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Stability Indicating HPLC Method for Simultaneous Determination of Several Angiotensin-II-Receptor Antagonists in Their Dosage Forms

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Abstract

A stability-indicating reversed-phase liquid chromatographic (RP-HPLC) method has been established for simultaneous determination of four angiotensin-II-receptor antagonists (Losartan potassium, Valsartan, Telmisartan and Irbesartan) in the presence of the degradation products generated in studies of forced decomposition. All drug substances were subjected to drastic condition of stress studies involving hydrolysis by acid and base, thermal decomposition by heat at 70°C, oxidation by hydrogen peroxide and photo degradation. Losartan potassium and Valsartan were degraded acidic conditions. Irbesartan was degraded basic conditions. Telmisartan showed good stability against all stress conditions. Successful separation of the drugs from the degradation products was achieved on ACE C18 column (250 mm×4.6 mm, 5 µm) with 65:35 % (v/v) potassium dihydrogen phosphate (0.025 M, pH 6.0): acetonitrile as a mobile phase at flow rate 1.5 ml/min with UV detection at 220 nm. The proposed method was validated in terms of linearity, accuracy, precision and limits of detection and quantitation. Statistical analysis proved the method enabled reproducible and selective quantification of these drugs as the bulk drug and in pharmaceutical dosage forms. Because the method effectively separates the drugs from their degradation products, it can be used as stability-indicating.

Keywords: Stability indicating HPLC; Losartan potassium; Irbesartan; Valsartan; Telmisartan

Introduction

Angiotensin antagonists are the first major innovation in essential hypertension management as a first-line treatment. Angiotensin II receptor antagonists (ARA II) have been developed to specifically and selectively block the AT1 receptor of the rennin angiotensin system by displacing angiotensin II from it [1]. Losartan potassium, Telmisartan, Irbesartan and Valsartan are highly selective, non-peptide angiotensin-II receptor antagonists (ARA-II). They are effective agents for the treatment of hypertension and heart failure either alone or together with diuretics [2].

Information on the stability of the drug substance is an integral part of the systematic approach to stability evaluation. Stress testing (or forced degradation studies) is a critical component of the drug development process [3]. The ICH guideline indicates that stress testing is designed to help "determine the intrinsic stability of the molecule by establishing degradation pathways in order to identify the likely degradation products and to validate the stability indicating power of the analytical procedures used [4]. Stress testing also is becoming increasingly important in testing new molecules. Methods developed by stress testing and the stability information gained from those methods can have a significant effect on the actual compound selected for development [3]. Literature review revealed that several methods have been reported for analysis of the drug in pharmaceutical formulation alone or with other drugs in combination. USP described a HPLC method for assay and impurities of Losartan potassium, Valsartan and their impurities B, C (RP-HPLC), Valsartan impurity A (normal phase HPLC) and Irbesartan (ion pair HPLC), ion pair HPLC for Telmisartan [5]. BP described a potentiometric titration for assay of Losartan potassium, Irbesartan, Telmisartan and Valsartan. For impurities BP used a gradient RP-HPLC for Losartan potassium and ion pair HPLC for Telmisartan [6]. Some methods have been published for simultaneous determination of studied ARA-II-drugs including Spectrophotometry [7], HPLC-DAD [2], HPLC with fluorimetric detection [1] and capillary zone electrophoresis [8,9]. Some stability studies were reported for Losartan [10-13], Irbesartan [14,15], Valsartan [16-18] and Telmisartan [19]. But no published method depends on optimum stress test conditions [20] and it is the first stability study which comprises the four ARA-II compounds together. So, our scope is achieving a stress testing study of ARA-II drugs including development of a powerful stability indicating method. New study depends on previous study has been established to reach the optimum stress conditions in drug development process and time points for a detailed study (solid-state stress). Developed stability indicating method is accurate, sensitive, rapid and simple. The developed method was validated according to (ICH) guidelines with respect to specificity, linearity, limit of detection, limit of quantification, accuracy, precision and robustness [21]. The method is proved to be robust with respect to change in flow rate, pH, organic phase composition and column temperature. It can be applied in majority of companies in the world.

Losartan potassium is 2-butyl-4-chloro-1-[[2'-(1H-tetrazol-5-yl)[1,1'-biphenyl]-4-yl]methyl]-1H-imidazole-5-methanol mono potassium salt, is the first member of a new class of non-peptide angiotensin II receptor antagonist. Irbesartan is 2-butyl-3-[p-(o-1H-

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tetrazol-5-ylphenyl) benzyl]-1, 3-diazaspiro [4.4] non-1-en-4-one. It is an orally active specific angiotensin II receptor antagonist used, as a hypotensive agent does not require biotransformation into an active form. Valsartan is N-(1-oxopentyl)-N-[[2-(1H-tetrazol-5-yl) [1, 1-biphenyl]-4-yl] methyl]-l-valine. Valsartan is a potent, highly selective, and orally active antagonist at the angiotensin II AT1- receptor. Telmisartan is 4-((2-n-propyl-4-methyl-6-(1-methylbenzimidazol-2-yl)-benzimidazol-1-yl) methyl) biphenyl-2- Carboxylic acid (Figure 1).

Experimental

Instrumentation

Balance: KERN model 870-13, Instrument Kern Balance, Supplied from Kern, Germany

High performance liquid chromatography: Consisting of instrumental

a. AGILENT 1200 Quaternary pump.

b. AGILENT 1200 Diode Array detector (DAD).

c. AGILENT 1200 Auto sampler (injector).

d. Column: ACE column (250×4.6) mm particle size 5 µ Supplied from ACE.

e. The system equipped by Agilent chemistation PC program.

pH meter: Metrohm model 713 pH meter, Supplied from Metrohm, Switzerland.

Stirrer: Heiedolph model MR 3002 S, Supplied from Heiedolph, Germany.

Chemicals and reagents

All reagents are analytical or HPLC grade. Potassium dihydrogen phosphate, orthophosphoric acid and sodium hydroxide (NaOH) were supplied by (Merck, Darmstadt, and Germany), acetonitrile and methanol (HPLC grade) were supplied by (Fischer scientific, U.K.) and distilled water.

(Note: The water used in all the experiments was obtained from Milli-RO and Milli-Q systems (Millipore, Bedford, MA).

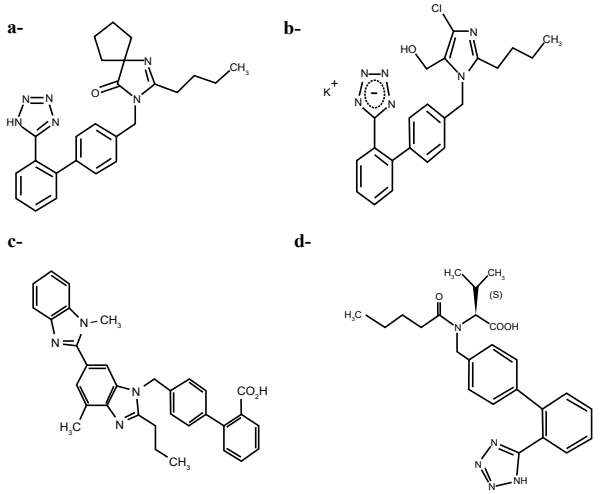


Figure 1: The structures of a- Irbesartan b- Losartan potassium c- Telmisartan d- Valsartan.

Irbesartan, Losartan potassium, Telmisartan and Valsartan working standard powders were kindly supplied by Egyptian international pharmaceutical industries company (EIPICO) (10th Ramadan, Egypt) and they were used without further purification.

Pharmaceutical preparation

X-tension tablets October pharma/EPCP (Egypt) contain (150 mg Irbesartan per tablet) B.NO: E0070211. *LosarMepha* tablets Medical union pharmaceuticals (Egypt) contain (50 mg Losartan potassium per tablet) B.NO:02862. *Micardis* tablets Boehringer Ingelheim (Germany) contain (80 mg Telmisartan per tablet) B.NO:104643. *Disartan* capsules Global Napi Pharmaceuticals (Egypt) contain (80 mg Valsartan per capsule) B.NO: 915003.

Chromatographic condition

Mobile phase: a mixture of 65% potassium dihydrogen phosphate buffer (0.025M, pH=6.0):35% acetonitrile (an isocratic technique).

Column: ACE RP-C18 supplied from ACE.

Detector was set at 220 nm. **Flow rate;** 1.5 ml/min.

Column temperature: 40°C. **Injection volume**: 50 µl.

Stock standard solutions

Stock standard solutions containing (1.5, 0.5, 0.8, 0.8 mg/ml) of Irbesartan, Losartan potassium, Telmisartan and Valsartan were prepared by dissolving (150, 50, 80, 80 mg) of them in methanol in 100 ml volumetric flask respectively. Solutions were sonicated for 15 minutes and the final volume of solutions was made up to 100 ml with methanol to get stock standard solutions.

Preparation of calibration plot (working standard solutions)

To construct calibration plots, The stock standard solutions were diluted with the mobile phase (freshly prepared) to prepare working standard solutions in the concentration ranges (30-180,10-60,16-96, 16-96 µl/ml) for Irbesartan, Losartan potassium, Telmisartan, Valsartan respectively. Each solution (n=5) was injected in triplicate and analyzed under the mentioned conditions above. Linear relationships were obtained when average drug standard peak area were plotted against the corresponding concentrations for each drug. Regression equation was computed.

Sample preparation

A composite of ten X-tension tablets, Losar Mepha tablets, Disartan capsules and Micardis tablets were prepared by grinding each type of tablets separately to a fine, uniform size powder, triturated using mortar and pestle. After calculating the average tablet weight, amounts of powder equivalent to 150, 50, 80 and 80 mg for Irbesartan, Losartan potassium, Telmisartan, and Valsartan respectively were accurately weighed and transferred separately of each type of tablets to 100 ml volumetric flasks respectively then complete with methanol up to 100 ml. The solutions were sonicated for 15 min and the solutions were then filtered through 0.45 µm Nylon membrane filters (Millipore, Milford, MA, USA). Aliquots of appropriate volume (10 ml) were transferred to 100 ml calibrated flasks and diluted to volume with mobile phase to obtain the mentioned concentration above. The diluted solutions were analyzed under optimized chromatographic conditions and chromatogram is showed in Figure 2.

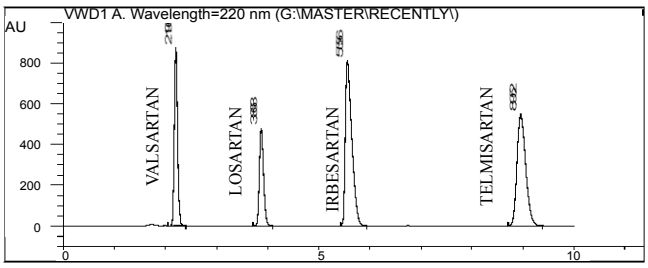


Figure 2: HPLC chromatogram of Valsartan , Losartan potassium, Irbesartan and Telmisartan respectively on ACE C-18 (25cm) Column and mobile phase consisted of (A) acetonitrile and (B) phosphate buffer pH=6.0 in ratio 35:65%.

Forced degradation of Irbesartan, Losartan potassium, Telmisartan, and Valsartan

To determine the proposed method as a stability-indicating method, Irbesartan, Losartan potassium, Telmisartan, and Valsartan respectively bulk powders were stressed under different conditions in forced degradation studies.

Acidic and alkaline degradation: Hydrochloric acid (HCl) (1 M, 10 ml) and sodium hydroxide (NaOH) (1 M, 10 ml) were separately added to 10 ml methanolic stock solutions of Irbesartan, Losartan potassium, Telmisartan, and Valsartan respectively. These mixtures were separately heated at 70°C for 14 days in the dark (to exclude the possible degradative effect of light). The solutions (1 ml) were then transferred to 10 ml volumetric flasks, neutralized by addition of 1 M NaoH or 1 M HCl, and diluted to final volume with mobile phase [4,20].

Oxidation: Hydrogen peroxide (H_2O_2; 3%, v/v, 10 ml) was added to 10 ml methanolic stock solutions of Irbesartan, Losartan potassium, Telmisartan, and Valsartan respectively. These solutions were separately set aside for 7 days at ambient temperature in the dark. The solutions (1 ml) obtained were then transferred to 10 ml volumetric flasks and diluted to final volume with mobile phase [4,20].

Neutral degradation (Thermal degradation): Methanolic stock solutions of Irbesartan, Losartan potassium, Telmisartan, and Valsartan respectively were heated at 70°C for 14 days in the dark to study the effect of thermal stress. Also the experiment was performed on solid-state samples which could be stressed under previous condition and then diluted with a known amount of mobile phase. The experiment was performed in the dark to exclude the possible degradative effect of light. The solutions (1 ml) obtained were then transferred to 10 ml volumetric flasks and diluted to final volume with mobile phase [4,20].

Photo stability: Methanolic stock solutions of Irbesartan, Losartan potassium, Telmisartan, and Valsartan respectively (10 ml) were exposed to light providing an overall illumination of not less than 1.2 million lux hours and an integrated near ultraviolet energy of not less than 200 watt hours/square meter. Also the experiment was performed on solid-state samples which could be stressed under previous condition and then diluted with a known amount of mobile phase. The solutions (1 ml) obtained were then transferred to 10 ml volumetric flasks and diluted to final volume with mobile phase [4,22].

Stress-testing conditions: According to ICH recommendations at least five conditions must be used. In this study the Conditions were chosen as Solid, Aqueous solution, 0.1N HCl solution, Aqueous Solution, 0.1N NaoH solution, 0.3% H_2O_2 solution (Table 1) [20].

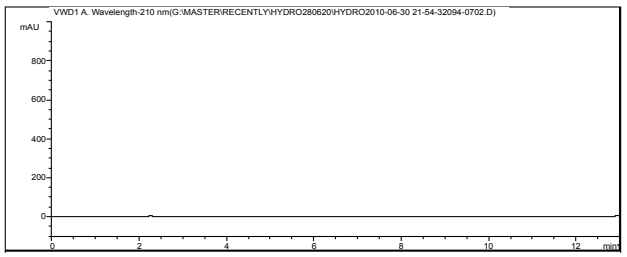
Method Validation

Specificity

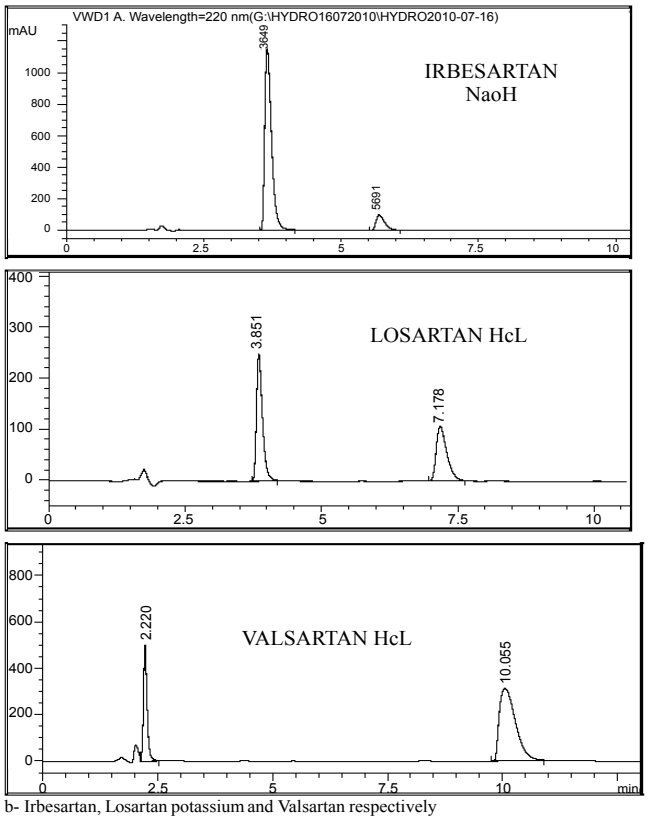
The Specificity of the method was evaluated by assessing whether

Sample condition	Storage condition	Time points for a detailed study
Solid	70°C	7, 14, 28 days
Solid	simulated sunlight	Exposure 2-3 times ICH guideline
Aqueous solution	simulated sunlight	Exposure 1-3 times ICH guideline
0.1N HCl solution	up to 70°**C**	3, 7, 14 days
Aqueous Solution	up to 70°C	3, 7, 14 days
0.1N NaOH solution	up to 70°C	3, 7, 14 days
0.3% H_2O_2 solution	up to 70°C	3, 7days

Table 1: Proposed storage conditions and time points for a detailed study (solid-state stress).



a- tablet placebo under optimized chromatographic conditions



b- Irbesartan, Losartan potassium and Valsartan respectively

Figure 3: Typical HPLC chromatograms obtained from 50 µl injections of Irbesartan, Losartan potassium and Valsartan respectively are obtained from stress studies involving acid, base as well as analysis of samples stored under ICH stability conditions under optimized chromatographic conditions isocratic method.

excipients present in the pharmaceutical formulations interfered with the analysis [23]. A placebo for each tablet was prepared by mixing the respective excipients. Solutions were prepared by following the procedure described in the section on sample preparation. The commonly used tablet excipients did not interfere with the method. The diluent chromatogram shows that the tablet diluent has negligible contribution after the void volume at the method detection wavelength of 220 nm. The method were also evaluated by assessing whether degradation products present in the pharmaceutical formulations interfered with the analysis, obtained from stress studies involving acid, base, peroxide, and heat as well as analysis of samples stored under ICH stability conditions (Figure 3).

Linearity and range

According to ICH recommendations [Q2R1] [21], at least five concentrations must be used. In this study five concentrations were chosen in the ranges (30-180, 10-60, 16-96, and 16-96 µl/ml) corresponding levels of 20-120% w/w of the nominal analytical concentration for Irbesartan, Losartan potassium, Telmisartan and Valsartan respectively. The linearity of peak area responses versus concentrations was demonstrated by linear least square regression analysis. The linear regression equations were {Y=116.98X+198.75 (r=1.0), Y=133.95X+68.63 (r=1.0), Y=193.2X+550.8 (r=0.9997), Y=113.1X+222.2 (r=0.9998)} for Irbesartan, Losartan potassium, Telmisartan and Valsartan respectively. Where Y is the peak area of standard solution and X is the drug concentration.

Precision

The precision of the method was investigated by measurement of both repeatability and Intermediate precision [21].

Repeatability: Repeatability is also termed intra-assay precision. Repeatability was investigated by injecting 6 determinations at 100% of the test concentration. RSD were calculated (Table 2).

Intermediate precision: In the inter-day studies, standard and sample solutions prepared as described above, were analyzed in triplicate on three consecutive days at 100% of the test concentration. RSD were calculated (Table 3).

Accuracy

Accuracy was assessed using 9 determinations over 3 concentration levels covering the specified range (80,100 and120%). Accuracy was reported as percent recovery by the assay of known added amount of analyte in the sample (Table 4).

Limits of detection and Limits of quantitation

In accordance with ICH recommendations [21], determination of limits of detection and quantitation was based on the standard deviation of the y-intercepts of regression lines (n=3) and the slope of the calibration plots. The values obtained are given in table 5.

Robustness

Robustness of an analytical procedure is a measure of its capacity to remain unaffected by small deliberate variations in method parameters and provides an indication of its reliability during normal usage. Robustness was assessed by studying the effect of changing mobile phase pH by ± 0.1, the amount of acetonitrile in the mobile phase by ± 2%, temperature ± 2°C, different column and flow rate ± 0.05 ml/min. Variations had no significant effect on the chromatographic resolution of the method.

Stability of analytical solution

Also as part of evaluation of robustness, Solution stability was evaluated by monitoring the peak area response. Standard solutions in methanol were analyzed after its preparation 1, 2 and 3 days after at 5°C and for a day at room temperature. The change in standard solution peak area response over 3 days was (0.56, 0.74, 0.53and 0.97%) for Irbesartan, Losartan potassium, Telmisartan and Valsartan respectively and no significant degradation was observed during this period. Their solutions were found to be stable for 3 days at 5°C (in the refrigerator) and for a day at room temperature at least.

Application on pharmaceutical Preparation

The proposed methods were successfully used to determine Irbesartan, Losartan potassium, Telmisartan and Valsartan respectively in their dosage forms e.g. *X-tension tablets, LosarMepha tablets, Micardis tablets and Disartan capsules* respectively.

Drug name	Average µg/ml	Average %	RSD
Irbesartan	150.06	100.04	0.17%
Losartan potassium	49.97	99.94	0.16%
Telmisartan	80.16	100.20	0.15%
Valsartan	79.96	99.95	0.13%

Table 2: Repeatability of Irbesartan, Losartan potassium, Telmisartan and Valsartan respectively.

Drug name	1st day µg/ml	2nd day µg/ml	3rd day µg/ml	Pooled average	Pooled average %	RSD
Irbesartan	150.06	151.83	150.48	150.79	100.52	0.61%
Losartan potassium	49.97	50.78	50.18	50.31	100.63	0.83%
Telmisartan	80.16	81.10	80.73	80.66	100.83	0.59%
Valsartan	79.96	81.01	79.67	80.21	100.26	0.88%

Table 3: Intermediate precision of Irbesartan, Losartan potassium, Telmisartan and Valsartan respectively.

Drug name	Recovery at 80% conc. (%)	Recovery at 100% conc. (%)	Recovery at 120% conc. (%)	Average Recovery (%)	RSD
Irbesartan	100.9	101.3	100.21	100.8	0.55%
Losartan potassium	100.88	101.65	100.35	100.96	0.65%
Telmisartan	101.03	101.50	100.43	100.98	0.53%
Valsartan	101.04	101.35	99.87	100.75	0.77%

Table 4: Recovery results for standard solution plus excipients for Hydrochlorothiazide, Irbesartan, Losartan potassium, Telmisartan and Valsartan respectively.

Item	Irbesartan	Losartan	Telmisartan	Valsartan
Linear range (µl/ml)	30-180	10-60	16-96	16-96
Detection limit (µg/ml)	0.007	0.06	0.01	0.13
Quantitation limit (µg/ml)	0.02	0.185	0.04	0.4
Regression data				
N	5	5	5	5
Slope (b)	116.98	133.95	193.23	113.1
Standard deviation of the slope	0.06	0.02	0.22	0.15
Intercept (a)	198.08	68.64	550.82	222.26
Standard deviation of the intercept	13.64	3.97	11.97	9.28
Correlation coefficient (r)	1.0	1.0	0.9997	0.9998
Standard error of regression	40.1	15.25	118	55.12

Table 5: Calibration data for analysis of the mixture by use of the proposed HPLC method of Irbesartan, Losartan potassium, Telmisartan and Valsartan respectively

Five replicate determinations were performed. Satisfactory results were obtained for each compound in good agreement with label claims (Table 6 and 7). The results obtained were compared statistically with those from published methods [15,23-25] by using Student's t-test (for accuracy) and the variance ratio F-test (for precision). The results in (Table 8) showed that the t and F values were smaller than the critical values. So, there were no significant differences between the results obtained from this method and published methods.

Results and Discussion

Optimization of parameters of HPLC method

To establish and validate an accurate method for analysis of these drugs in pharmaceutical formulations, preliminary tests were performed with the objective of selecting optimum conditions. The main problems encountered during these investigations were lack of resolution between of Irbesartan and Valsartan and excessive retention of Telmisartan. To solve these problems, ACE column (25 cm), Hypersil C18 (15 cm), Hypersil cyano (CN) and Hypersil CPS columns were tried for simultaneous determination of the drugs.

The effect of mobile phase composition were also studied (a) aqueous phase e.g. ammonium acetate buffer, citrate buffer and phosphate buffer (b) organic modifier e.g. acetonitrile and methanol (c) pH of aqueous phase e.g. 2.5, 3.5, 4.5, 6.0.

Changing pH of mobile phase from 3.5 to 6.0 affects on eluting of Valsartan because It is ionizable compound containing carboxylic group (COOH), pH of mobile phase greater than pKa of Valsartan (4.9) by more one unit and at pH's above the pKa of the analyte, the acidic analyte carries a negative charge and behaves as an extremely polar molecule [26].

The optimum wavelength for detection was 220 nm at which much better detector responses for four drugs were obtained. The best resolution with reasonable retention time was obtained at 65% phosphate buffer pH 6.0 and 35% acetonitrile as organic modifier. A major reason for using a concentration of 25 mM was achieving maximum sensitivity of UV detection at low wavelengths.

Optimization of parameters of stability indicating method

According to current good manufacturing practice (cGMP), all drugs must be tested under a stability-indicating method before release. Stress testing of drug substance can help in identifying the likely degradation products which can establish the degradation pathways and the intrinsic stability of the molecule. The nature of the stress testing will depend on the individual drug substance. These studies provide valuable information on drugs inherent stability and help in the validation of analytical methods to be used in stability studies. Because of the special nature of separation requirement during analysis of stability samples, chromatographic methods have taken precedence over the conventional methods of analysis. Other than separation of multiple components, the merits of chromatographic methods are that possess greater accuracy and sensitivity for even small quantities of degradation products produced [4,20].

Degradation studies: The chromatograms obtained from samples treated with acid, base, hydrogen peroxide, heat and photo degradation were examined. The chromatograms showed that both Losartan potassium and Valsartan were degradated under acidic conditions (Figure 4 and 5). Irbesartan also was degradated to large extent under basic conditions (Figure 6) unlike Telmisartan wasn't degradated under all previous condition (Figure 7). In this study, thermal degradation had no effect and acidic and alkaline degradation had some extent effect but if acidic and alkaline degradation undergoes under effect of heat at 70°C, the extent of degradation increased for Valsartan, Losartan and Irbesartan (Figure 8-10).

The ratio of degradation of Losartan potassium, Valsartan and Irbesartan respectively had been calculated by normalization method (The percentage content of a component of the substance to be examined is calculated by determining the area of the corresponding peak as a percentage of the total area of all the peaks, excluding those due to solvents or reagents or arising from the mobile phase or the sample matrix and those at or below the disregard limit) [5] (Table 9).

Losartan potassium was degradated by acidic hydrolysis (1M HCl at 70°C) to give impurity E and F [12]. The impurity E was the major. It was found that many compounds will undergo dimerization reactions: those containing olefins, alcohols, and carboxylic acids (or other carbonyl chemistry e.g. aldol condensation reactions). Indoles have been shown to dimerize under acidic conditions. The dimerization is presumed to occur via protonation and nucleophilic attack of a second indole (Figure 11).

Impurity E is ((-(1′-[1-[[2-butyl-4-chloro-1-[[2′[2-butyl-1-[[2H-tetrazol-5-yl)biphenyl-4-yl]methyl]-1H-imidazol-5-yl]methyl]-1H-tetrazol-5-yl]biphenyl-4-yl]methyl]-4-chloro-1H-imidazol-5-yl]

Drug name	Irbesartan (%)	Losartan (%)	Telmisartan (%)	Valsartan (%)
Test 1	97.97	99.52	98.58	98.85
Test 2	99.99	99.96	99.18	98.59
Test 3	99.52	100.3	101.6	99.14
Test 4	99.38	100.8	102.1	98.7
Test 5	99.45	101.1	101.8	99.63
SD	0.76	0.63	1.64	0.42
Average	99.26	100.33	100.65	98.98
R.S.D	0.77	0.63	1.63	0.42

Table 6: Results from determination of Irbesartan, Losartan potassium, Telmisartan and Valsartan respectively in their dosage forms by proposed method.

Drug name	Irbesartan (%)	Losartan (%)	Telmisartan (%)	Valsartan (%)
Test 1	97.04	99.82	98.54	99.94
Test 2	99.44	99.73	99.06	99.63
Test 3	99.49	99.99	98.4	100.6
Test 4	99.28	99.97	101.2	99.89
Test 5	99.67	100.8	101.2	98.89
SD	1.1	0.43	1.41	0.62
Average	98.98	100.06	99.68	99.79
R.S.D	1.1	0.43	1.41	0.61
Method number	15	23	24	25

Table 7: Results from determination of Irbesartan, Losartan potassium, Telmisartan and Valsartan respectively in their dosage forms by reported method.

Drug name	Recovery ± SD		Calculated t-values	Calculated F-values
	Proposed methods	Reference method		
Irbesartan (%)	99.26 ± 0.76	98.98 ± 1.10	$1.36^{(2.57)}$	$0.48^{(5.05)}$
Losartan (%)	100.33 ± 0.63	100.06 ± 0.43	$1.53^{(2.57)}$	$2.21^{(5.05)}$
Telmisartan (%)	100.65 ± 1.64	99.68 ± 1.41	$1.68^{(2.57)}$	$1.36^{(5.05)}$
Valsartan (%)	98.98 ± 0.42	99.79 ± 0.62	$2.05^{(2.57)}$	$0.46^{(5.05)}$

(Where the Tabulated t-values and F-ratios at p=0.05 are 2.57 and 5.05)

Table 8: Statistical comparison of the proposed and published methods for determination of Irbesartan, Losartan potassium, Telmisartan and Valsartan respectively in their dosage forms by reported method (T-student test) and (F-test for variance).

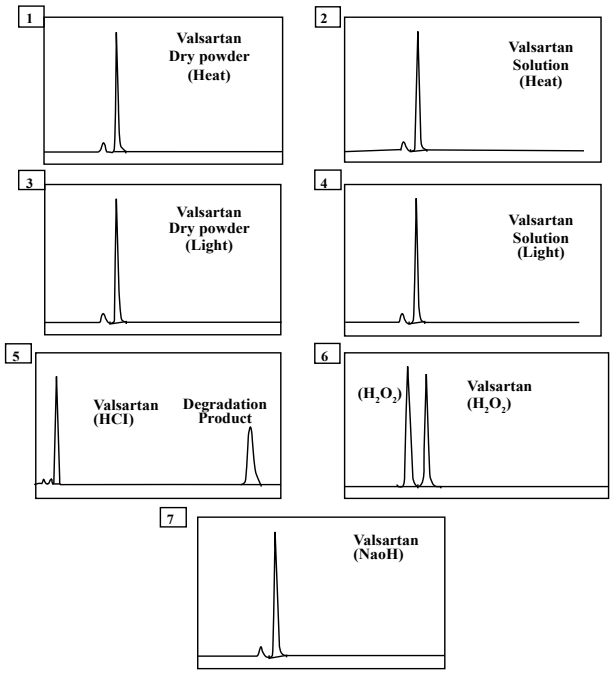


Figure 4: Typical HPLC chromatograms obtained from Valsartan under stress conditions:
1- Dry powder under heat. 2- Methanolic solution under heat.
3- Dry powder under light. 4- Methanolic solution under light.
5- Methanolic solution under 1.0 M HCl at 70°C.
6- Methanolic solution under 3.0 % H_2O_2 at 25°C.
7- Methanolic solution under 1.0 M NaOH at 70°C.

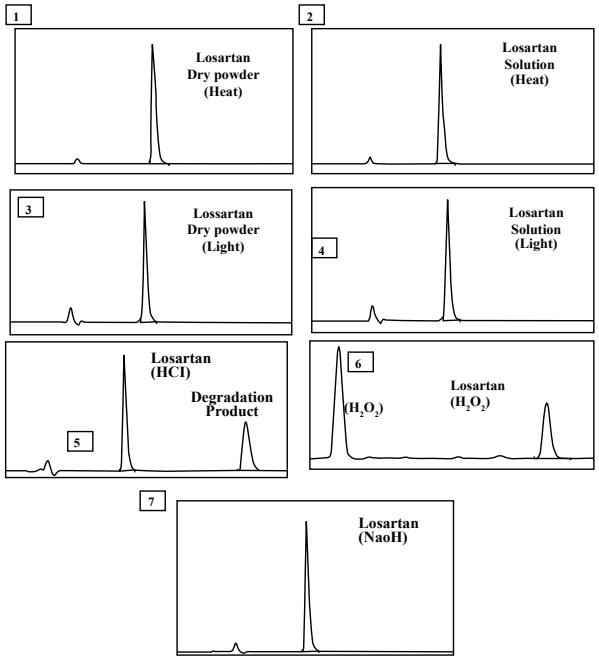


Figure 5: Typical HPLC chromatograms obtained from Losartan under stress conditions:
1- Dry powder under heat. 2- Methanolic solution under heat.
3- Dry powder under light. 4- Methanolic solution under light.
5- Methanolic solution under 1.0 M HCl at 70°C.
6- Methanolic solution under 3.0 % H_2O_2 at 25°C.
7- Methanolic solution under 1.0 M NaOH at 70°C.

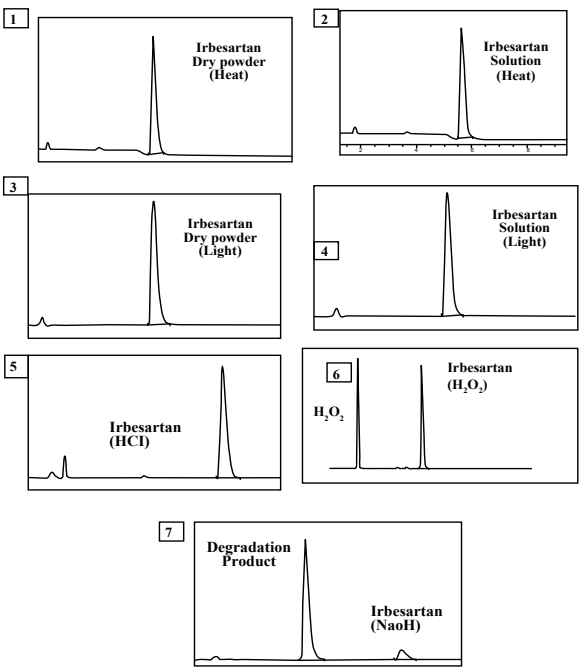


Figure 6: Typical HPLC chromatograms obtained from Irbesartan under stress conditions:
1- Dry powder under heat. 2- Methanolic solution under heat.
3- Dry powder under light. 4- Methanolic solution under light.
5- Methanolic solution under 1.0 M HCl at 70°C.
6- Methanolic solution under 3.0 % H_2O_2 at 25°C.
7- Methanolic solution under 1.0 M NaOH at 70°C.

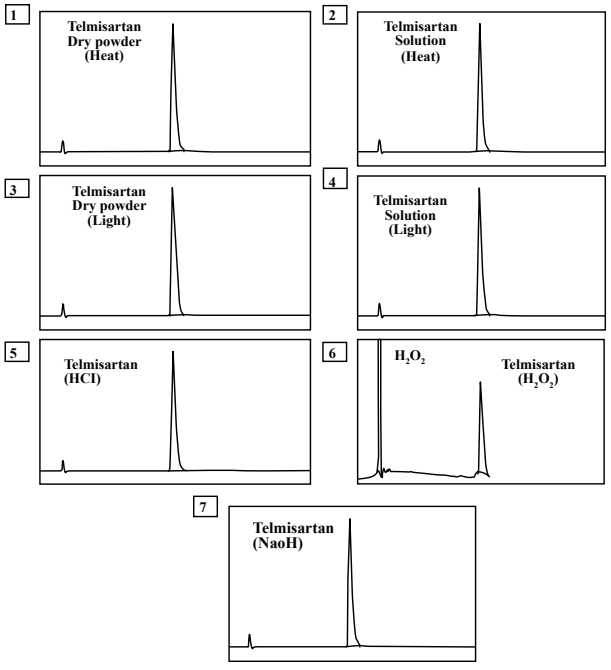


Figure 7: Typical HPLC chromatograms obtained from Telmisartan under stress conditions:
1- Dry powder under heat. 2- Methanolic solution under heat.
3- Dry powder under light. 4- Methanolic solution under light.
5- Methanolic solution under 1.0 M HCl at 70°C.
6- Methanolic solution under 3.0 % H_2O_2 at 25°C.
7- Methanolic solution under 1.0 M NaOH at 70°C.

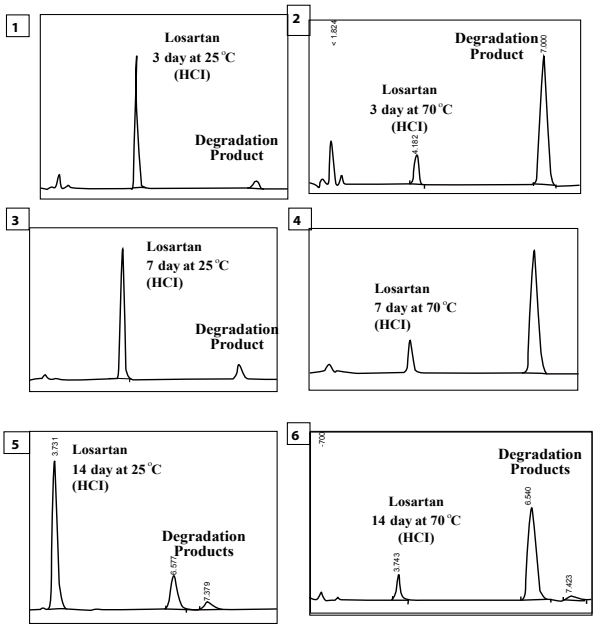


Figure 8: Typical HPLC chromatograms obtained from Losartan under acidic stress conditions:
1- Methanolic solution under 1.0 M HCl at 25°C after 3 days.
2- Methanolic solution under 1.0 M HCl at 70°C after 3 days.
3- Methanolic solution under 1.0 M HCl at 25°C after 7 days.
4- Methanolic solution under 1.0 M HCl at 70°C after 7 days.
5- Methanolic solution under 1.0 M HCl at 25°C after 14 days.
6- Methanolic solution under 1.0 M HCl at 70°C after 14 days.

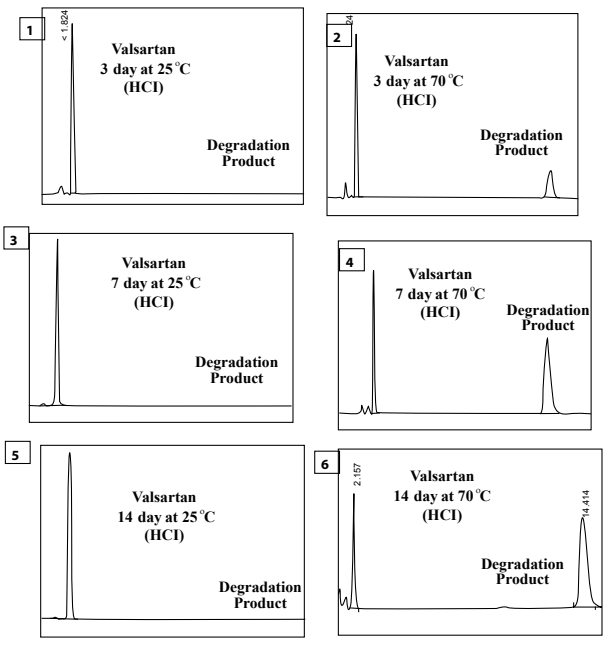


Figure 9: Typical HPLC chromatograms obtained from Valsartan under acidic stress conditions:
1- Methanolic solution under 1.0 M HCl at 25°C after 3 days.
2- Methanolic solution under 1.0 M HCl at 70°C after 3 days.
3- Methanolic solution under 1.0 M HCl at 25°C after 7 days.
4- Methanolic solution under 1.0 M HCl at 70°C after 7 days.
5- Methanolic solution under 1.0 M HCl at 25°C after 14 days.
6- Methanolic solution under 1.0 M HCl at 70°C after 14 days.

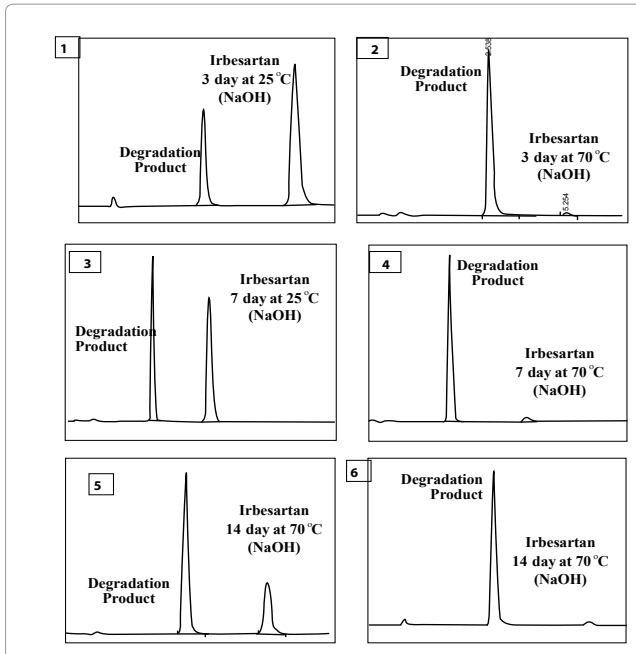


Figure 10: Typical HPLC chromatograms obtained from Irbesartan under stress alkaline conditions:
1-Methanolic solution under 1.0 M NaOH at 25°C after 3 days.
2-Methanolic solution under 1.0 M NaOH at 70°C after 3 days.
3-Methanolic solution under 1.0 M NaOH at 25°C after 7 days.
4-Methanolic solution under 1.0 M NaOH at 70°C after 7 days.
5-Methanolic solution under 1.0 M NaOH at 25°C after 14 days.
6-Methanolic solution under 1.0 M NaOH at 70°C after 14 days.

Losartan potassium	Principle peak	Degradation peak	Sum of peak	Principle peak %	Degradation peak %
3 days (25°C)	4801.31	483.62	5284.93	90.85	9.151
3 days (70°C)	493.96	3597.9	4091.86	12.07	87.93
7 days (25°C)	3028.6	538.87	3567.47	84.89	15.11
7 days (70°C)	455.12	3228.05	3683.17	12.36	87.64
14 days (25°C)	2765.89	1180	3945.89	70.1	29.9
14 days (70°C)	453.8	3071	3524.8	12.87	87.13
Valsartan	**Principle peak**	**Degradation peak**	**Sum of peak**	**Principle peak %**	**Degradation peak %**
3 days (25°C)	3896.52	45.48	3942	98.85	1.154
3 days (70°C)	2282.36	1427.87	3710.23	61.52	38.48
7 days (25°C)	4954.97	95.73	5050.7	98.1	1.895
7 days (70°C)	1938.32	2943.72	4882.04	39.7	60.3
14 days (25°C)	5110.7	150	5260.7	97.15	2.851
14 days (70°C)	1176.08	3045.26	4221.34	27.86	72.14
Irbesartan	**Principle peak**	**Degradation peak**	**Sum of peak**	**Principle peak %**	**Degradation peak %**
3 days (25°C)	6521.5	2749.24	9270.74	70.34	29.66
3 days (70°C)	283.69	8453.58	8737.27	3.247	96.75
7 days (25°C)	5001.21	4289.9	9291.11	53.83	46.17
7 days (70°C)	314.76	9286.84	9601.6	3.278	96.72
14 days (25°C)	2751.72	6374.8	9126.52	30.15	69.85
14 days (70°C)	169.15	9116.94	9286.09	1.821	98.18

Table 9: Percentages of degradation by normalization method for Losartan potassium, Valsartan and Irbesartan respectively.

methanol))Impurity F is -(1′-[2-[[2-butyl-4-chloro-1-[[2′((([2-butyl-1-[[2*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]-1*H*-imidazol-5-yl]methyl]-2*H*-tetrazol-5-yl]biphenyl-4-yl]methyl]-4-chloro-1*H*-imidazol-5-yl] methanol)) (Figure 12).

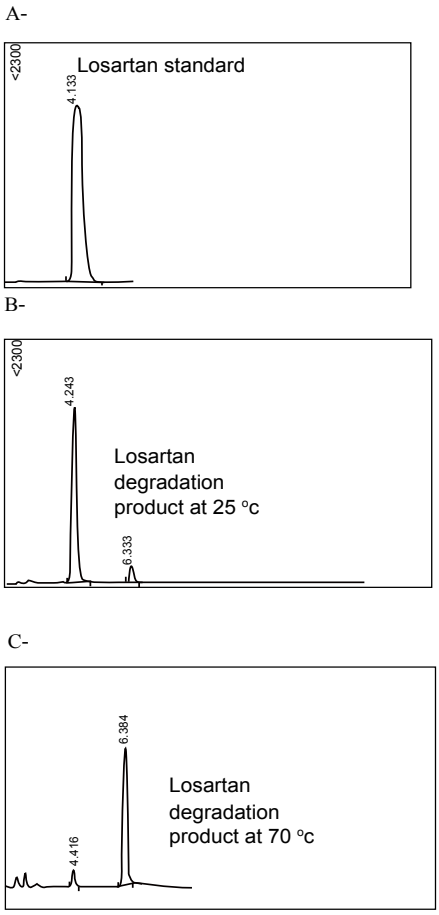


Figure 11: Typical HPLC chromatograms obtained from (a) Losartan standard and (b, c) Losartan under acidic stress conditions (1.0 M HCl at 70°C) according to method [12].

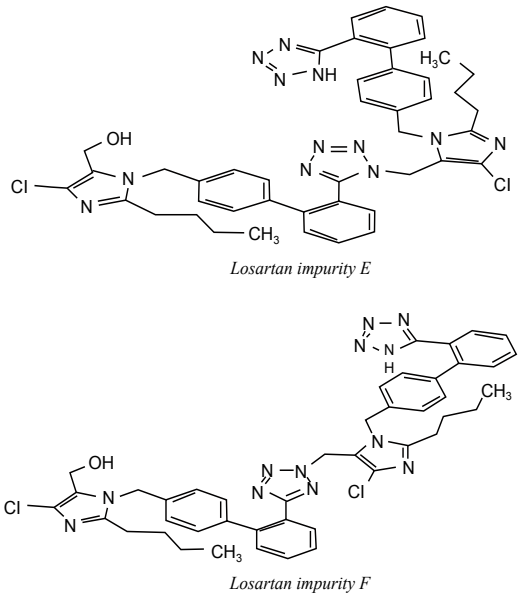


Figure 12: Chemical structure of Losartan impurity E and F.

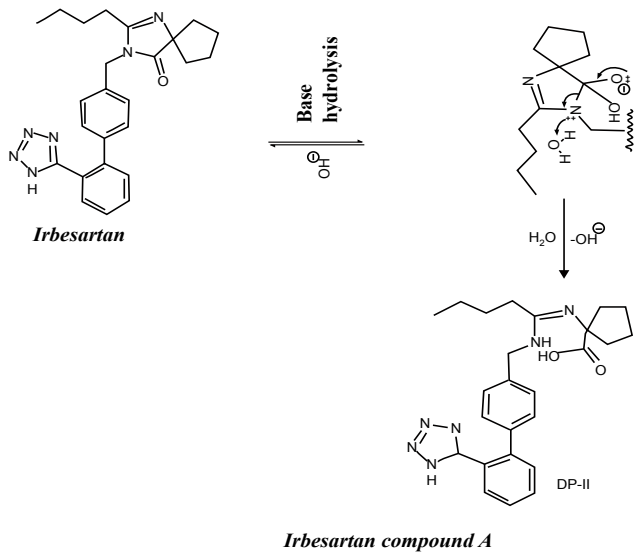


Figure 13: Schematic diagram shows formation of Irbesartan compound A.

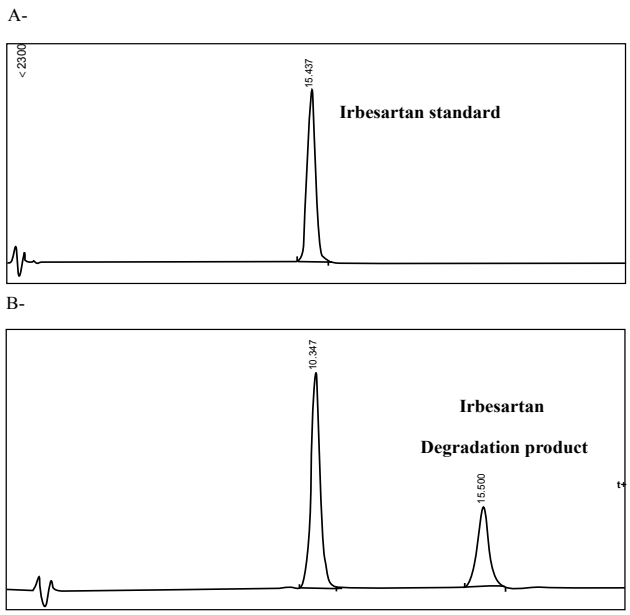


Figure 14: Typical HPLC chromatograms obtained from (a) Irbesartan standard and (b) Irbesartan under alkaline stress conditions (1.0 M NaOH at 25°C) according to USP [5].

Impurity E and F are isomer resulting from dimerization of two molecules of Losartan potassium by formation of a bond between nitrogen atom in tetrazole ring and carbon atom in 5- methanol in imidazole ring producing dimer and water.

Irbesartan was degradated by alkaline hydrolysis (1M NaoH at 70°C) to give Irbesartan compound A [5]. It hydrolyzed by rupture of amide link in 1, 3-diazaspiro [4.4] non-1-en-4-one. Irbesartan compound A was identified as ((1-(1-((2′-(1H-tetrazol-5- yl) biphenyl-4-yl) methyl amino) pentylidene amino) cyclopentane carboxylic acid)) (Figure 13).

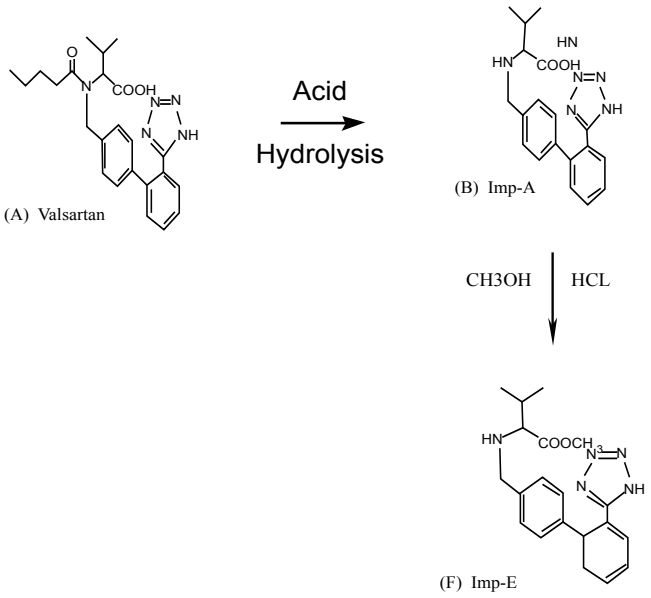


Figure 15: Schematic diagram shows formation of Valsartan related compound.

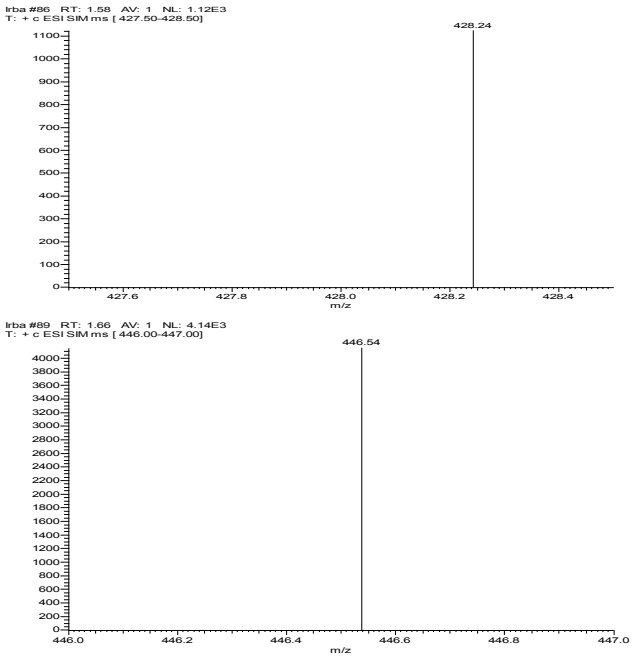


Figure 16: Representative LC-SIM chromatograms for a- Irbesartan at m/z=428 b- Irbesartan related compound A at m/z=446 in positive mode.

Valsartan was degraded by acidic hydrolysis (1M HCl at 70°C) to give impurity (E). There is a question, why was impurity E not impurity A the formed under acidic hydrolysis as degradation product? (Figure14).

It was found that carboxylic acids typically have pKa's in the range of 2-5.5 (depending on the nature of the substituents). Below the pKa (as in acidic hydrolysis), the carboxylic group is protonated and therefore the carbonyl carbon is more electrophilic. The carbonyl can undergo nucleophilic attack to form esters, amides, thioesters, etc.

In the case of attack by an alcohol, the reaction product is an ester and the reaction is called an esterification reaction. This can occur as an artifact reaction when acid/base hydrolysis reactions are performed using an alcohol co-solvent system such as methanol. Esters of the parent compound can also be observed as process-related impurities especially when alcohol solvents are used in the re-crystallization step.

Above the pKa, the carboxylate is anionic and the charge is resonance stabilized; the group is therefore less electrophilic and does not have a good leaving group. Therefore, reactions with nucleophiles are significantly suppressed when compared to the protonated form. Carboxylic acids are not prone to oxidative degradation (Figure 15).

For more investigation, samples containing related compound of

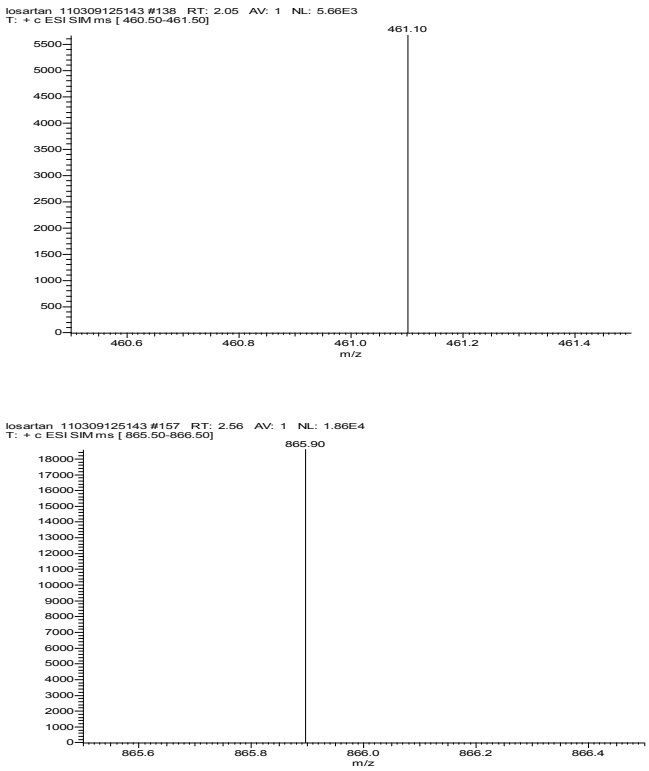


Figure 17: Representative LC-SIM chromatograms for a- Losartan at m/z =461 b- Losartan related dimer E at m/z=865 in positive mode.

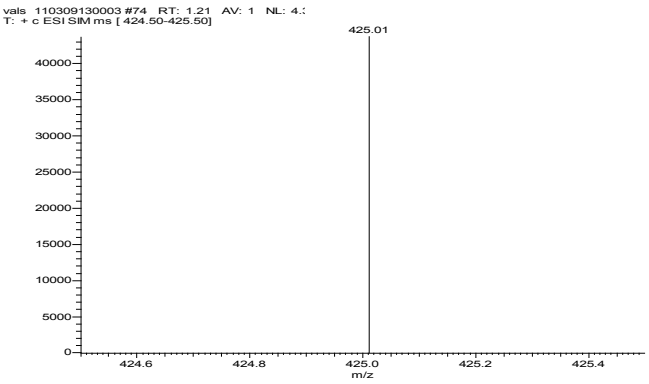


Figure 18: Representative LC-SIM chromatograms for a- Valsartan at m/z=425 in positive mode.

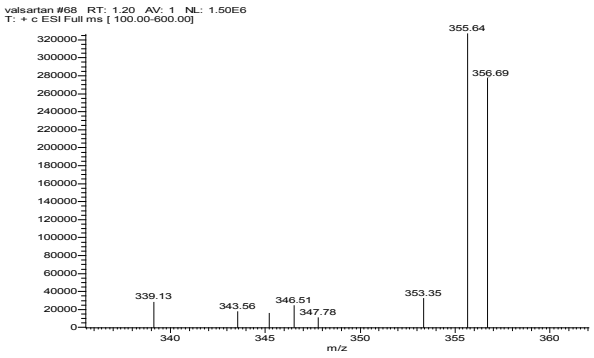


Figure 19: Full-scan product ion mass spectra of $[M - H]^+$ ions of Valsartan related compound A at m/z=355 in positive mode.

the three drugs subjected to liquid tandem mass analysis by using full scan and selective ion monitoring (SIM) and the results from HPLC method were confirmed by that of LC-MS

A. Irbesartan and its related compound A: (Figure 16)

B. Losartan and its dimers (degradated compound E): (Figure 17)

C. Valsartan and its related compound A: (Figure 18 and 19).

Conclusion

A simple, rapid, accurate, precise, robust and reliable HPLC method has been established for simultaneous determination and stability indicating HPLC method for Irbesartan, Losartan potassium, Telmisartan and Valsartan either alone, in synthetic mixture or in their formulations. The method has several advantages, including

1. It depends on previous study has been established to reach the optimum stress condition in drug development process and time points for a detailed study (solid-state stress). It is a practical scientific standardized guide for stress condition. (ICH did not explain stress condition in details).

2. Resulting degraded compound elucidated by mass spectroscopy and provided by mechanisms of the reactions.

3. It is the first study for stress condition comprises these four ARA-II compounds together.

4. HPLC with UV detection becomes the most available apparatus and is a low cost instrument in comparison with HPLC coupling with mass spectroscopy and capillary electrophoresis.

5. Rapid analysis (isocratic elution), simple sample preparation, does not use polluting reagents.

6. A simple mobile phase does not affect on column reproducibility (not like ion pair mobile phase)

7. Improved sensitivity LOD and LOQ ranged from (0.007-0.13) - (0.02-0.4) µg/ml.

It is suitable for analysis of antihypertensive agents in their formulations in a single isocratic run, in contrast with previous methods. This makes the method suitable for routine analysis in quality control laboratories.

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26. Preparing Buffered Mobile Phases for Reversed Phase HPLC.