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DEVELOPMENT AND VALIDATION OF AN RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF AMLODIPINE BESYLATE AND INDAPAMIDE IN BULK AND TABLET DOSAGE FORM

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Abstract

Objective: *An isocratic reversed-phase liquid chromatograpic assay method was developed for the quantitative determination of amlodipine besylate (AML) and indapamide (IND) in bulk and tablet dosage form.*

Material and method:*A Inertsil C8 (150mm x 4.6mm) column with a mobile phase containing KH_2PO_4 buffer: Acetonitrile (70:30) total pH-adjusted to 3 using o-phosphoric acid was used. The flow rate was 1.0 mL min^{-1}: 1.5 ml/min and effluents were monitored at 242 nm.*

Results and conclusion: *The retention times of both amlodipine besylate and indapamide were 14 minutes. The proposed method was validated with respect to system suitability, Specificity and selectivity, Stability of analytical solutions linearity, accuracy, precision, and robustness. The method was successfully applied to the estimation of amlodipine besylate and indapamide in bulk and tablet dosage form.*

Keywords: *RP-HPLC, Amlodipine, Indapamide, Tablet, Validation*

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INTRODUCTION:

Amlodipine besylate is chemically 3-ethyl 5-methyl 2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-6-methyl-1, 4-dihydropyridine-3, 5-dicarboxylate benzenesulfonate. It is a long-acting calcium channel blocker (dihydropyridine class) used as an anti-hypertensive and in the treatment of angina. It is official in IP, BP. Indapamide is chemically 4-chloro-N-(2-methyl-2, 3-dihydro-1H-indol-1-yl)-3-sulf-amoylbenzamide. It is a non-thiazide sulphonamide diuretic drug, generally used in the treatment of hypertension, as well as decompensated cardiac failure. At present, these drugs are available in combination therapy. The rationale behind use of this drug combination is that in treatment of hypertension in patients whose blood pressure is not adequately controlled by monotherapy. Oral administration of amlodipine besylate and indapamide has been found to be more effective than use of either drug alone. Combination treatment with Amlodipine besylate and indapamide effectively reduces blood pressure in elderly patients with essential hypertension [1]. Literature survey revealed various spectrophotometric, HPTLC and HPLC (Anthony et al 2004) methods have been reported for estimation of AML individually or in combination with other drugs. Different spectrophotometric (Indian Pharmacopoeia 2007) HPTLC and HPLC methods have been reported for estimation of IND individually or in combination with other drugs. To the best of our knowledge, no analytical methods have been reported for analysis of AML and IND in pharmaceutical formulations (Sharma et al 2005). Hence the aim of the present study is to establish an accurate and sensitive RP-HPLC method and, after validation in accordance with International Conference on Harmonization (ICH) guidelines, to use the method for analysis of the drug content of both in tablet dosage form [2].

Chemicals and Reagents

Amlodipine Besilate (Active Pharmaceutical Ingredient) and working standard were supplied by Cadila Healthcare Limited Ankleshwar, India whereas Indapamide (Active Pharmaceutical Ingredient) and working standard were supplied by Ami Lifesciences Limited Baroda, India. Ortho-Phosphoric Acid was obtained from Spectrochem Pvt. Ltd., India. Acetonitrile was obtained from Spectrochem Pvt. Ltd, India. Methanol was obtained from Spectrochem Pvt. Ltd., India. Milli-Q Wateras produced by In-house production of company. Triethylamine was obtained from Spectrochem Pvt. Ltd, India.

Chromatographic System

The HPLC system (Shimadzu Corporation, Japan), model Shimadzu VP, consisted of a sy stem controller (CLASS-VP), on-line degasser (LC 2010C, Shimadzu), low pressure gradient valve (LC 2010C, Shimadzu), solvent delivery module (LC 2010C, Shimadzu), auto injector (LC 2010C, Shimadzu), column oven (LC 2010C, Shimadzu), and CLASS – VP software version = SPI, binary pump, auto injector (SIL-10AD VP, Shimadzu), column oven (CTO-10AS VP, Shimadzu) and PDA detector (PDA-SPD-M10A VP, Shimadzu Diode Array Detector) and Chem station (software).

Parameters for method development with Specifications are given in table 1

Table 1: Parameters for method development

Parameters	Specifications
Stationary Phase	Inertsil C8 (150mm x 4.6mm)
Mobile Phase	KH_2PO_4 buffer (pH 3): Acetonitrilse (70:30)
Diluent	MilliQ Water: Acetonitrile (40:60)
ssFlow rate	1.5 ml/min
Injection volume	10µl
Detection	240 nm
Temperature	30˚C
Run time	14min (Amlodipine, Indapamide)
Buffer	KH_2PO_4 + Triethylamine in milliQ water and its pH 3.0 made by dilute orthophosphoric acid.

Selection Criteria

Working Standard & sample from reliable source in pure form was collected. Solubility was determined of Amlodipine Besylate and Indapamide in appropriate solvent or their mixture of solvents. On the basis of solubility studies and literature survey, the mobile phase composition for further development work was decided. The λmax for Amlodipine Besylate and Indapamide was obtained with the help of UV Spectroscopy. Concentration or µg/ml solution was prepared for standard by help of their label claim mentioned. Label claim for Amlodipine Besylate and Indapamide are 5+ 1.5 mg. Selection of column carried out on the basis of previous work on individual drugs or combination with other drugs, Mainly C-8 & C-18 column. The column was selected on the basis of their retention time, area, peak shape and asymmetry. Isocratic mode for the analysis was decided by primary run on HPLC system. Injection volume was determined on the basis of their symmetry and resolution in chromatogram by several run on HPLC method. Run time was determined on the basis of the retention time of both mentioned components. Optimization was performed by changing the proportion of mobile phase or adjusts the pH of mobile phase, as well as trials made on different grade column. The mobile phase was selected on the basis of resolution, asymmetry, peak shape and area.

Method Development

Amlodipine Besilate and Indapamide showed λ_{max} at 238 nm and 242 nm, respectively. Overlay UV Spectra for Amlodipine Besilate and Indapamide was taken at 240nm. Proper selection of the HPLC method depends upon the nature of the sample (ionic or ionizable or neutral molecule), its molecular weight and solubility. RP-HPLC was selected for the initial separation because of its simplicity and suitability. [3]. To optimize the chromatographic conditions the effect of chromatographic variables such as mobile phase, pH, flow rate and solvent ratio were studied and the chromatographic parameters such as capacity factor, asymmetric factor, and resolution and column efficiency were calculated. The condition was chosen that gave the best resolution and symmetry was selected for estimation. The sensitivity of HPLC method that uses UV detection depends upon proper selection of detection wavelength. An ideal wavelength is the one that gives good response for the drugs that are to be detected [4]. In the present study, standard solution of Amlodipine Besilate and Indapamide were scanned over the range of 200–400 nm wavelengths. The both drugs have shown absorbance maxima nearer 240 nm. So the 240 nm wavelength was selected for simultaneous estimation of Amlodipine Besilate and Indapamide in solid dosage forms. For RP-HPLC method, various columns are available but our main aim to resolve the drugs in the presence of degradation products and other impurities. So the C-8 column was selected over the other columns. For Amlodipine Besilate and Indapamide, Inertsil C-8 Select (150 x 4.6 mm 5.0 µm) column was chosen to give good peak shape and high resolution as compared to other C- 8 columns. This column has an embedded polar group and which are more stable at lower pH and carbon loads, which provide high peak purity and more retention to polar drugs and facilitates the separation of impurity peaks within a very short run time. Stationary Phase-Inertsil C8 (150mm x 4.6mm), Mobile Phase-KH_2PO_4 buffer (pH 3)-Acetonitrilse (70:30), Diluent-Milli Q Water: Acetonitrile (40:60), ssFlow rate -1.5 ml/min, Injection volume-10µl, Detection- 240 nm, Temperature - 30˚C, Run time-14min (Amlodipine, Indapamide), Buffer-KH_2PO_4 + Triethylamine in milliQ water and its pH- 3.0 made by dilute orthophosphoric acid[5].

Method Validation

Validation was done as per ICH guideline Q2 (R1). The developed RP-HPLC methods were validated with respect to parameters such as linearity, precision, accuracy, specificity, ruggedness, robustness and solution stability [6].

System Suitability

System suitability is the checking of a system to ensure system performance before or during the analysis of unknowns. Parameters such as plate count, tailing factors, resolution and reproducibility (% RSD, retention time and area for six repetitions) are determined and compared against the specifications set for the method. These parameters are measured during the analysis. The Assymetry for analyte peak should be not more than (NMT) 1.2 and % RSD of five replicate standared injections should be NMT 2.0 [7].

System Precision

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of homogenous samples. This is usually expressed as the standard deviation or the relative standard deviation (coefficient of variation). Precision is a measure of the degree of reproducibility or of the repeatability of the analytical method under normal operating circumstances [8]. Repeatability involves analysis of replicates by the analyst using the same equipment, method and conducting the precision study over short period of time while reproducibility involves precision study at different occasions, different laboratories, and different batch of reagent, different analysts and different equipments. The Standard Solution is prepared at

working Concentration and analyzed in replicate. The % RSD of five replicate standard injection is NMT 2.0.

Linearity and Range

The linearity of an analytical method is its ability to elicit test results that are directly (or by a well defined mathematical transformation) proportional to the analyte concentration in samples within a given range. Linearity usually expressed in terms of the variance around the slope of regression line calculated according to an established mathematical relationship from test results obtained by the analysis of samples with varying concentrations of analyte [9]. The linear range of detect ability that obeys Beer's law is dependent on the compound analyzed and the detector used. Linearity was determined at five levels over the range of 10% to 150% of test concentration. Standard linearity solutions were prepared to different concentration of 10%, 20%, 50%, 80%, 100%, 120%, and 150% of the test concentration. Each linearity solution was injected in duplicate. The correlation coefficient is should be not less than (NLT) 0.995.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

Limit of Detection

The limit of detection is the parameter of limit tests. It is the lowest level of analyte that can be detected, but not necessarily determined in a quantitative fashion, using a specific method under the required experimental conditions. The limit test thus merely substantiates that the analyte concentration is above or below a certain level [10]. A signal-to-noise ratio of 2:1 or 3:1 is generally accepted. The signal-to-noise ratio is determined by dividing the base peak by the standard deviation of all data points below a set threshold. Limit of detection is calculated by taking the concentration of the peak of interest divided by three times the signal-to-noise ratio. The standard deviation of the intercept (Sa) which may be related to LOD and the slope of the calibration curve, b, by: LOD = 3.3 Sa / b.

Limit of Quantification

Limit of Quantification is a parameter of quantitative assays for low levels of compounds in sample matrices such as impurities in bulk drugs and degradation products in finished pharmaceuticals. The limit of quantification is the lowest concentration of analyte in a sample that may be determined with acceptable accuracy and precision when the required procedure is applied. It is measured by analyzing samples containing known quantities of the analyte and determining the lowest level at which acceptable degrees of accuracy and precision are attainable. The standard deviation multiplied by a factor (usually 10) provides an estimate of the limit of quantification [11]. In many cases, the LOQ is approximately twice the limit of detection. Sa is the standard deviation of the intercept which may be related to LOQ and the slope of the calibration curve, b, by: LOQ = 10 Sa / b.

Stability of Analytical Solution

Stability of the sample, standard and reagents is required for a reasonable time to generate reproducible and reliable results. For example, 24 hour stability is desired for solutions and reagents that need to be prepared for each analysis. System suitability test provide the added assurance that on a specific occasion the method is giving, accurate and precise results [12]. System suitability test are run every time a method is used either before or during analysis. Solution stability period for standard and sample preparation was determined by keeping the solution for 12 hour at room temperature. At interval 2, 4, 6, 8, 10, and 12 hour the solutions were analysed. The insignificant changes (<2%) were observed for the chromatographic responses for the solution analysed, relative to freshly prepared standard. The peak areas of analyte in standard and sample solution not differ by more than 2% from initial peak area for the accepted storage time.

Accuracy

The accuracy of an analytical method may be defined as the closeness of the test results obtained by the method to the true value. It is the measure of the exactness of the analytical method developed. Accuracy may often express as percent recovery by the assay of a known amount of analyte added. Accuracy may be determined by applying the method to samples or mixtures of excipients to which known amount of analyte have been added, both above and below the normal levels expected in the samples. Accuracy is then calculated from the test results as the percentage of the analyte recovered by the assay. The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. The accuracy of the method was carried out at three levels in the range of 50-150% of the working concentration of sample. Calculated amount of Amlodipine besilate and Indapamide working standards were added in placebo containing volumetric flasks to prepare 50%, 100% and 150% level of the working concentration [13]. Each level was prepared in triplicate manner and each preparation was injected in duplicate. The recovery at each level should be 98%-102% and the % RSD NMT 2.0.

Specificity and Selectivity

The selectivity of an analytical method is its ability to measure accurately and specifically the analyte

of interest in the presence of components that may be expected to be present in the sample matrix. If an analytical procedure is able to separate and resolve the various components of a mixture and detect the analyte qualitatively the method is called selective. On the other hand, if the method determines or measures quantitatively the component of interest in the sample matrix without separation, it is said to be specific Specificity is a procedure to detect quantitatively the analyte in the presence of components that may be expected to be present in the sample matrix. While selectivity is the procedure to detect qualitatively the analyte in presence of components that may expected to be present in the sample matrix [14]. Specificity of developed method was established by determining peak purity of active component in standard preparation, test preparation and spiked sample preparation using PDA detector.

Interference from Blank and Placebo

A blank preparation, standard preparation, placebo preparation, sample preparation of Amlodipine besilate and Indapamide and placebo spiked with targeted concentration of both API were prepared and injected. There is no interference from placebo with analyte and peak purity of analyte in sample solution is NLT 0.995.

Robustness and Ruggedness

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variation in method parameters and provides an indication of its reliability during normal usage. The determination of robustness requires that methods characteristic are assessed when one or more operating parameter varied. The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test conditions such as different laboratories, different analysts, using operational and environmental conditions that may differ but are still within the specified parameters of the assay [15]. The testing of ruggedness is normally suggested when the method is to be used in more than one laboratory. Ruggedness is normally expressed as the lack of the influence on the test results of operational and environmental variables of the analytical method. % RSD of five replicate standard injections should be NMT 2.0 [16].

RESULT AND DISCUSSION:

System Suitability

Table 2: System Suitability

Sr. No.	Parameters (n= 5)	Amlodipine Besilate	Indapamide
1	Retention Time (min)	6.123	9.99
2	Theoritical Plates	4353	6380
3	Asymmetry	1.01	0.90
4	% RSD	0.67	0.63

According to above table the all parameters like theoretical plates, assymetry and %RSD was within the limit so system is suitable for method.

System Precision

Table 3: System Precision

System precision Injection	Area	
	Amlodipine besilate (mV*sec)	**Indapamide (mV*sec)**
Injection 1	1733551	662488
Injection 2	1743575	678399
Injection 3	1744042	686980
Injection 4	1758714	676302
Injection 5	1760903	671609
Injection 6	1735062	668345
% RSD	**0.7**	**1.3**

According to table the all parameters like theoretical plates, asymmetry and %RSD was within the limit so system is precise for method.

Linearity

The linearity of developed method was achieved in the range of 10 – 150µg/ml (r^2=1.0) for Amlodipine besilate and 3 – 45µg/ml (r^2=0.99997) for Indapamide, The results show that all validation parameters of method lie within its specific acceptance crieteria.

Table 4 : Linearity Data of Amlodipine Besilate

Linearity Range	Stock solution to be taken in ml	Dilute to volume (ml)with diluents	Final concentration in µg/ml Amlodipine besilate	AUC mV*sec	Response Ratio mV*sec/ (µg/ml)
10%	1.0	50	10.0	2970466	297046.6
20%	2.0	50	20.0	5735302	286765.1
50%	5.0	50	50.0	14334699	286693.98
80%	8.0	50	80.0	23373555	292169.43
100%	10.0	50	100.0	29213733	292137.33
120%	12.0	50	120.0	34833612	290280.10
150%	15.0	50	150.0	43728746	291524.97

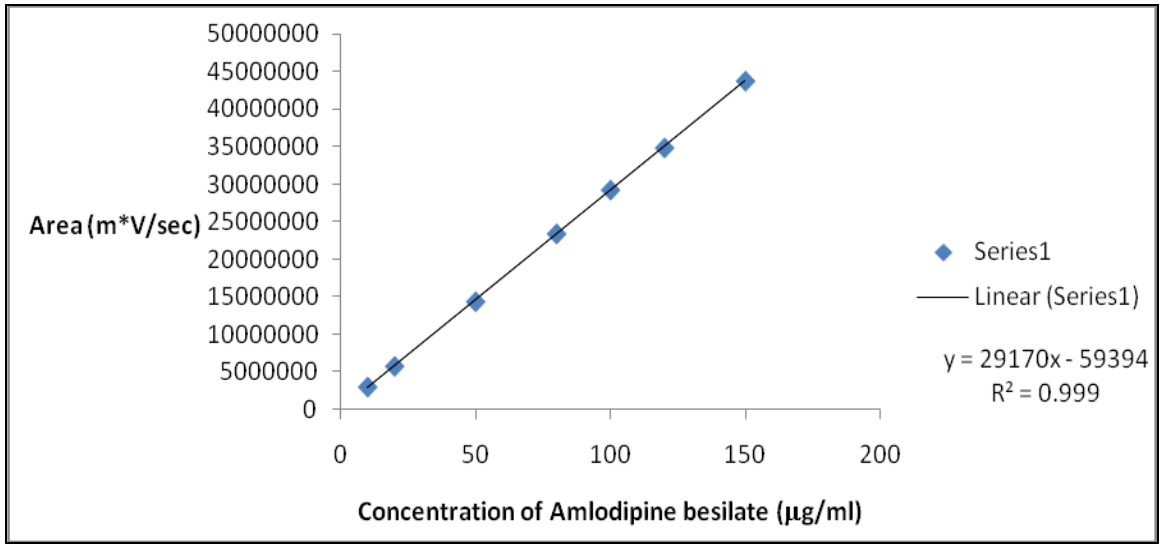

Fig 1 : Linearity Curve for Amlodipine besilate

Table 5: Linearity Data of Indapamide

Linearity Range	Stock solution to be taken in ml	Dilute to volume (ml)with diluents	Final concentration in µg/ml Indapamide	AUC mV*sec	Response Ratio mV*sec/ (µg/ml)
10%	1.0	50	3.0	1211864	404954.66
20%	2.0	50	6.0	2357033	392838.83
50%	5.0	50	15.0	5864778	390985.20
80%	8.0	50	24.0	9596021	39834.20
100%	10.0	50	30.0	11948348	398278.26
120%	12.0	50	36.0	14249657	395823.80
150%	15.0	50	45.0	17843877	396530.6

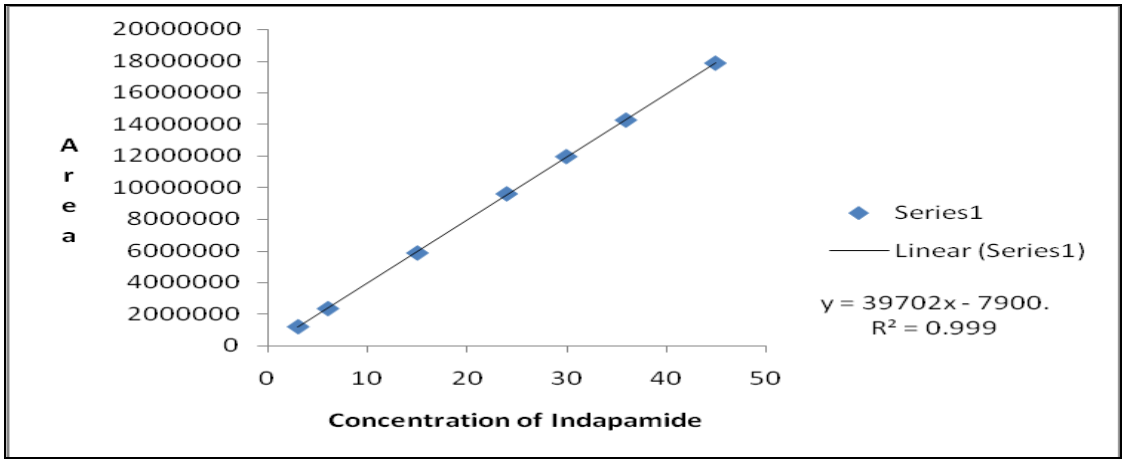

Figure2: Linearity curve of Indapamide

The mean area at each level was calculated and a graph of mean area versus concentration was plotted. The correlation co-efficient, Y intercept and slope of regression line were calculated.

LOD and LOQ

Table 6: LOD and LOQ

Parameters	Amlodipine besilate	Indapamide
Linearity equation	193368,21283x+931.8342	244280.20134x+300.9722
Correlation coefficient	0.0998	0.0999
LOD	0.015µg/ml	0.004µg/ml
LOQ	0.048µg/ml	0.012µg/ml

The above data shows that a micro gram quantity of both drugs can be accurately and precisely determined.

Stability of Analytical Solution

Table 7: Results of Standard Solution Stability

Time (hours)	Area(mV*sec)		% Difference	
	Amlodipine Besilate	Indapamide	Amlodipine besilate	Indapamide
0 (Initial)	1807795	694245	-----	------
2	1816711	693003	0.5	-0.2
4	1816558	696255	0.5	0.3
6	1809207	697248	0.1	0.4
8	1820324	695273	0.7	0.1
10	1817613	697624	0.5	0.5
12	1817853	699094	0.6	0.7

Solution stability lie within its specific acceptance criteria for 12 hrs.

Table 8: Results of Sample Solution Stability

Time (hours)	Area (mV*sec)		% Difference	
	Amlodipine Besilate	Indapamide	Amlodipine besilate	Indapamide
0 (Initial)	1703338	706672	------	------
2	1704216	702701	0.1	-0.6
4	1710046	702277	0.4	-0.6
6	1699414	694231	-0.2	-1.8
8	1728171	711129	1.5	0.6
10	1723531	707694	1.2	0.1
12	1680273	699585	-1.4	-1.0

The solution stability of standard and sample was performed and the percentage difference was not more than 2%.

Precision

Method Precision (Repeatability)

Table 9: Method Precision Data of Amlodipine Besilate and Indapamide

Set No.	% Assay		% Assay Mean		%RSD	
	Amlodipine	Indapamide	Amlo.	Inda.	Amlo.	Inda.
1	102.9	101.1				
2	100.8	101.5	101.1	100.7	1.20	1.7
3	102.3	103.6				
4	100.1	99.9				
5	100.2	99.8				
6	100.3	98.5				

Individual % assay, mean % assay and % RSD were calculated. The % RSD is 0.6 for Amlodipine besilate & 0.1 for Indapamide which indicate that the method is precise.

Intermediate Precision (Ruggedness)

Table 10: Intermediate Precision Data of Amlodipine besilate and Indapamide

Set No.	% Assay		% Assay Mean		%RSD	
	Amlodipine	Indapamide	Amlo.	Inda.	Amlo.	Inda.
1	100.1	97.5				
2	100.9	99.5				
3	100.7	100.4	100.6	99.3	0.4	1.0
4	101.2	100.2				
5	100.3	99.2				
6	100.6	99.3				

Individual % assay, mean % assay and % RSD were calculated and recorded in Table10. The % RSD is 0.4 for Amlodipine besilate & 1 for Indapamide which indicate that the method is rugged.

Specificity and Selectivity

Table 11: Peak Purity in Specificity Study of Amlodipine besilate and Indapamide

Sample	% Assay		Peak purity	
	Amlodipine Besilate	Indapamide	Amlodipine Besilate	Indapamide
Standard Solution	100.50	98.21	0.9960	0.9998
Test Solution	101.87	98.42	0.9976	0.9985
Spiked Sample	101.65	98.03	0.9989	0.9978

The peak purity index for the main peak in all the standard preparation, sample and placebo preparation was determined there is no interference in main peak.

Robustness

Table 12: Robustness

Compound	% RSD (n= 5)		
	Normal Condition	Changed Condition	
Temperature	**Normal**	**(-5°C)**	**(+5°C)**
Amlodipine besilate	0.10	0.5	1.9
Indapamide	0.10	0.9	1.6
pH	**Normal**	**(-0.2 unit)**	**(+0.2 unit)**
Amlodipine besilate	0.10	0.5	0.6
Indapamide	0.10	0.7	0.9
Flow Rate	**Normal**	**(-10%)**	**(+10%)**
Amlodipine besilate	0.10	0.3	0.3
Indapamide	0.10	1.3	1.8
Mobile phase ratio	**Normal**	**(-2%)**	**(+2%)**
Amlodipine besilate	0.10	0.7	0.4
Indapamide	0.10	0.6	1.4

The low % RSD value (< 2%) reveals that the proposed method is robust for this variation. The Summary of validation parameters is given in table 13.

Table 13: Summary of validation parameters by RP-HPLC method

Parameter	Acceptance Crieteria	Amlodipine Besilate	Indapamide
System Precision	RSD < 2%	0.7	1.3
Solution Stability	> 12 hour	Complies	Complies
Linearity Range Correlation Coefficient	Correlation coefficient r^2 > 0.999 or 0.995	10 – 150µg/ml r^2 = 0.999	3.75-45µg/ml r^2 = 0.999
LOQ	S/N > 10	0.048µg/ml	0.012µg/ml
LOD	S/N > 2 or 3	0.015µg/ml	0.004µg/ml
Precision (Repeatability)	RSD < 2%	%RSD = 0.15	%RSD = 0.15
Method Precision	RSD < 2%	%RSD = 0.5	% RSD = 0.7
Accuracy/ Recovery	RSD< 2%	Complies	Complies
Robustness	RSD NMT 2% in modified condition	Complies	Complies
System Suitability	RSD < 2% Theoritical plates< 2000 Asymmetry is 0.9 to 1.2	0.67 4353 1.01	0.63 6380 0.90

CONCLUSION:

This developed and validated method for simultaneous analysis of amlodipine besylate and indapamide in pharmaceutical preparations is very simple, rapid, accurate and precise. The method was successfully applied for determination of AML and IND in its pharmaceutical formulations. Moreover, it has advantages of short run time and the possibility of analysis of a large number of samples, both of which significantly reduce the analysis time per sample. Hence, this method can be conveniently used for routine quality control analysis of AML and IND in their pharmaceutical formulations.

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