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RESEARCH ARTICLE

New Stability Indicating Method for Quantification of Impurities in Amlodipine and Hydrochlorothiazide Tablets by Validated HPLC

Eranki RJV[1 *], Inti G[1], Jayaraman V[1], Vidiyala SR[1], Jadi S[2]

[1]*Quality & Analytical Development Department, InvaGen Pharmaceuticals, Inc. Hauppauge, NY 11788 USA.*

[2]*Department of Chemistry, Sri Krishnadevaraya University, Anantapur, India.*

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ABSTRACT

A stability indicating LC method was developed for the simultaneous determination of amlodipine and hydrochlorothiazide in pharmaceutical dosage form. Efficient chromatographic separation was achieved on Zorbax SB Phenyl stationary phase with simple combination of a mixture contained mobile phase (1 ml of methane sulfonic acid in to 1000 ml of DI Water) and Acetonitrile in the ratio of 65:35 v/v respectively, delivered in an Isocratic mode and quantification was carried out using ultraviolet detection at 210 nm at a flow rate of 1.0 mL min^{-1} with Injection Volume of 10 µl and Column temperature at 40°C. In the developed Isocratic method the separation was achieved the between all the specified analytes (Hydrochlorothiazide, Amlodipine and the potential degradation products Amlodipine Impurity-A and Hydrochlorothiazide Impurity-A) and were found to have greater than resolution 1.0 and correlation coefficient of greater than 0.999 was found for the specified known impurities (using Amlodipine and Hydrochlorothiazide standard responses). This method was capable to detect both the drug components of Amlodipine and Hydrochlorothiazide in presence of their degradation products (Amlodipine Imp-A and Hydrochlorothiazide Impurity-A) with the detection level of 0.05 %. The developed HPLC method was validated with respect to linearity & range, accuracy, precision and robustness.

KEYWORDS

Column liquid chromatography, Method validation, Stability indicating study, Amlodipine and Hydrochlorothiazide

INTRODUCTION

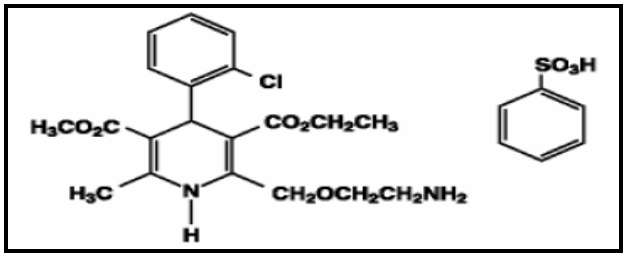
The amlodipine besylate component of Amlodipine/Hydrochlorothiazide tablets is chemically described as 3-ethyl-5-methyl (±)-2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-1, 4-dihydro-6-methyl-3,5-pyridinedicarboxylate, monobenzenesulphonate[1].

Amlodipine is a dihydropyridine calcium antagonist (calcium ion antagonist or slow-channel blocker) that inhibits the movement of calcium ions into vascular smooth muscle cells and cardiac muscle cells. Hydrochlorothiazide is a type of medicine called a diuretic. It works by reducing the amount of excess fluid in the body. Using these two medicines together will lower your blood pressure more effectively than using either one unit.

Amlodipine besylate is a white to pale yellow crystalline powder, with a molecular weight of 567.1, and is slightly soluble in water and sparingly soluble in ethanol.

***Address for Correspondence:**
Rama Joga Venkata Eranki
Quality & Analytical Development,
InvaGen Pharmaceuticals, Inc. Hauppauge, NY 11788, USA .
E-Mail Id: ramjoga@yahoo.com

Amlodipine besylate has empirical formula of $C_{20}H_{25}ClN_2O_5 \cdot C_6H_6O_3S$, and its structural formula is as in Scheme 1.

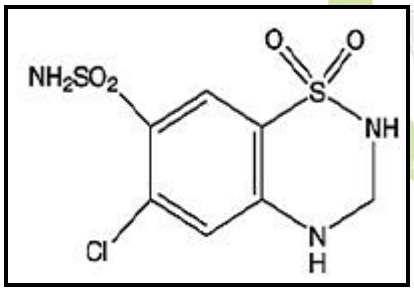


Scheme 1: Amlodipine Chemical Structure

Hydrochlorothiazide is chemically described as 6-chloro-3, 4-dihydro - 2H - 1, 2, 4-benzothiadiazine-7-sulfonamide 1, 1-dioxide[2].

Hydrochlorothiazide is a white, or practically white, crystalline powder with a molecular weight of 297.72, which is slightly soluble in water, but freely soluble in sodium hydroxide solution.

Hydrochlorothiazide has empirical formula of $C_7H_8ClN_3O_4S_2$ and its structural formula is as in Scheme 2.

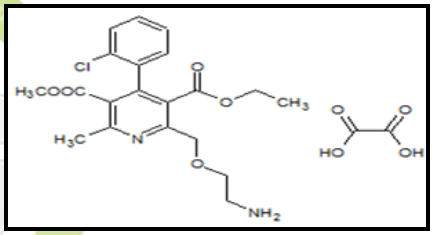


Scheme 2: Hydrochlorothiazide chemical Structure

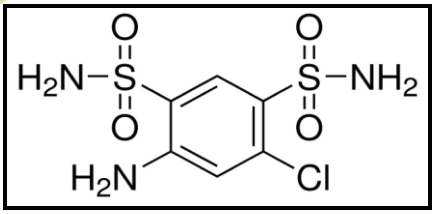
Amlodipine/Hydrochlorothiazide Tablets Brand name called as AMLONG-H[3]. Amlong-H contains 2 of the #1 prescribed high blood pressure medicines of their classes: amlodipine, a calcium channel blocker (CCB), and Hydrochlorothiazide, an angiotensin receptor blocker (ARB). In clinical studies, Amlong-H was proven to be more effective in lowering high blood pressure than either of its components alone. Amlodipine and Hydrochlorothiazide are official in USP[4,5] but their combination drug product is not official in any pharmacopoeia. Amlong-H was proven to significantly lower high blood pressure in adults, regardless of age or gender. The blood pressure-lowering effects of Amlong-H have been studied in several clinical trials. AMLONG-H is available in a variety of dosage combinations that can be adjusted to best fit your needs. The best dosage combination is given as 5/12.5 mg Tablets.

Amlodipine and Hydrochlorothiazide Tablets are developed in reference to (Brand) Amlong-H Tablets in 5/12.5 mg. The inactive ingredients and colorants are standard excipients used in generic products.

Since this combination drug product has not been published in USP, this method has been developed and validated as per ICH guideline and monitoring of these impurities with good separation of peaks and quantification of impurities in Amlodipine / Hydrochlorothiazide tablets.



Scheme 3: Amlodipine Impurity-A Chemical Structure



Scheme 4: Hydrochlorothiazide Impurity-A Chemical Structure

Stability-indicating methods have been reported for assays of various drugs in drug products containing only one active drug substance. Only few stability-indicating methods are reported for the Impurity assay of combination drug products containing two or more active drug substances[6,7,8]. The objective of this work was to develop an analytical LC procedure, which would serve as stability-indicating Impurity assay method for combination drug products of Amlodipine and Hydrochlorothiazide.

The literature survey reveals that several methods were reported for the individual estimation of Amlodipine and Hydrochlorothiazide. Various methods using HPLC, RP-HPLC, HPTLC, LC-MS, LCMS / MS and simultaneous UV spectrophotometric methods[9-23] are reported for the estimation of Amlodipine alone or in combination with other antihypertensive agents. None of the reported analytical procedures describe a stability indicating method for simultaneous determination of Amlodipine and Hydrochlorothiazide in combined pharmaceutical dosage form in the presence of their degradants. In the present study attempts were made to develop a rapid, economical, precise and accurate method for the simultaneous estimation of the ingredients of this combination in the presence of their degradants.

EXPERIMENTAL

Chemicals & Reagents

Samples of Amlodipine Impurity-A and the Hydrochlorothiazide Impurity-A were synthesized and characterized at Hetero Drugs and Hetero Labs Limited, India. HPLC grade acetonitrile & Methanol was procured from Honeywell: Burdick & Jackson, Muskegon, MI 49442 and analytical grade Methane Sulphonic Acid was procured from Sigma Aldrich Company, 3050 spruce Street, St. Louis, MO-63103, High purity water was generated in-house from Siemens water purification system.

Chromatographic Conditions

The chromatographic system used was Shimadzu LC 2010 HPLC system comprised of degasser, quaternary pump, auto injector, column compartment, UV detector and the system was controlled through EZ chrome software. Zorbax SB Phenyl column (i.d. 4.6 · 250 mm, 5.0 µm, Advance Chromatography, USA), maintained at 40 °C using a column oven, eluted with mobile phase at the flow rate of 1.0 mL min^{-1} with Isocratic program.

Mobile Phase: 1 ml of methane sulfonic acid in to 1000 ml of DI Water and Acetonitrile in the ratio of 65:35(v/v) respectively. Filtered through 0.45 µm nylon membrane filter and degassed.

Measurements were made with injection volume 10µl and ultraviolet (UV) detection at 210 nm.

For standard and sample solution were prepared using the diluents consist of Acetonitrile and DI water in the ratio of 50:50 v/v.

For analysis of forced degradation samples, the photodiode array detector (Model No. 2998) and Empower Software was used in scan mode with a scan range of 200–400 nm. The peak homogeneity was expressed in terms of peak purity and was obtained directly from the spectral analysis report using the above-mentioned software.

Standard Stock Solutions

Standard solutions were prepared by dissolving the drugs in the diluent and diluting them to the desired concentration.

Amlodipine

50.0 mg Amlodipine Standard was accurately weighed, transferred into a 100 mL volumetric flask, and dissolved with diluent.

Hydrochlorothiazide

125.0 mg Hydrochlorothiazide standard was accurately weighed, transferred into a 100mL volumetric flask, and dissolved with diluent.

Low Level Standard Preparation

The concentration of Low level standard Preparation contains 0.00125 mg / mL of Amlodipine Besylate and 0.00625 mg/mL of Hydrochlorothiazide.

Detectability Level standard preparation

The concentration of Detectability level standard Preparation contains 0.000125 mg / mL of Amlodipine Besylate and 0.000625 mg/mL of Hydrochlorothiazide.

Preparation of Sample

Twenty tablets were weighed for average weight and finely powdered. A quantity of powder equivalent to 125.0 mg of Hydrochlorothiazide was transferred into a 100 mL volumetric flask.

To this flask, 75 mL of diluent were added, and the solution was sonicated for about 15 min with intermittent shaking and with mechanical shaking for about 15min. The solution was cooled to ambient temperature. Then the volume was made up with diluent and centrifuged at 10,000 rpm for about 15 min. Then the solution was used for injection.

Analytical Method Validation

The developed chromatographic method was validated for selectivity, linearity, range, precision, accuracy, sensitivity, robustness and system suitability.

Selectivity/Specificity

Selectivity of the developed method was assessed by performing forced degradation studies. According to ICH stress testing of the drug substance can help the intrinsic stability of the molecule and validate the stability indicating power of the analytical procedure used. Photo stability testing should be an integral part of stress testing. The standard conditions for photo stability testing are described in ICH Q1B.

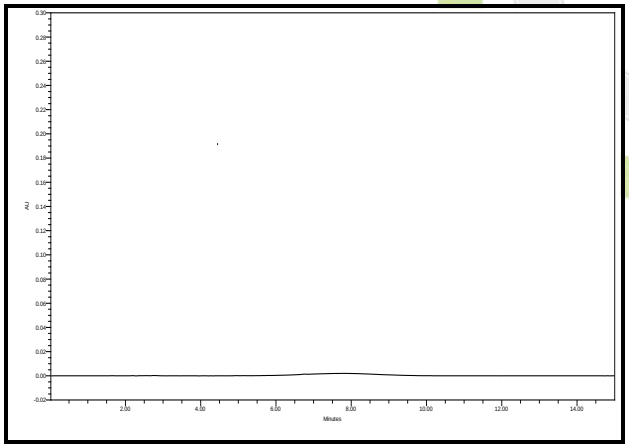


Figure 1: Chromatogram of Blank sample of 5/12.5 mg

The specificity of the developed LC method for Amlodipine and Hydrochlorothiazide was determined in the presence of its related compounds Amlodipine impurity-A, Hydrochlorothiazide impurity-A. The stress conditions employed for degradation study includes photolysis (ICH Q1B), thermal (80°C), acid hydrolysis (1N HCl), base hydrolysis (1N NaOH) and oxidation (30% H_2O_2). For thermal study period was 7 days at 80°C and whereas for acid, base and oxidation it was 2 hours.

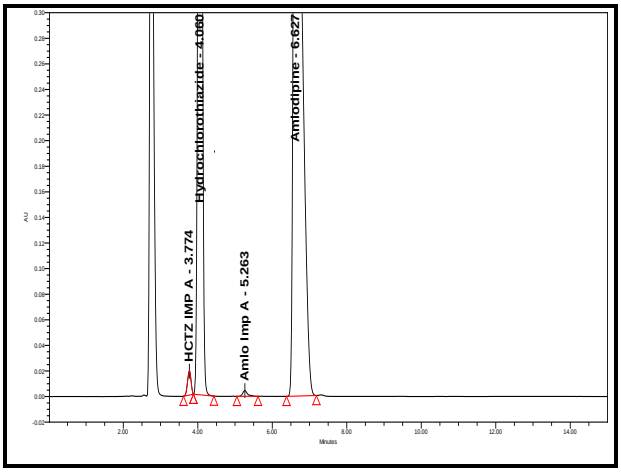


Figure 2: Chromatogram of Spiked sample of 5/12.5 mg

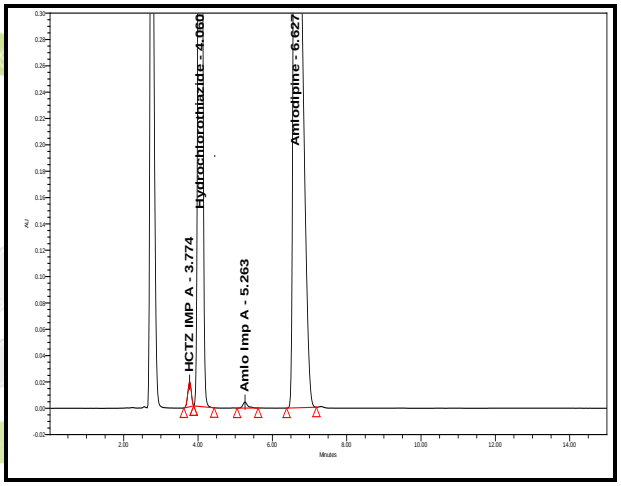


Figure 3: Chromatogram of as such sample of 5/12.5 mg

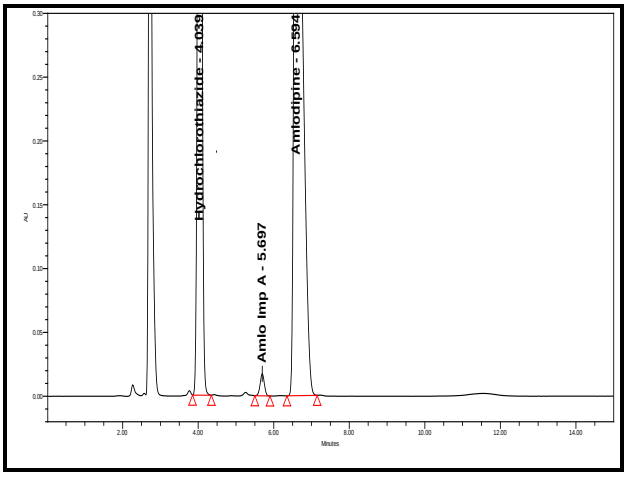


Figure 4: Chromatogram of sample of 5/12.5 mg on acid degradation

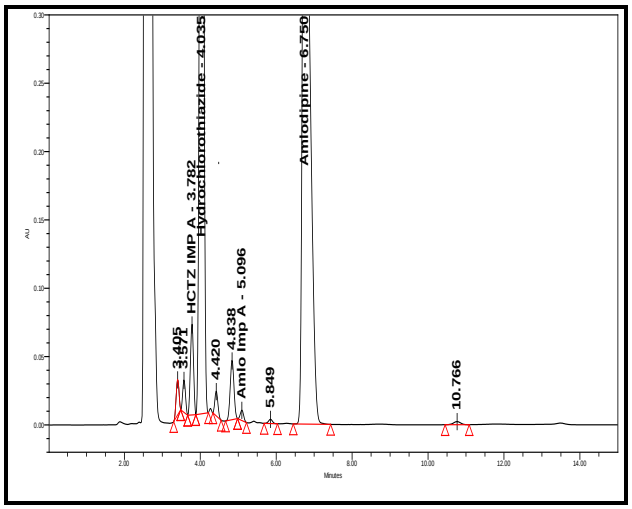


Figure 5: Chromatogram of sample of 5/12.5 mg on base degradation

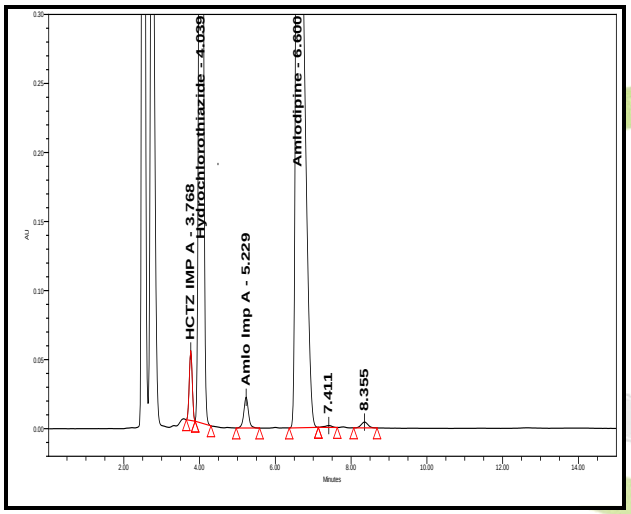


Figure 6: Chromatogram of sample of 5/12.5 mg on Oxidation

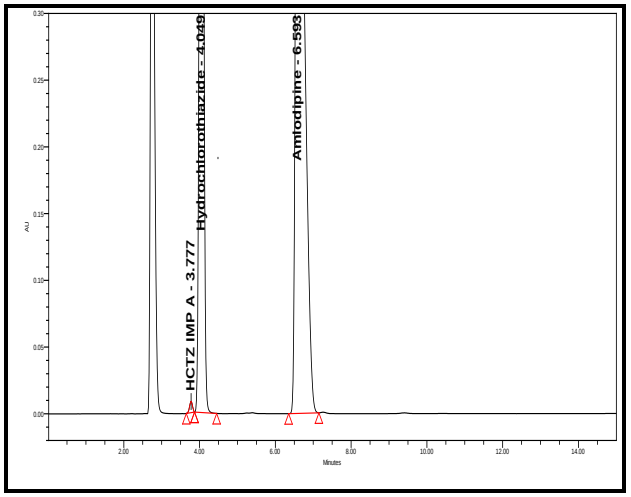


Figure 7: Chromatogram of sample of 5/12.5 mg on UV treatment

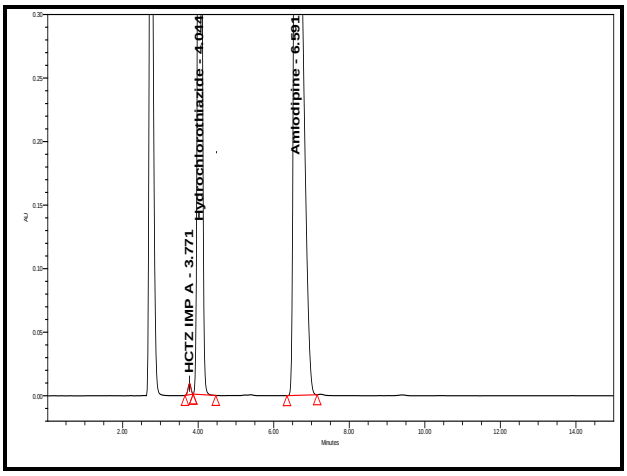


Figure 8: Chromatogram of Placebo and sample of 5/12.5 mg on Thermal Treatment

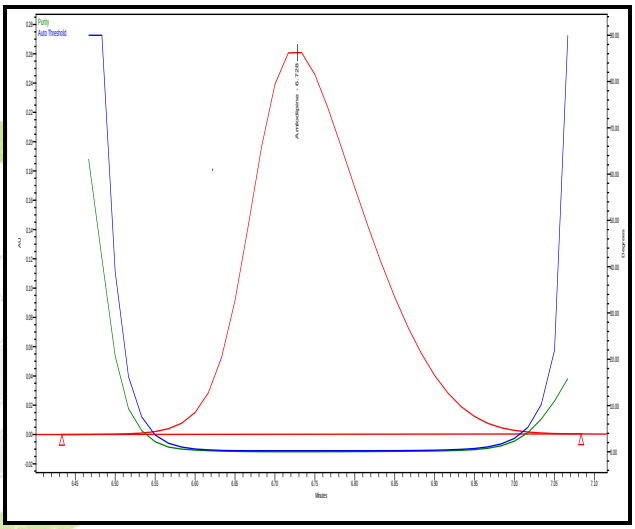


Figure 9: Purity plot of Amlodipine in Amlodipine and Hydrochlorothiazide Tablets 5/12.5mg

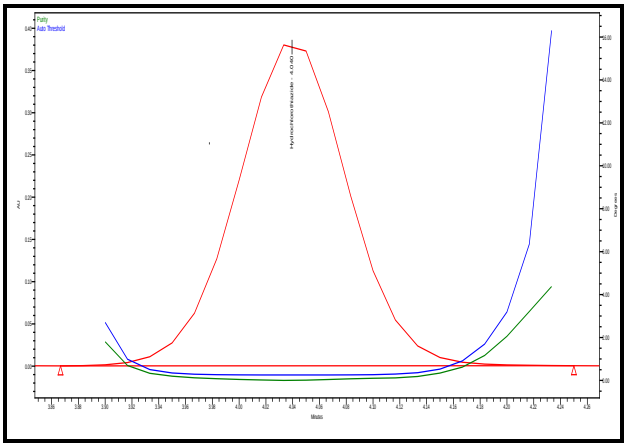


Figure 10: Purity plot of Hydrochlorothiazide in Amlodipine and Hydrochlorothiazide Tablets 5/12.5 mg

Table 1a: Results of analysis of forced degradation study samples using the proposed method, indicating percentage of degradation of Amlodipine and Hydrochlorothiazide

Degradation In	Degradation Sample	Amlodipine Imp-A (%)	Hydrochlorothiazide Imp-A (%)	Maximum Individual Unknown Impurity (%)	Total Impurities (%)
As such sample	Placebo +Amlodipine and Hydrochlorothiazide	ND	0.2	ND	0.20
1N HCl (5 mL)	Placebo +Amlodipine and Hydrochlorothiazide	0.78	ND	ND	0.78
1N NaOH (5 mL)	Placebo +Amlodipine and Hydrochlorothiazide	0.29	2.41	1.89	7.25
30 %H_2O_2 (5 mL)	Placebo +Amlodipine and Hydrochlorothiazide	1.15	1.68	0.32	3.26
UV Light	Placebo +Amlodipine and Hydrochlorothiazide	ND	0.21	ND	0.21
Thermal Condition at 80°C	Placebo +Amlodipine and Hydrochlorothiazide	ND	0.25	ND	0.25

ND: Not Detected BRL: Below Reporting level (BRL=0.05%)

Table 1b: Results of analysis of forced degradation study samples indicating peak purity of Amlodipine and Hydrochlorothiazide

Degradation Samples	Peak Area	Peak Area	Retention Time (min)	Purity Angle	Purity Threshold
Placebo + Drug Degradation in 5 mL of 1N HCl	Amlodipine	9143108	6.59	0.041	0.234
	Hydrochlorothiazide	7917180	4.04	0.059	0.259
Placebo + Drug Degradation in 5 mL of 1N NaOH	Amlodipine	7084654	6.75	0.087	0.356
	Hydrochlorothiazide	7479460	4.03	0.099	0.339
Placebo + Drug Degradation in 5 mL of 30 % H_2O_2	Amlodipine	8359288	6.60	0.025	0.221
	Hydrochlorothiazide	7647830	4.04	0.061	0.256
Placebo + Drug Degradation in UV Light	Amlodipine	9653228	6.59	0.062	0.259
	Hydrochlorothiazide	7792994	4.05	0.052	0.267
Placebo + Drug Degradation in Thermal Condition at 80°C	Amlodipine	9640795	6.59	0.056	0.262
	Hydrochlorothiazide	7783040	4.04	0.051	0.267

RESULTS AND DISCUSSION

Optimization of the Chromatographic Conditions

To develop the stability-indicating method different stationary phases like C18, CN, different mobile phases containing buffers like phosphate, ammonium acetate and trifluoroacetic acid with different pH (3–5) and organic modifier (acetonitrile) were used.

Our objective of the chromatographic method development was to achieve a peak tailing factor <2, Signal to Noise Ratio should be above 10, Theoretical plates should be above 1000 for Amlodipine and Hydrochlorothiazide and %RSD for 5 consecutive injection should be less than NMT 10.0 % and very good separation between amlodipine Impurity-A, Hydrochlorothiazide Impurity-A along with drug peak amlodipine and Hydrochlorothiazide.

The test solution was found stable in diluent for 48 hrs. The drug was subjected to stress conditions as per ICH guidance, considerable degradation was found to occur in oxidative stress conditions.

The stress samples were assayed against a qualified reference standard and the mass balance was found close to 30%. Signal to Noise ratio of Identification solution for Amlodipine and Hydrochlorothiazide were found to be more than 10. Theoretical plates for Amlodipine and Hydrochlorothiazide were 12460.23 and 617503.71. Tailing factor for Amlodipine and Hydrochlorothiazide was 1.034 and 1.204, %RSD for five replicate injections of Amlodipine and Hydrochlorothiazide were found 0.85 and 0.89 Amlodipine / Hydrochlorothiazide and their combination drug product were exposed to thermal, photolytic, hydrolytic and oxidative stress conditions, and the stressed samples were analysed by the proposed method.

Peak homogeneity data of Amlodipine and Hydrochlorothiazide is obtained using photodiode array detector, in the stressed sample chromatograms, demonstrated the specificity of the method for their estimation in presence of degradants. The described method shows excellent linearity over a range of 0.05-1.0% for Amlodipine, Amlodipine Impurity-A and 0.05-1.0 % for Hydrochlorothiazide and Hydrochlorothiazide Impurity-A. The correlation coefficient for Amlodipine and Hydrochlorothiazide are 0.9999. The relative standard deviation for five measurements in two sets of each drug in tablets is less than 2%. The proposed method was found to be suitable and accurate for quantitative determination and the stability study of Amlodipine and Hydrochlorothiazide in pharmaceutical preparations.

The chromatographic separation was achieved using a Zorbax SB Phenylcolumn (i.d. 4.6·250 mm, 5 µm). Changing the composition of mobile phase optimized the chromatographic method. Segregation of all 2 peaks (Amlodipine and Hydrochlorothiazide) was observed on any C_{18} or CN column but it was difficult to separate both drug degradants on these columns (amlodipine Impurity-A and Hydrochlorothiazide Impurity-A). The Zorbax SB Phenyl column shows better performance as compared to other columns.

From the development studies, it was determined that using mobile phase: 1 ml of methane sulfonic acid in to 1000 ml of DI Water and Acetonitrile in the ratio of 65:35 v/v respectively with Isocratic flow rate of 1.0 mL/min and temperature at 40°C .The analytes of this combination had adequate retentions, peak shape, less tailing, more resolution between drug and its degradants and the chromatographic analysis time was about 20 min.

In optimized conditions Amlodipine, Hydrochlorothiazide and their degradants were well separated. Typical retention times of Amlodipine and Hydrochlorothiazide were about 6.627 and 4.040 minutes respectively.

Even the retention time looks low but the separations were achieved to greater extent and the methods prove to be stability indicating. During the initial forced degradation experiments, it was observed that oxidation was

a fast reaction for Amlodipine and Hydrochlorothiazide tablets and almost complete degradation occurred when 5 mL of 30 % H_2O_2 solution was used. Both drugs showed extensive degradation in alkali hydrolytic and oxidative condition and indicating homogeneous peaks and thus establishing the specificity of the Impurity assay method.

Calibration and Linearity

Calibration curve obtained by the least square regression analysis between average peak area and concentration showed linear relationship with a regression coefficient of 0.999 over the calibration ranges tested.

The results of linearity and range obtained for the two potential impurities were tabulated. Linear calibration plot for this chromatographic method was obtained over the calibration ranges tested, i.e. 0.05 % to 1.0 % for Amlodipine impurity-A and 0.05% to 1.0 % for Hydrochlorothiazide impurity-A.

The correlation coefficient obtained was greater than 0.999 for the two impurities and the major compounds Amlodipine and Hydrochlorothiazide (Figure 11 & Figure 12).

The Linearity data of the Amlodipine and Hydrochlorothiazide impurities was presented in the below Table. The method exhibited good linearity with correlation coefficient values greater than 0.999.

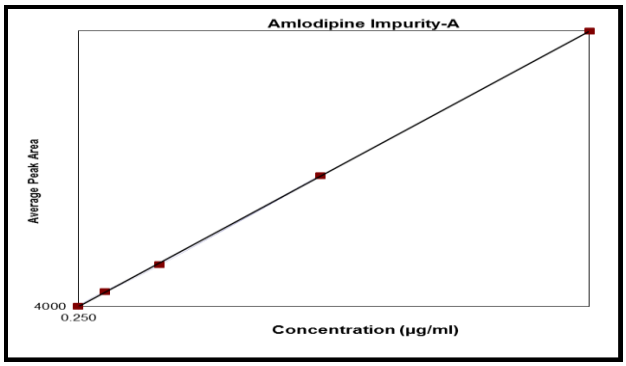


Figure 11: Linearity Plot of Amlodipine Impurity-A

Slope (m) = 15626.286
Intercept=327.00
Correlation Co-efficient (r) = 0.9998237

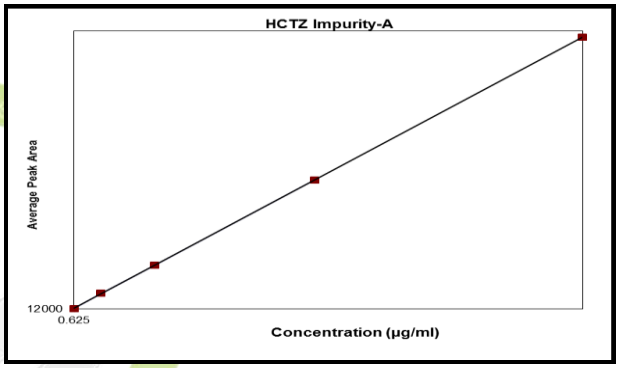


Figure 12: Linearity Plot of Hydrochlorothiazide Impurity-A

Slope (m) = 17966.057

Intercept (c) = 1320.500

Correlation Co-efficient (r) = 0.9999836

Table 2: Results of the Precision Study

% RSD of six (6) replicate injections of each standard should be less than 10.0, theoretical plates should be NLT 1000 and tailing factor should be NMT 2.0 for system precision. % RSD of six (6) sample preparations for each impurity should be less than 10.0 for method precision.	**System Precision**			
	Amlodipine		**Hydrochlorothiazide**	
	% RSD	0.85	% RSD	0.89
	Theoretical Plates (N)	12460.23	Theoretical Plates (N)	617503.71
	Tailing Factor (T)	1.034	Tailing Factor (T)	1.045
	Method Precision 5/12.5 mg			
	Amlodipine Impurity-A % RSD	1.06	Hydrochlorothiazide Impurity-A % RSD	0.83

Table-3: Percentage Recovery results of both analysts at different concentrations

Amlodipine Imp-A					Hydrochlorothiazide Imp-A				
Conc^{n} µg/mL	% Spiking Level	Avg. Peak Area	% Recovery	Mean Recovery %	Concentration µg/mL	% Spiking Level	Average Peak Area	% Recovery	Mean Recovery %
0.25	0.05	4021	94.0	101.5	0.625	0.0	12318	105.2	105.4
2.5	0.5	40215	96.3		6.25	0.5	118205	105.5	
5.0	1.0	80159	101.5		25.0	1.0	472820	105.4	

Table 4: S/N Ratio of Amlodipine, Hydrochlorothiazide and composite Impurities at LOQ (0.05%) Level

Component Name	Actual Conc. (µg/mL)	% RSD	Signal to Noise Ratio (S/N)
Amlodipine Impurity-A	0.25	5.43	75.672
Amlodipine	0.25	0.70	100.156
Hydrochlorothiazide Impurity-A	0.625	0.71	910.987
Hydrochlorothiazide	0.625	0.66	997.428

Precision (Repeatability)

The precision of the method was studied by determining the concentrations of each0.85 and 0.89. The results of the precision study indicate that the method is reliable (RSD% < 10) as shown in Table 2.

Accuracy (Recovery Test)

The percentage recovery was established for all the analytes throughout the range concentration as explained under linearity studies and obtained results are tabulated below in Table-3.

Determination of Limit of Quantification

Prepared Amlodipine and Hydrochlorothiazide LOQ solution as per the method containing the concentration of about 0.25 µg/mL of amlodipine and 0.625 µg/mL of Hydrochlorothiazide. Made five (5) replicate injections and recorded % RSD. Calculated S/N ratio of 0.05 % to establish LOQ in Table 4.

CONCLUSION

The Isocratic RP-LC method developed for the analysis of binary mixtures of Amlodipine and Hydrochlorothiazide in their pharmaceutical preparations is precise, accurate and with less run time. The method was fully validated showing satisfactory data for all the method validation parameters tested. The developed method is stability-indicating, separates degradants and can be conveniently used by the quality control department to determine

Impurity assay of pharmaceutical preparations and also stability sample analysis.

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