 10.00–100.00 µg/mL for Atenolol. The linearity was evaluated by linear regression analysis, which was calculated by least square method. It is depicted in (**Fig- 3**).

Fig-2: Chromatograms shown below indicate limit of Detection (LOD) above and Limit of Quantitation (LOQ) below.

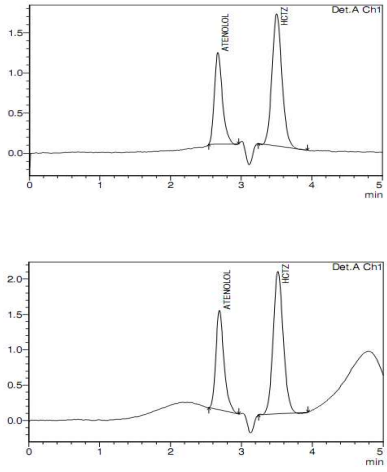


Fig-3a: Linear calibration curve of HCTZ

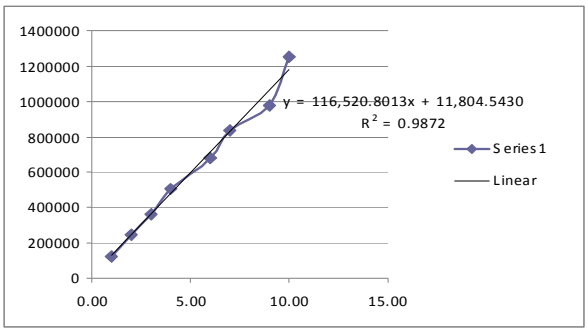
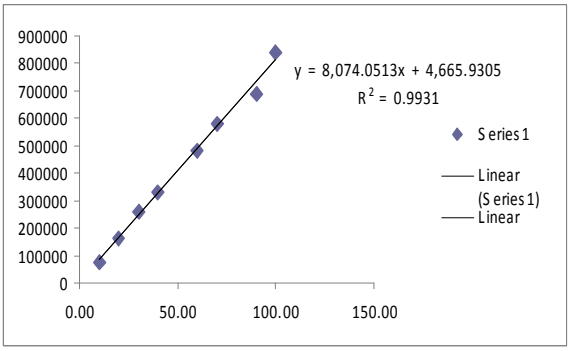


Fig-3b: Linear calibration curve of Atenolol.



2.5.4 Accuracy and Precision

Accuracy of assay method was determined for both intra-day and inter-day variations using triplicate analysis of the QC samples. Precision of the assay was determined by repeatability (intra-day) and intermediate

precision (inter-day). Repeatability refers to the use of the analytical procedure within the laboratory over the shorter period of the time that was evaluated by assaying the QC samples during the same day. Intermediate precision was assessed by comparing the assays on different days (2 days).

2.5.5 Specificity

For demonstration of specificity, 4 samples namely Blank sample, sample containing Diazepam alone, sample containing Propranolol HCl alone and sample containing the mixture of Diazepam and Propranolol HCl were prepared separately. Specificity of the method was determined by comparing results of all the samples (**Fig-4**). The developed method is said to be specific if the % interference calculated as peak area (if any) at the retention time of each of the analytes in the blank sample is less than 20% of peak area at the corresponding retention times of each of the drugs in the lowest calibration standard. Sample Specificity is also observed in the degradation study of the drug. None of the degraded products must interfere with the quantification of the drug.

2.5.6 Stability

The stability of the drug is determined by placing the MQC samples for the short term stability at room temperature up to 12 hours and then comparing the obtained peak area with that of the similarly prepared fresh sample. Further, auto-sampler stability for up to 24 hrs was studied and established.

2.5.7 Stress Degradation Studies

For Stress Degradation Analysis, 1 mL aliquots (in duplicate) of samples containing MQC level concentration are treated separately with 100 µL of 0.1N HCl (Acid stress), 0.1N NaOH (Alkaline stress), 5% v/v Hydrogen Peroxide (Oxidative Stress), for 24 Hrs. Samples for Photolytic stress are placed in a transparent glass vial & placed in a UV chamber for 24 Hrs. Samples are then injected for analysis. The results of analysis are then compared with similarly prepared fresh samples. The analysis is performed in triplicate.

3.0 RESULTS AND DISCUSSION

3.1 Method Development and Validation

The HPLC procedure was optimized with a view to develop a stability indicating assay method. Functional character to the molecules. Therefore we evaluated the chromatographic behavior at different pH values ranging from pH 3.0 to pH 6.5 using various columns like Hypersil-BDS-C18, Symmetry C18, Ymc-pack C18, Ymc-pack pro, Spherisorb C18, Phenomenex C18 have been tried with different buffer salts such ammonium Formate, ortho phosphoric acid, di-potassium hydrogen orthophosphate, in combination with acetonitrile, methanol and tetrahydrofuran. However less tailing and high theoretical plates are obtained with Phenomenex Prodigy column C18 150 X 4.6 cm 5µm column.. . Mobile phase composition consisted of (50:50 v/v) of Methanol and 20mM Orthophosphoric acid (pH adjusted to 3.0 ± 0.1 with glacial acetic acid) on isocratic mode. The flow rate of the method is 0.6 ml/min. Calibration standards were prepared in diluents solution containing 50:50 % v/v of Methaol and Milli-Q water. The wavelength of detection is 271nm. The column temperature is maintained at 25 ^{O}C. At the reported flow rate, peak shape was excellent; however increasing or decreasing the flow rate resulted in unacceptable tailing factor and poor peak shape. Hence 0.6 ml/min was optimized flow rate decreasing the consumption of the mobile phase, which in turn proves to be cost effective for long term routine quality control analysis. To evaluate the feasibility of the experiment under regular lab conditions, we assessed the stability of Hydrochlorothiazide and Atenolol under room temperature and under normal light conditions.

3.2 Method Validation

3.2.1 System Suitability

The % RSD of the peak area for both drugs is within the acceptable criteria (**Table-1**). The efficiency of the column was expressed as the number of theoretical plates for the six replicate injections was around 7709 ±82.4 for Hydrochlorothiazide and 6860 ± 72.60 for Atenolol. The USP tailing factor for Atenolol is 1.31±0.02 while that of hydrochlorthiazide is 1.15 ±0.02.

3.2.2 Determination and Quantification Limits (Sensitivity)

Fig-2 represents the chromatogram of limit of detection and limit of quantification. The method is found to be sensitive which can be determined from the data

group analysis revealed the presence of acidic

3.2.3 Linearity

The linearity was demonstrated in triplicate. The results of the best fit line (y = mx + c) for the triplicate analysis is given in **Table 3**. The accuracy of the calibration standards was evaluated from the back calculated concentrations **(Table 4)**. All the standards were found to be within the range atenolol is 90.39-105.82% and hydrochlorothiazide is 92.43-106.16 %.

3.2.4 Accuracy and Precision

Accuracy and precision calculated for the QC samples during the intra- and inter –day run are given the (**Table-5)**. The intra-day (day-1) accuracy for Atenolol ranged from 96.75- 110.15 % while that of Hydrochlorothiazide ranged from 95.27 – 103.75 %. The intra-day (day-1) precision for Atenolol ranged from 1.39- 1.71 % while that of Hydrochlorothiazide ranged from 2.10 – 2.83 %. The inter-day (day-2) accuracy for Atenolol ranged from 98.34- 107.95 % while that of Hydrochlorothiazide ranged from 96.73– 103.90 %. The inter-day (day-2) precision for Atenolol ranged from 0.72-1.89 % while that of Hydrochlorothiazide ranged from 1.00– 2.47 %. The results obtained from intermediate precision (inter-day) also indicated a good method precision. All the data were within the acceptance criteria.

3.2.5 Specificity

Specificity was determined by comparison of the Blank chromatogram with that of the Standard chromatogram (**Fig-4**)

3.2.6 Room Temperature Stability

Stability studies were done for short term stability up to 12 hrs on the bench top for the MQC levels conditions. Stability is calculated as the ratio of the mean peak area of the stability sample to the mean peak area of the fresh sample and expressed as the percentage (n=6). The room temperature stability was found to be 103.19 % for Atenolol and 101.56 % for Hydrochlorothiazide. The results are tabulated in **Table-6**.

3.2.7 Stress Degradation

Stress studies revealed that Atenolol is not susceptible to degradation under acid, light (UV) and oxidative stress conditions (**Fig 5**). However, in alkaline conditions (0.1N NaOH), the drug was instable and the degradation peak eluted and accompanied with a drastic peak distortion obtained from the (**Table-2)**.

drug content was within 97 –104 % for all stress conditions indicating the stability and specificity of the analytical method to differentiate the degradation peaks.

Stress studies on Hydrochlorothiazide indicated instability under alkaline conditions. This has been clearly demonstrated by the help of overlap spectra of all the stress samples as compared with that of freshly prepared sample of similar concentration **(Fig 5)**.

3.2.8 Robustness study

Robustness is the measure of method capacity to remain unaffected by deliberate small changes in the chromatographic conditions. The experimental conditions were deliberately altered to evaluate the robustness of the method. The impact of flow-rate (0.6 ± 0.1 ml/min), and effect of mobile-phase composition (± 5%) on chromatographic parameters such as retention time, theoretical plates, and tailing factor, were studied**.** At lower flow rate, the retention time of Hydrochlorothiazide was 4.19 ± 0.01 minutes (n=5) while that of Atenolol was 3.22 ± 0.01 minutes. At lower flow rate, the tailing factor for Hydrochlorothiazide increased to 1.163 ± 0.01 while that of Atenolol increased to 1.380±0.02. At higher flow rate, tailing factor for both Atenolol and hydrochlorothiazide is decreased to 1.273±0.01 and 1.120±0.01 respectively as compared to normal flow. The elution was earlier at higher flow rate; Hydrochlorothiazide and Atenolol eluted at 3.0 ± 0.01 and 2.5 ± 0.41 minutes respectively. The retention time of and 25 parts of 20m orthophosphoric acid (pH 3.0).

3.3 Application of the method to dosage forms

The HPLC method developed is sensitive and specific for the quantitative determination of Atenolol and Hydrochlorothiazide. Also the method is validated for different parameters; hence it has been applied for the simultaneous estimation in pharmaceutical dosage forms. ATEN-H was evaluated. The % assay of Atenolol in the tablet is 99.63 % and % assay of Hydrochlorothiazide is 101.12 %. None of the tablets ingredients interfered with the analyte peak. The spectrum of Atenolol and Hydrochlorothiazide the extracted tablet was matching with that of standard compounds indicating the purity of the compounds in the tablets.

Conclusions

The method gave accurate and precise results in the

and increased tailing. Except for alkaline conditions, the

Hydrochlorothiazide and 10.00 to 100.00µg/mL for Atenolol. The mobile phase composition consists of (50:50 v/v) of Methanol and orthophosphoric acid (pH adjusted to 3.0 with glacial acetic acid), at the flow rate of 0.6 ml/min. The retention time of Atenolol is 2.68 ± 0.1 minutes and that of Hydrochlorothiazide is 3.49 ± 0.03 minutes. The column is Phenomenox Prodigy C_{18} column (150 X 4.6 mm id, ODS 2, 5µm. A rapid sensitive and specific method for the simultaneous estimation of Hydrochlorothiazide and Atenolol in the pharmaceutical tablet formulations has been developed and validated.

concentration range of 1.00 – 10.00 µg/mL for

Table 1. System Suitability test for Diazepam (above) and Proprtanolol (below)

ATENOLOL				
Sample ID	**Peak Retention Time**	**Peak Area**	**Theoretical Plates**	**Tailing Factor**
1	2.68	431601	6782	1.32
2	2.67	416312	6942	1.3
3	2.68	389296	6782	1.31
4	2.67	400936	6936	1.3
5	2.68	379928	6888	1.31
6	2.66	404366	6830	1.31
MEAN	**2.673**	**403739.8**	**6860.0**	**1.308**
STDEV	**0.0082**	**18537.45**	**72.60**	**0.0075**
%CV	**0.31**	**4.59**	**1.06**	**0.58**

HYDROCHLORTHIAZIDE (HCTZ)				
Sample ID	**Peak Retention Time**	**Peak Area**	**Theoretical Plates**	**Tailing Factor**
1	3.49	634191	7636	1.15
2	3.49	626426	7658	1.15
3	3.49	595988	7775	1.13
4	3.48	577177	7640	1.15
5	3.49	533850	7839	1.15
6	3.48	581167	7706	1.14
MEAN	**3.487**	**591466.5**	**7709.0**	**1.145**
STDEV	**0.0052**	**36600.34**	**82.41**	**0.01**
%CV	**0.15**	**6.19**	**1.07**	**0.00**

Table 2. Sensitivity

ATENOLOL
LOQ

SR NO	DRUG	
	Retention Time	Peak Area
1	2.69	11222
2	2.69	11759
3	2.68	9280
MEAN	**2.7**	**10753.7**
ST DEV	**0.01**	**1304.17**
% CV	**0.21**	**12.13**

HYDROCHLOROTHIAZIDE
LOQ

SR NO	DRUG	
	Retention Time	Peak Area
1	3.51	19841
2	3.52	20905
3	3.51	17675
MEAN	**3.5**	**19473.7**
ST DEV	**0.01**	**1646.03**
% CV	**0.16**	**8.45**

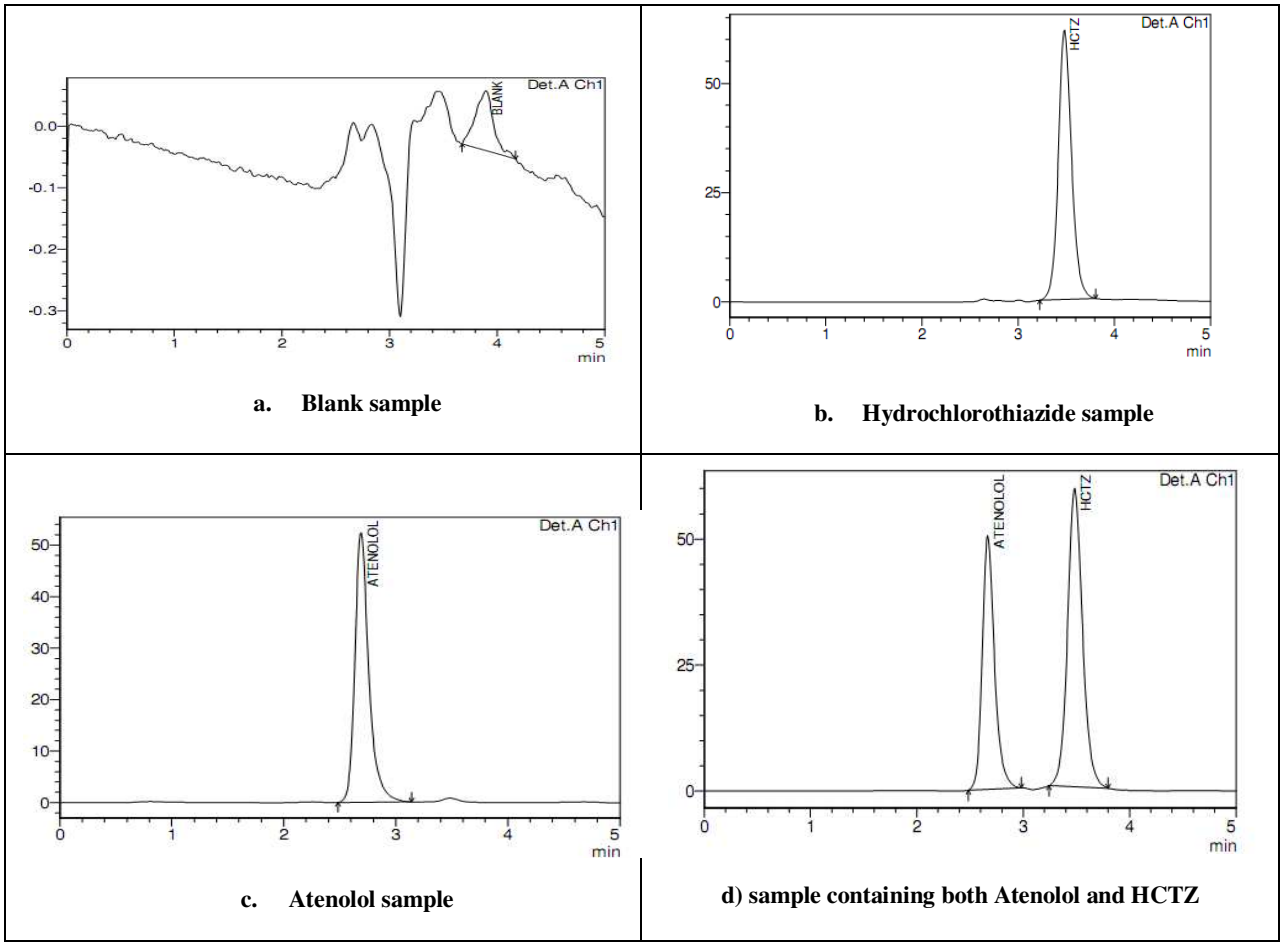
ATENOLOL
LOD

SR NO	DRUG	
	Retention Time	Peak Area
1	2.68	9395
2	2.67	8941
3	2.68	8612
MEAN	**2.7**	**8982.7**
ST DEV	**0.01**	**393.16**
% CV	**0.22**	**4.38**

HYDROCHLOROTHIAZIDE
LOD

SR NO	DRUG	
	Retention Time	Peak Area
1	3.51	17087
2	3.5	16145
3	3.52	14538
MEAN	**3.5**	**15923.3**
ST DEV	**0.01**	**1288.88**
% CV	**0.28**	**8.09**

Fig-4: Comparison of (a)Blank Chromatogram, (b) Hydrochlorothiazide (c) Atenolol and (d) sample containing both Atenolol and Hydroclorothiazide



a. Blank sample

b. Hydrochlorothiazide sample

c. Atenolol sample

d) sample containing both Atenolol and HCTZ

Table 3. Results of best-fit line for triplicate analysis for Atenolol (above) and Hydrochlorothiazide (below)

Atenolol			
Curve	**Slope**	**Intercept**	**r^2**
1	8074.05	4665.93	0.9931
2	8101.25	3955.23	0.9967
3	8066.41	5896.40	0.9980
Mean	**8107.24**	**4839.18**	**0.9959**

Hydrochlorothiazide			
Curve	**Slope**	**Intercept**	**r^2**
1	116520.80	11804.54	0.9872
2	117943.38	4251.27	0.9900
3	119158.36	153.70	0.9932
Mean	**117874.18**	**5403.17**	**0.9902**

Table 4. Linearity and Range for Atenolol (above) and Hydrochlorothiazide (below) demonstrating accuracy, carryover effect and specificity of the method (Curve 1).

ATENOLOL					
Sample ID	**Concentration (Microgram/mL)**	**Retention Time**	**Peak Area**	**Back Calc Concentration**	**% Accuracy**
Blank	0	**NA**	**0**	NA	
CC - 01	10.00	2.55	77650	9.04	90.39
CC - 02	20.00	2.68	164082	19.74	98.72
CC - 03	30.00	2.68	260974	31.74	105.82
CC - 04	40.00	2.69	331831	40.52	101.30
CC - 05	60.00	2.67	481566	59.07	98.44
CC - 06	70.00	2.68	582136	71.52	102.17
CC - 07	90.00	2.68	687147	84.53	93.92
CC - 08	100.00	2.68	843043	103.84	103.84
Blank	**0**	**NA**	0	**NA**	**NA**

- NA - Not applicable

HYDROCHLOROTHIAZIDE					
Sample ID	**Concentration (Microgram/mL)**	**Retention Time**	**Peak Area**	**Back Calc Concentration**	**% Accuracy**
Blank	0.00	**NA**	**0**	NA	#VALUE!
CC - 01	1.00	3.37	123806	0.96	96.12
CC - 02	2.00	3.5	246394	2.01	100.66
CC - 03	3.00	3.5	366044	3.04	101.34
CC - 04	4.00	3.51	506611	4.25	106.16
CC - 05	6.00	3.5	678725	5.72	95.39
CC - 06	7.00	3.51	837930	7.09	101.28
CC - 07	9.00	3.51	981082	8.32	92.43
CC - 08	10.00	3.5	1247718	10.61	106.07
Blank	0	**NA**	**0**	NA	**NA**

NA - Not applicable

Table 5a. Results of inter and intra-day accuracy & precision for Atenolol

	Nominal Concentration (µg/mL)		
	25.00	50.00	75.00
DAY 1			
MEAN (n=6)	27.54	48.38	77.39
SD	0.47	0.81	1.08
% CV	1.71	1.67	1.39
DAY 2			
MEAN (n=6)	26.99	49.17	75.84
SD	0.46	0.93	1.06
% CV	1.79	1.89	1.39
DAY 3			
MEAN (n=6)	26.45	49.66	75.47
SD	0.45	0.94	0.54
% CV	1.72	1.89	0.72

Table 5b. Results of inter and intra-day accuracy & precision for HCTZ

	Nominal Concentration (µg/mL)		
	2.50	5.00	7.50
DAY 1			
MEAN (n=6)	2.59	4.76	7.44
SD	0.07	0.11	0.16
% CV	2.83	2.35	2.10
DAY 2			
MEAN (n=6)	2.60	4.84	7.45
SD	0.06	0.12	0.14
% CV	2.44	2.45	1.82
DAY 3			
MEAN (n=6)	2.56	4.86	7.47
SD	0.06	0.09	0.07
% CV	2.47	1.75	1.00

Fig-5a: Overlay Chromatogram showing the influence of various stress conditions on Atenolol; Data 1-Fresh Sample Data 2-Acid Stress, Data 3 – Oxidative Stress; Data 4 –Photolytic Stress; Data 5 – Alkaline Stress. Data 5 clearly indicates the spectral degradation of Atenolol due to alkaline instability.

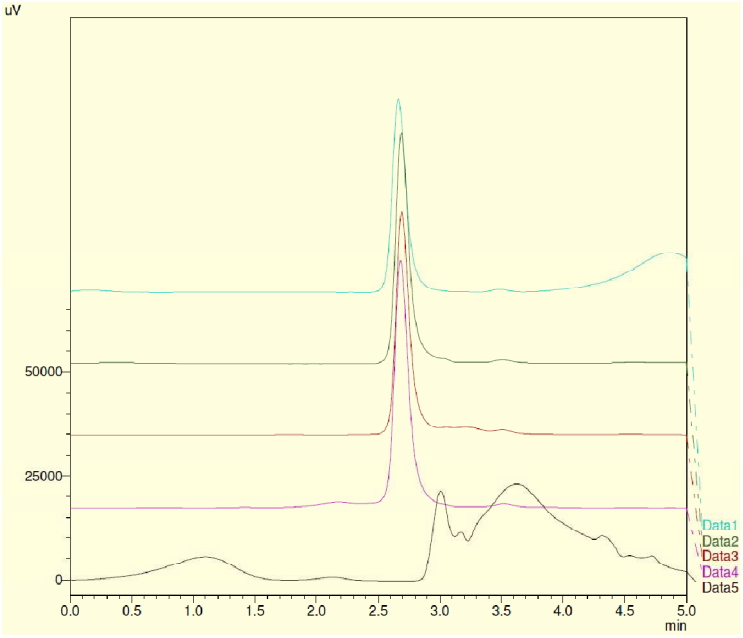


Fig-5b: Overlay Chromatogram showing the influence of various stress conditions on Hydrochlorothiazide; Data 1-Fresh Sample Data 2-Acid Stress, Data 3 – Alkaline Stress; Data 4 –Photolytic Stress; Data 5 – Oxidative Stress. Data 3 clearly indicates the spectral degradation of Hydrochlorothiazide due to alkaline instability.

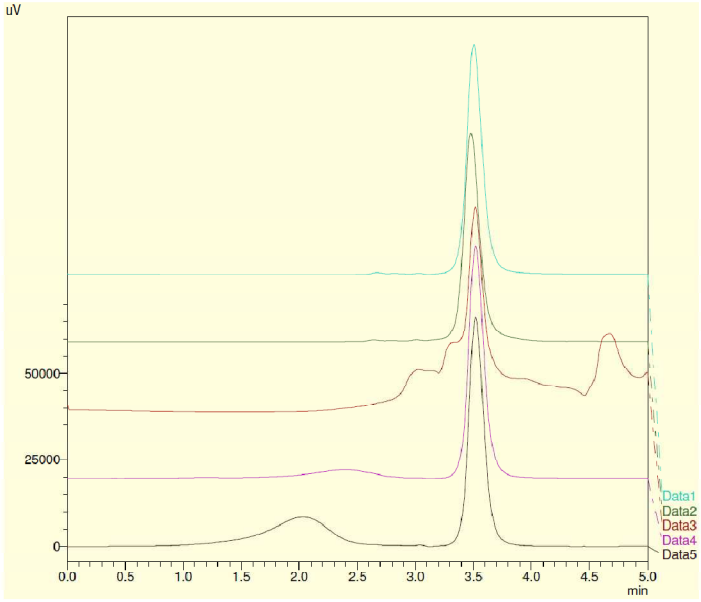


Table 6a. Room Temperature Stability of Atenolol (n = 6).

ATENOLOL

FRESH SAMPLE

SR NO	SAMPLE ID	CONC (µg/mL)	DRUG	
			Rt	PEAK AREA
1	FRESH	50.00	2.69	452991
2	FRESH	50.00	2.68	406443
3	FRESH	50.00	2.69	472123
4	FRESH	50.00	2.68	465435
5	FRESH	50.00	2.69	467687
6	FRESH	50.00	2.68	485330
Mean				**458334.83**
Stdev				**27488.35**
%				**6.00**

STABILITY SAMPLE

SR NO	SAMPLE ID	CONC (µg/mL)	DRUG	
			Rt	PEAK AREA
1	STABILITY	50.00	2.68	475082
2	STABILITY	50.00	2.7	483449
3	STABILITY	50.00	2.68	489437
4	STABILITY	50.00	2.68	480644
5	STABILITY	50.00	2.68	435325
6	STABILITY	50.00	2.67	473862
Mean				**472966.50**
Stdev				**19299.04**
% Cv				**4.08**

% Stability 103.19

Table 6b. Room Temperature Stability of HCTZ (n = 6).

Diazepam

FRESH SAMPLE

SR NO	SAMPLE ID	CONC (µg/mL)	DRUG	
			Rt	PEAK AREA
1	FRESH	5.00	3.52	627802
2	FRESH	5.00	3.49	579016
3	FRESH	5.00	3.50	631307
4	FRESH	5.00	3.49	628719
5	FRESH	5.00	3.50	636412
6	FRESH	5.00	3.50	649676
MEAN				**625488.67**
STDE				**24139.99**
% CV				**3.86**

STABILITY SAMPLE

SR NO	SAMPLE ID	CONC (µg/mL)	DRUG	
			Rt	PEAK AREA
1	STABILITY	5.00	3.49	637897
2	STABILITY	5.00	3.51	644102
3	STABILITY	5.00	3.49	646868
4	STABILITY	5.00	3.49	643340
5	STABILITY	5.00	3.49	607116
6	STABILITY	5.00	3.49	632044
MEAN				**635227.83**
STDE				**14748.48**
% CV				**2.32**

% Stability 101.56

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