International Journal of Advances in Pharmaceutical Analysis
IJAPA Vol. 5 Issue 2 (2015) 36-41

Isolation and characterization of acid and base degradation products in Atenolol and Hydrochlorothiazide and a validated selective stability-indicating HPLC–UV method for their quantification

Bhattacharyya Indrani[*1], Souvik Basak[1], Arindam Maity[1] and Dr S.K Ghosh

[1]*Dr B.C Roy College of Pharmacy and AHS, Dr. Meghnad Saha Sarani, Bidhan Nagar Pin-713206, Durgapur, West Bengal, India*
[2]*Department of Pharmaceutical Technology, Dibrugarh University, Assam, India*

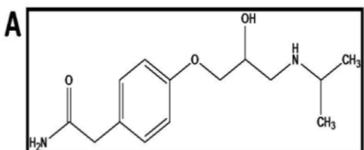
Abstract

Atenolol ((*RS*)-2-{4-[2-Hydroxy-3-(propan-2-ylamino)propoxy]phenyl}acetamide) and Hydrochlorothiazide (6-chloro-1,1-dioxo-3,4-dihydro-2*H*-1,2,4-benzothiadiazine-7-sulfonamide) are beta$_1$ (β_1) receptor blocker and diuretic drug respectively; however the combination dosage regime are used for cardiovascular therapy. Thus a forced degradation study was carried out upon this combination drug regime under acidic and basic environment in order to deconvolute the possible degradation product under specified stressed conditions. Under acidic conditions atenolol and hydrochlorothiazide were cleaved into 2-(4-(3-amino-2- oxopropoxy) phenyl) acetamide and 6-sulphamido benzothiazide. However, under basic conditions, the drugs were spliced into 2-(4-(2-hydroxypropoxy) phenyl) acetamide and 2-chloro 4-amino 1, 6-dihydro benzene sulphonamide respectively. The degradant peaks were elucidated by HPLC using C18 column with methanol: phosphate buffer (70:30 v/v) with a flow rate of 0.5ml/min (UV detection at 226nm).. For quantitative method validation, linearity was observed over product concentration range 2μg/ml - 100 μg/ml (r^2 0.9992) with regression equation y=43432x. The products were first identified by LC-MS and further confirmed by FT-IR and $_1H^1$ NMR. A specific and sensitive stability-indicating assay method for the simultaneous determination of the drugs, its process related impurities and degradation products was developed.

Keywords: Stability indicating method, Atenolol, hydrochlorothiazide, Forced degradation

1.Introduction

Atenolol is a selective β_1 receptor antagonist, a drug belonging to the group of beta blockers and primarily used in cardiovascular diseases [1]. Atenolol [Fig-1A] is one of the most widely used β-blockers in the United Kingdom and was once the first-line treatment for hypertension [2-5]. Hydrochlorothiazide is a diuretic drug of the thiazide class that acts by inhibiting the kidneys ability to retain water [6]. This reduces the volume of the blood, decreasing blood return to the heart and thus cardiac output and, by other mechanisms, is believed to lower peripheral vascular resistance. Hydrochlorothiazide [Fig. 1B] is a calcium-sparing diuretic, meaning it can help the body get rid of excess water while still keeping calcium. The only HPLC method of the combination formulation of Atenolol and Hydrochlorothiazide in stability indicating method suffers the drawback of splitting of the peak and sometimes tailing and fronting effect occurred. Literature survey reveals no RP-HPLC method for simultaneous estimation and stability study of Atenolol and Hydrochlorothiazide in combined dosage forms [7-9]. In the present study we developed a simple, precise, accurate, selective and robust liquid chromatographic method for the determination of Atenolol and Hydrochlorothiazide in the combined tablet dosage form. Forced degradation studies were carried out on the drugs in order to generate the potential degradation products under acidic and basic stress conditions as per ICH guidelines. Four potential degradation products that were formed were isolated and characterized. The structural characterization of the degradation products were determined using Mass Spectroscopy (MS), Fourier Transform Infrared Spectroscopy (FTIR), and Proton-Nuclear Magnetic Resonance (1H NMR). Structural characterization of the major degradation products enables one to establish the degradation pathway under which the degradation products are formed. In addition, synthesis of the drug assists in identifying the process related impurities. This further helps with the quantitative determination of the drug, in the presence of its process related impurities and degradation products. In the present work, intrinsic stability of Atenolol and hydrochlorothiazide were found and a selective, precise and accurate HPLC method was developed for simultaneous determination of the drugs and its process related impurities and degradation products.

A


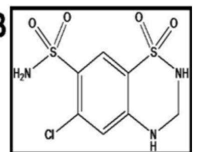
B


Fig-1 A chemical structure of Atenolol
B chemical structure of Hydrochlrothiazide

2. Experimental

2.1 Chemical and apparatus

Atenolol (ATN) working standard has been supplied by Cadila Healthcare Pvt. Ltd. (Sikkim, India) and Hydrochlorothiazide (HCZ) working standard has been supplied by Cadila Healthcare Pvt. Ltd. (Sikkim, India). Water (HPLC grade), Methanol (HPLC grade), Chloroform, Di-sodium hydrogen phosphate, Concentrated hydrochloric acid, Sodium hydroxide, Hydrogen peroxide, Glacial acetic acid were purchased from Merck India. All chemicals were of analytical grade and used as received .Combined formulation of Atenolol and Hydrochlorothiazide was available as tablet with brand name ATN-H with label claimed 50mg Atenolol and 25mg Hydrochlorothiazide. System configuration of waters HPLC system consisted of Water 2489 UV/Visible detector was used throughout the analysis. Data analysis and interpretation were carried out using EMPOWER software. The analyses were carried out on a Phenomenex (Torrance, CA) Luna C18 column (250 mm 4.6 mm 10 mm). The measurements were carried out at a wavelength of 226 nm for the analytes.

Corresponding Author*: Indrani.das@rediffmail.com

2.2 Experimental Methods

2.2.1 High Performance Liquid Chromatography (HPLC)

2.2.1.1 Standard solutions and fortification: 20 mg of Atenolol and 10 mg of hydrochlorothiazide were dissolved in 5ml methanol to produce 2000µg/ml and 1000µg/ml Atenolol and Hydrochlorothiazide respectively. The solution was diluted 1:10 with methanol before subjecting to HPLC.

2.2.1.2 Calibration of Atenolol and Hydrochlorothiazide: Standard stock solutions were prepared with concentrations of 20:10 µg/ml (v/v), 40:20 µg/ml (v/v), 60:30 µg/ml (v/v), 80:40 µg/ml (v/v) and 100:50 µg/ml (v/v) of ATN and HCZ respectively and subsequently subjected to HPLC to record "Area under curve" (AUC).. The standard curve was constructed by plotting AUC vs. concentration.

2.2.1.3 Estimation of ATN and HCZ in tablet formulation by RP-HPLC method: From the stock solution of formulation 1ml was pipetted out and the volume was made up to 10ml with HPLC grade methanol and the solution was 100:50 µg/ml (w/v). And from this, 2ml solution was pipetted out and transferred to 10ml volumetric flask and the volume was made up to the mark with HPLC grade methanol. Finally the solution was 20:10 µg/ml (v/v). This solution was injected in HPLC system and the AUC were recorded. From the regression equation, the percentage purity of Atenolol and Hydrochlorothiazide was calculated.

3. Validation of RP-HPLC method

3.1 Accuracy: To study the accuracy, 10 tablets were weighed and powdered. Analysis of the same was carried out. Recovery studies are carried out by standard addition method by adding known amount of Atenolol and hydrochlorothiazide (reference standard) separately to the reanalyzed sample at three different concentration levels i.e. 80%, 100% and 120% of assay concentration and percentage recovery were calculated.

3.2 Precision: The precision of analytical method was studied by performing intra-day and inter-day precision.

3.2.1 Intra-day Precision: Variations of results within same day were analyzed. Intra-day precision was determined by analyzing the sample solution of formulation 20:10 µg/ml (v/v) in linearity range at three different time intervals on same day.

3.2.2 Inter-day Precision: Variations of results between the days were analyzed. Inter-day precision was determined by analyzing the sample solution of formulation 20:10 µg/ml (v/v) in linearity range on three consecutive days.

3.3 Repeatability

The sample solutions of formulation 20:10 µg/ml (v/v) was prepared and analyzed. The solution was analyzed six times and standard deviation was calculated.

3.4 Reproducibility

The sample solutions of formulation 20:10 µg/ml (v/v) was prepared and analyzed. The solution was prepared and analyzed by Analyst 1 and Analyst 2 separately. The values obtained were evaluated by using F – test and t – test to verify their reproducibility.

3.5 Linearity and Range

The concentration ranges 10, 20, 30, 40, 50, 60 and 100 µg/ml for ATN were selected as linearity range. Similarly, the concentration ranges 5, 10, 15, 20, 25, 30 and 50 µg/ml for HCZ were selected as linearity range.

3.6 Limit of Detection and Limit of Quantization

Detection limit and Quantization limit were determined based on the standard deviation of y – intercepts of six calibration curves and average slope of six calibration curves.

3.7 Robustness

The effect of change in the pH of mobile phase and flow rate on the retention time, tailing factor, theoretical plates and resolution were studied. The sample solutions of formulation were prepared and analyzed at different pH (3.9, 4.1) of the mobile phase and at different flow rate (0.45, 0.55ml/min).

4. Forced degradation studies of tablets

4.1 For acid degradation:

Ten tablets were weighed, crushed and powdered. Then amount equivalent to 50mg of Atenolol and 25mg of Hydrochlorothiazide was taken in 100ml round bottomed flask. About 50ml of 1N Hydrochloric acid was added to the flask and refluxed on heating mantle for 6 hours at 60ºC.

4.2 For basic degradation

Ten tablets were weighed, crushed and powdered. Then amount equivalent to 50mg of Atenolol and 25mg of Hydrochlorothiazide was taken in 100ml round bottomed flask. About 50ml of 5N Sodium Hydroxide was added to the flask and refluxed on heating mantle for 6 hours at 60ºC.

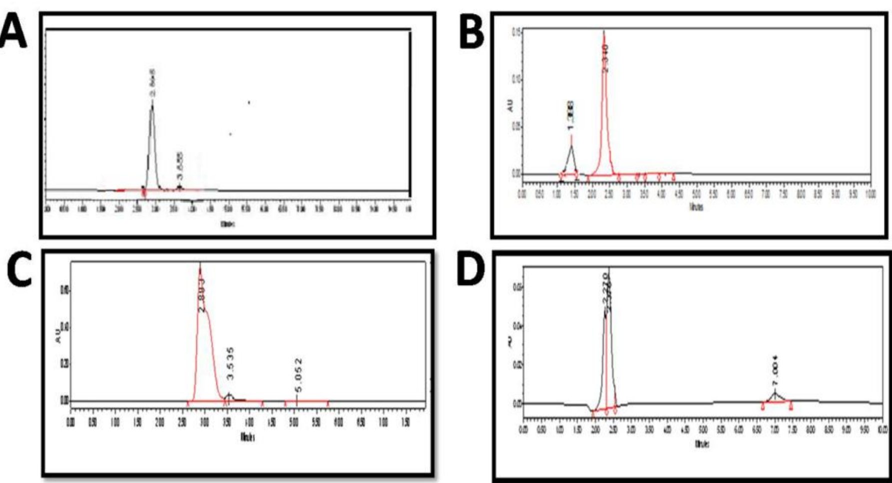


Fig-2 (A) Acid hydrolysis of Hydrochlorothiazide (B) Acid hydrolysis of Atenolol (C) Base hydrolysis of Hydrochlorothiazide (d) Base hydrolysis of Atenolol

5. Results and discussion

5.1 Method validation

This method was validated in accordance to ICH guidelines. Recovery studies were carried out by standard addition method by adding the known amount of Atenolol and hydrochlorothiazide separately to the formulation solution at three different concentration levels i.e. 80%, 100% and 120% of assay concentration and percentage recoveries were calculated. Percentage recoveries of ATN and HCZ were found in the range from 100.07 – 100.75 % and 104.6 – 105.9 % respectively (Supplementary Table 1). Precision of the method was determined by %RSD found among intra-day precision, inter-day precision and repeatability. It was found to be less than 1 % (Supplementary Table 2-5). Reproducibility was determined by preparing and measuring the standard solution of ATN (20µg/ml) and HCZ (10µg/ml) by Analyst 1 and Analyst 2, separately. The values obtained were evaluated using F-test and t-test to verify their reproducibility (Supplementary Table 6-7). Calculated value for t-test was found to be less than the tabulated (standard) value hence no significant difference was observed in the result of analysis. Limit of Detection and Limit of Quantization were determined from their standard deviation of y-intercepts of calibration curves and slopes of calibration curves. LOD and LOQ of ATN were found to be 0.0007 and 0.0002 µg/ml at 226nm respectively. LOD and LOQ of HCZ were found to be 0.000062 and 0.000187 µg/ml at 226nm respectively.

For Robustness study, the effect of change in the pH of mobile phase and flow rate on retention time, tailing factor, theoretical plates and resolution were studied. The sample solutions of formulation were prepared and analyzed at different pH (3.9, 4.1) of the mobile phase and at different flow rate (0.5, 0.55 ml/min). Percentage RSD of each peak was found to be less than 1% (Supplementary Table 8, 9).

5.2 Forced degradation study

Atenolol and Hydrochlorothiazide combined formulation was exposed to stress condition such as acidic and basic and refluxed on heating mantle for 6 hours. The developed RP-HPLC method was used for Quantization of drug in presence of degradation products. The amounts of degradation products for Atenolol were found to be 9.45% (in acidic condition), 34.9% (in basic condition). The amounts of degradation products for hydrochlorothiazide were found to be 0.5% (in acidic condition), 8.0% (in basic condition). The relative retention time of the degradants has been shown in Table1.

Table 1 Acid and base degradation products of ATN and HCZ

Degradant	Molecular weight	Condition of degradation	Relative retention time (R_t)	Parent drug	
				ATN	CZ
2-(4-(3-amino-2-oxopropoxy)phenyl) acetamide	222	Acid	1.388 min	✓	
6-sulfamido Benzothiazide	264	Acid	3.855 min		✓
2-(4-(2-hydroxypropxy)phenyl) acetamide	209	Base	2.230min	✓	
2-chloro 4-amino 1, 6-dihydro Benzene sulphonamide	209	Base	3.535min		✓

5.3 Characterization of acid degradation products

The acid and base degradation of drugs yielded four degradants. Actually subjecting each drug under single stress produced one degradant (Table 1). The four degradants were found as 2-(4-(3-amino-2-oxopropoxy) phenyl) acetamide, 6-sulfamido benzothiazide, 2-(4-(2-hydroxypropxy)phenyl) acetamide and 2-chloro 4-amino 1, 6-dihydro benzene sulphonamide. The compound structures were deconvoluted gradually through MS, NMR and FT-IR spectra.

5.3.1 Atenolol

The acid degradation products of Atenolol were characterized by Mass, IR and NMR spectra Mass spectra of the degraded product showed a molecular weight of 222 (determined from the molecular ion peak, which is significantly lower than the parent molecule (molecular weight 246). A fragmentation based database search suggested the compound structure to be a free amine. IR spectra (KBr, cm^{-1}) (Supplementary Fig. S1) of the degraded compound elicited structural conformation to the compound as mentioned. For example, it showed

peaks at 3421 cm^{-1} ($-NH_2$ str, H bonded), 3249 cm^{-1} ($-NH_2$ wk, free), 1508 cm^{-1}(aryl –CH, med-wk), 1560 cm^{-1} (med-wk, -C=C conjugated, aryl), 1648 cm^{-1} (str, $-CONH_2$). No peak for $-CH_3$-CH-CH_3- group in the product molecule suggests degradation. NMR spectra also depict the absence of $-CH_3$-CH-CH_3- group in the product which also indicates degradation (Table 2).

Table 2: NMR peaks of the degradants from ATN

	Acid degradant		Base degradant	
Sl. No.	**Proton**	**Chemical Shift (ppm)**	**Proton**	**Chemical Shift (ppm)**
1.	2 (-NH)	2.351	3 ($-CH_3$)	1.35
2.	2 ($-CH_2$)	3.474	2 ($-CH_2$)	2.20
3.	2 ($-CH_2$)	3.597	1 (–OH)	2.35
4.	2 ($-CH_2-$)	4.264	2 ($-CH_2-$)	3.47
5.	2 (aromatic proton)	7.103	1 (–CH–, aliphatic)	4.257
6.	2 (aromatic proton)	7.360	2 (aromatic C–H) (J = 5.1Hz)	7.10
7.	2(amide amine)	7.536	2 (aromatic C–H) (J = 4.8Hz)	7.36
			2 ($-NH_2$)	7.56

5.3.2 Hydrochlorothiazide

The mass spectra for stressed compound suggested its molecular mass as 264 whereas IR spectra (Supplementary Fig. S2) revealed it is a free amine with no chlorine atom: (KBr, cm^{-1}) 3365.85 ($-NH_2$, wk), 3275.87, 3175.00 (-NH-, wk), 2924.24 (aliphatic –CH, str), 1604.85, 1546.37 (heterocyclic ring, med), 1346.39, 1167.85 and 1320.19, 1152.94 ($-SO_2NH_2$, str). The NMR spectra also interpreted the compound structure to be a sulphoxide derivative of quinazoline (Table 3).

Table 3: NMR peaks of the degradants from HCZ

	Acid degradant		Base degradant	
Sl. No.	**Proton**	**Chemical Shift (ppm)**	**Proton**	**Chemical Shift (ppm)**
1.	1H (aromatic NH)	6.097	$-CH_2$	2.503
2.	2H (Aromatic proton)	6.618	– free NH_2	3.359
3.	2H (aromatic proton)	7.357	2($-ArSO_2NH_2$)	6.618
4.	2H(Sulphonamide)	7.506	1 (Vinylic –CH– conjugated)	7.357
5.	1H(Aromatic)	8.158	1 ($-CH_2-$, near free NH_2, undergoing hyperconjugation)	7.506
6.			1 ($-CH_2-$, near SO_2NH_2, undergoing hyperconjugation)	8.514

5.4. Characterization of basic degradation products

5.4.1 Atenolol

The mass spectra showed an m/z ratio of 209 as its molecular weight. The reduction of molecular weight from 266 to 209 clearly suggests degradation. IR spectra (KBr, cm^{-1}, (Supplementary Fig. S3) showed peaks at 3630.05 (free –OH, wk), 3552.31 (H bonded –OH, wk), 3465.95 (H bonded –NH. med), 2929.28 (-CH, str), 1741.96 (-C=O-NH_2), 1560.06 (conjugated –C=C, med-wk), 847.44 (-C=CH_2, med-wk) which exhibited no peak for –NH–C–$(CH_3)_2$ that was present in original Atenolol molecule. This has been further reinforced by NMR spectra which also revealed no peak for –NH–C–$(CH_3)_2$ cluster (Table 2).

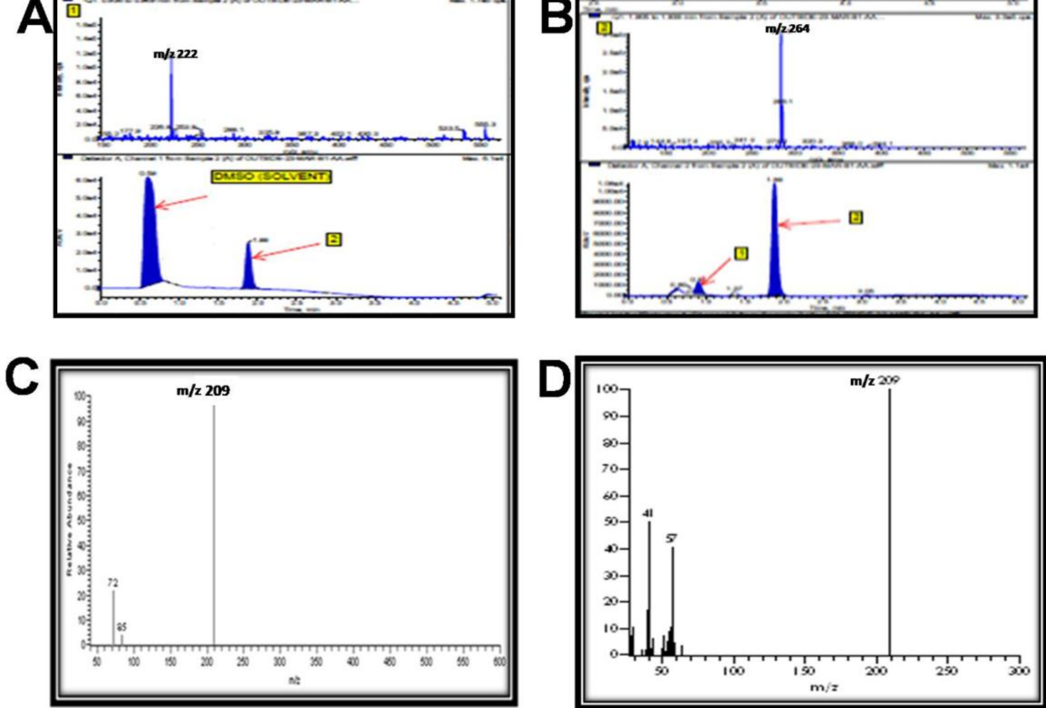


Fig-3 (A) mass spectra of acid degradants of atenolol (B) mass spectra of acid degradants of Hydrochlorothiazide (C) mass spectra of basic degradants of atenolol (D) mass spectra of basic degradants of Hydrochlorothiazide

5.4.2 Hydrochlorothiazide

The m/z peak showed in the mass spectra deduced the molecular weight of the product to be 209 which implied a large reduction of mass from the parent compound (Hydrochlorothiazide, molecular weight 297). This elicited degradation under basic condition. Furthermore IR spectra (KBr, cm^{-1}, Supplementary Fig. S4) depicted absence of ring bound [–SO_2NHCH_3-] group in the product compared to the parent; 3379.14 and 3482.73 (free -NH_2, wk, 2 bands), 3090.86 (=C-H, med), 1596.53 (-C=C in ring), 2924.56 and 1638.10 (-CH_2, str), 588.41 (-Cl, str), 1321.84 and 1145.52 (SO_2NH_2). In addition, structure assignment (Table 3) from NMR spectra also suggested cleavage of [–SO_2NHCH_3-] together with addition of two hydrogen atoms at 5, 6.

5.5 Formation of degradation products

5.5.1Acid degradant of ATN

ATN undergoes acid guided β-oxidation to get converted into β-keto ether which subsequently absorbs proton to yield primary amine from secondary one by proton induced bond cleavage (Fig. 4A).

5.5. 2 Acid degradant of HCZ

HCZ undergoes free radical [Cl] guided dehydrogenation, thus forming carbanion. The unpaired electron eventually leads to formation of π bonding with the vicinal carbon atom creating unsaturation in the ring. The ring again undergoes dehydrohalogenation to yield 6-sulfamido benzothiazide.

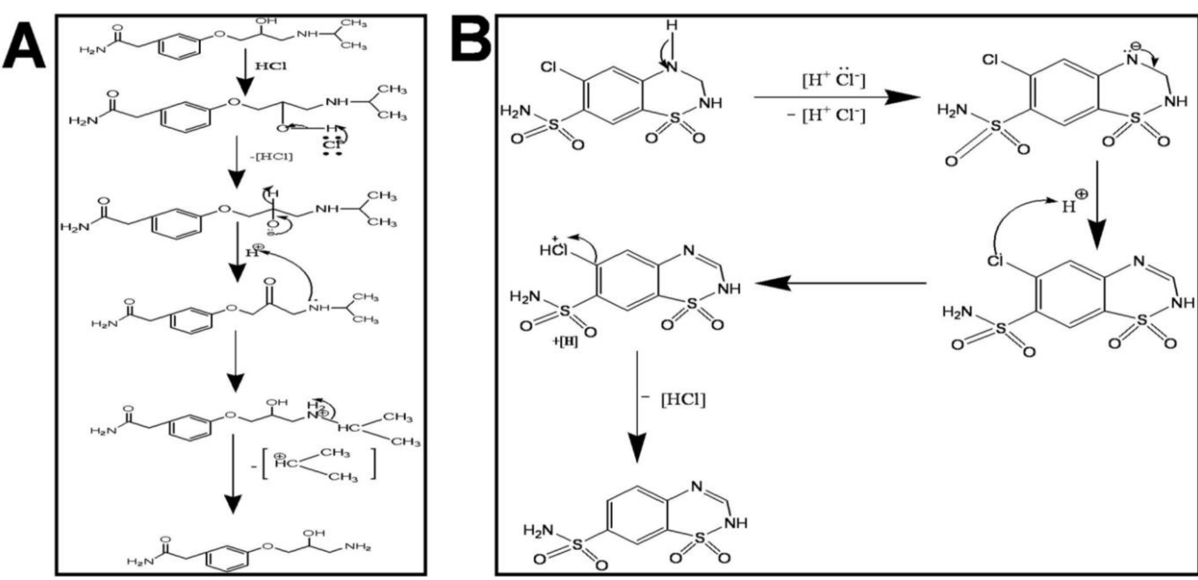


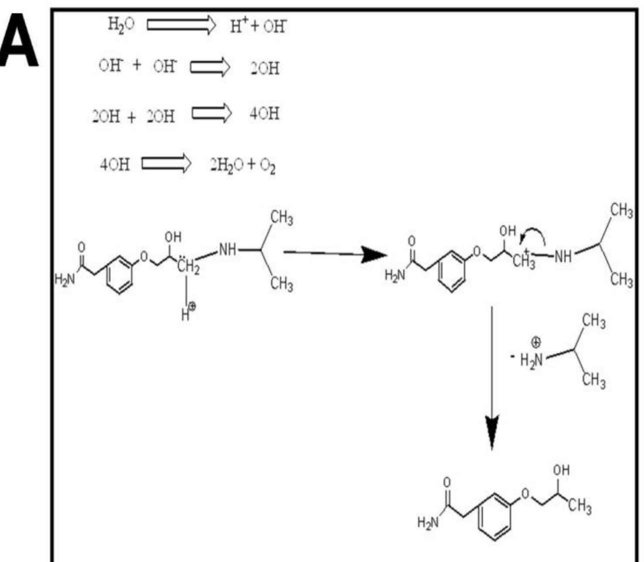
Fig-4: A degradation pathway of acid hydrolysis of Atenolol, B Degradation pathway of acid hydrolysis of Hydrochlorothiazide

5.5.3 Base degradant of ATN

Refluxing with alkali, the aqueous medium ionizes to generate proton which forms carbonation with the α-carbon attached to the amine terminal. In order to maintain the ring stability, the bond pair of electrons gets shifted to yield 2-(4-(2-hydroxypropxy) phenyl) acetamide as the degradant (Fig. 5A).

5.5. 4 Base degradant of HCZ

HCZ undergoes base catalyzed degradation in two major steps. First, nitrogen undergoes Lewis base type interaction to absorb proton that has been generated due to alkali guided reflux. This leads to ring exposure to generate free amine. Second, the hydroxyl radical attacks the –S terminal to undergo auto-oxidation and subsequent release of -SO_3H terminalized carbonations. Lastly a pair of reduction leads to 2-chloro 4-amino 1, 6-dihydro Benzene sulphonamide (Fig. 5B).



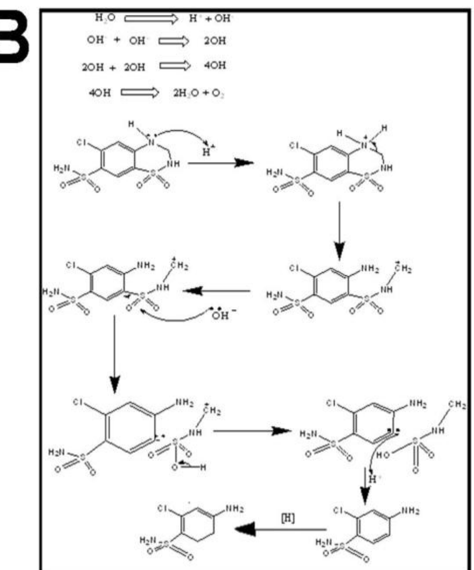


Fig-5: A degradation pathway of basic hydrolysis of Atenolol, B Degradation pathway of basic hydrolysis of Hydrochlorothiazide

6. Conclusion

A force degradation study of atenolol and hydrochlorthiazide combination was performed in acid and alkaline stress condition to reveal one characteristic degradation product from each parent under individual condition. Altogether, four degradants were found characterized as 2-(4-(3-amino-2-oxopropoxy) phenyl) acetamide, 6-sulfamido benzothiazide, 2-(4-(2-hydroxypropxy)phenyl) acetamide and 2-chloro 4-amino 1, 6-dihydro benzene sulphonamide. The structures were elucidated by LC-MS, $_{1}H^{1}$-NMR and FT-IR spectroscopy. A stability indicating RP-HPLC method was also developed to quantitate the component drugs in tablet dosage form as well as its degradation products. On the whole, the methods are found specific, precise, linear, accurate, sensitive, robust and suitable.

References

[1] The British Pharmacopoeia 2007. The Stationery Office, London; Electronic version.

[2] Martindale, Pharmaceutical Press, Edn 34, 2005, 865, 933, 947.

[3] The Merck Index, Merck & Co., Inc., Edn 14, Whitehouse Station. *NJ*, 2006; 859, 4781, 5583.

[4] Prasad CV, Parihar C, Sunil K, Parimoo P. Simultaneous determination of amiloride HCl, hydrochlorothiazide and atenolol in combined formulations by derivative spectroscopy. *J Pharm Biomed Anal* 1998; 17:877-84.

[5] Sivakumar T, Venkatesan P, Manavalan R, Valliappan K. Development of a HPLC method for the simultaneous determination of Losartan potassium and Atenolol in tablets. Indian *J Pharm Sci* 2007; 69:154-57.

[6] Parthiban C, Bhagavan RM, Sudhakar M. Simultaneous estimation and validation of Atenolol, hydrochlorothiazide and Losartank in dosage form by RP-HPLC method. *Int J Pharm & Ind* 2011; 1(4).

[7] Nevin E. Spectrophotometric analysis of Hydrochlorothiazide and Valsartan. *Analytical Letters* 2002; 35:283-302.

[8] Bhushan R, Deepak G, Shravan KS. Liquid chromatographic separation and UV determination of certain antihypertensive agents. *Biomed Chromato* 2005; 20:217-224.

[9] Zaveri M, Khandhar A. Development and Validation of a RP-HPLC for the Simultaneous estimation of Atenolol and Hydrochlorothiazide in Pharmaceutical Dosage Forms. *International Journal of Advances in Pharmaceutical Sciences* 2010; 167-171.