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Development and Validation of Novel RP-HPLC Method for the Simultaneous Estimation of Amlodipine and Hydrochlorothiazide in Combined Dosage Form

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ABSTRACT

A simple, specific, accurate, rapid, inexpensive Reversed Phase High Performance liquid chromatographic method has been developed and validated for the simultaneous determination of Amlodipine and Hydrochlorothiazide in pharmaceutical tablet dosage form. Separation was achieved using a C18 column with mobile phase consisting of Phosphate Buffer (pH 3.4) and Methanol (55:45 v/v) in isocratic mode at 1 ml/min flow rate. Column effluent was monitored at 238 nm using a UV detector. The retention time for Amlodipine and Hydrochlorothiazide was found to be 2.7 and 3.4 min respectively the method was validated for System Suitability, Specificity, linearity, accuracy, precision, robustness. The method demonstrated excellent linearity for Amlodipine and Hydrochlorothiazide with regression coefficients of 0.9999 and 0.9998, respectively. Linearity was established for Amlodipine besylate in the range of 20-80 µg / mL and Hydrochlorothiazide in the range of 50-200 µg / ml. The accuracy of the method is evaluated in triplicate at three concentration levels i.e. 50%, 100% and 150% of target test concentration. The method was found to be sensitive with quantification limits of 1.05 and 2.82 µg/ml for Amlodipine and Hydrochlorothiazide. The method was successfully employed for the determination of Amlodipine and Hydrochlorothiazide in commercially available tablets.

Keywords: Amlodipine, Hydrochlorothiazide, RP-HPLC, UV-Vis Detector, Validation

INTRODUCTION

AML is chemically as (RS)-3-ethyl 5-methyl 2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-6-methyl-1, 4- dihydropyridine-3, 5-dicarboxylate [figure 1]. Amlodipine is a dihydropyridine with calcium antagonist activity. It is used with or without other medications to treat high blood pressure. Lowering high blood pressure helps prevent strokes, heart attacks, and kidney problems. It works by relaxing blood vessels so blood can flow more easily. It is official in BP. HCTZ chemically is 6-chloro- 1,1- dioxo- 3,4-dihydro-2*H*- 1,2,4-benzothiadiazine -7- sulfonamide (Figure 2) is a first-line diuretic drug, which acts by inhibiting the kidneys' ability to retain water. It is frequently used in the treatment of hypertension, congestive heart failure, symptomatic edema, diabetes insipidus, renal tubular acidosis, and for the prevention of kidney stones [1-5] Extensive literature survey did not reveal a simple, selective and sensitive analytical method for simultaneous determination of AML and HCTZ. Most of the methods are reported for the determination of either AML or HCTZ, separately. Literature survey revealed the availability of several methods for determination of both AML and HCTZ includes UV [6-8] and HPLC [9-13] as alone or in combination with other drugs. However, several reported methods are developed for biological applications such as serum, plasma, urine, bile and other tissues. Moreover, most of the methods either demonstrate limitations such as poor sensitivity, selectivity, repeatability or use

sophisticated analytical techniques such as HPTLC, [14-17] thus making them unsuitable for routine analysis. Present work emphasizes on the determination of AML and HCTZ in combined dosage form by RP-HPLC.

MATERIALS AND METHODS

AML (assay 99.95%), HCTZ (assay 99.95%) were kindly gifted by NATCO Laboratories Ltd., India. Potassium dihydrogen phosphate, HPLC-grade solvents, methanol, were purchased from Spectrochem, India. All other chemicals used in the analysis were of analytical-reagent grade. One commercially available tablet of AML and HCTZ was selected from local Indian market.

Chromatographic system and conditions

An HPLC system (Waters Model NO.2690/5 series Compact System) Consisting of Inertsil-C18 ODS column and LABINDIA UV 30000+ UV/Vis detector was used for chromatographic determinations. Optimised mobile phase consisting of Phospate buffer (pH 3.4) and methanol in the ratio of (55:45, v/v) was degassed under vacuum and delivered in isocratic elution mode at a flow rate of 1 ml/ min. The chromatographic separations were carried out using Inertsil-C18 ODS column (250×4.6 mm, 5 µm, 100 Å). The quantification was carried out using UV-detector at 238 nm with injection volume of 20 µl. All experiments were carried out at ambient temperature after baseline stabilisation.(Table 1)

Stock solutions and standards

Stock solutions of 100µg/ml and 250 µg/ml were prepared by dissolving 10 mg of AML and 25 mg of HCTZ in Mobile phase 100 mL volumetric flask and sonicated for 20min.Secondary stock solution of 10 and 25 µg/ml were prepared by diluting 1.0 ml of each standard stock solutions to 10 ml in mobile phase for AML and HCTZ, respectively.

Preparation of sample Solution

Twenty tablets of AMLONG-H (containing 12.5mg of HCTZ and 5mg of AML) were weighed accurately and a quantity of tablet powder equivalent to 25mg Hydrochlorothiazide and 10 mg Amlodipine was weighed and dissolved in the 70 mL mobile phase with the aid of ultrasonication for 20 min. The content was diluted to 100 mL with mobile phase. This solution was filtered through a 0.45 µm Nylon syringe filter. The filtrate was appropriately diluted with mobile phase for further analysis.

Method validation

The developed analytical method was validated as per ICH guidelines and the studied parameters are presented below.

Linearity

A Series of solutions of HCTZ (50µg/ml to 200 µg/ml) and AML (20µg/ml to80 µg/ml) were prepared. The calibration curves were obtained by plotting mean peak area against the concentration using linear regression analysis [Table 2]

Determination of AML and HCTZ in tablets

The proposed method was employed for the determination of AML and HCTZ drug content in real world samples such as marketed tablet formulation (AMLONG_H; Micro labs Pvt. Ltd., Karnataka, India containing 5 mg of AML and 12.5 mg of HCTZ). For the estimation of drug content in tablet, the average weight of twenty tablets was noted, tablets were powdered and mixed uniformly. A quantity of powder equivalent to one tablet was accurately weighed and processed as per procedure described in sample standards section. Finally, 100µg/ml of HCTZ and 40µg/ml of AML of resulting solution was injected in triplicates and analysed by proposed method. The mean drug content for both the drug was determined using calibration.

System suitability

For system suitability study, six replicate injections of a mixed Standard solution (100µg/ml of HCTZ and 40µg/ml of AML) was prepared and injected and capacity factor (K′), asymmetry factor (A_s), number of theoretical plates (N), height equivalent

to theoretical plates (HETP) and resolution (R_s) were calculated for both the drugs.

SPECIFICITY:

Solutions of mixed standard and sample were prepared and are injected into chromatographic system.[Table 4]

Parameter	Chromatographic conditions	
Instrument	Waters Model NO.2690/5 series	
Column	Insertial C18 Column (4.6 X 250 mm, 5 µm)	
Detector	LABINDIA UV 30000+ UV/Vis detector	
Diluents	Phosphate Buffer (pH-3.4): Methanol (55 : 45 v/v)	
Mobile phase	Phosphate Buffer (pH-3.4): Methanol (55 : 45 v/v)	
Flow rate	1 mL.min^{-1}.	
Detection wave length	By UV at 238 nm.	
Run time	6 minutes	
Column back pressure	155-158 kgf	
Temperature	Ambient temperature (25^{o}C)	
Volume of injection loop	20 µL	
Retention time (R_t)	Amlodipine	Hydrochlorothiazide
	2.790 minutes	3.48 minutes
Theoretical plates per meter [t.p / m]	8742.60	5941.25
Tailing factor (asymmetry factor)	1.109	1.238

Table 2: Linearity Data of the Proposed HPLC Method of Amlodipine and Hydrochlorothiazide

S. No	Concentration, µg/ml		Peak area.	
	AML	HCT	AML	HCT
1	20	50	412977	523467
2.	30	75	605369	829544
3.	40	100	857564	1139272
4.	50	125	1007428	1448018
5.	60	150	1210925	1728926
6.	70	175	1409560	2089505
7	80	200	1627087	2407574

Table 3: Assay Results of Amlodipine and Hydrochlorothiazide Formulation

S.No	Formulation	Labeled amount (mg)		Amount found (mg)		% Assay ± RSD*	
1	Amlong-H	HCT	AML	HCT	AML	HCT	AML
		12.5	5	12.76	5.39	102.13 ± 1.6	107.89 ± 2.5

* Average of 6 determinations.

Table 4: Specificity Study

S. No	Name of the solution	Retention time (R_t) minutes
1	Mixed standard solution of Hydrochlorothiazide (100 µg) and Amlodipine 40µg)	2.789min and 3.480min for Hydrochlorothiazide and Amlodipine Respectively.
2	Sample Solution containing Hydrochlorothiazide (100 µg) and Amlodipine 40µg)	2.789min and 3.480min for Hydrochlorothiazide and Amlodipine Respectively.

Table 5: Results of Precision Study

S. No	Validation parameter	% RSD (acceptance criteria < 2.0)	
		Amlodipine besylate	Hydrochlorothiazide
1	System precision	0.223	0.562
2	Method precision	0.251	0.562

Table 6: Recovery Data of the Amlodipine and Hydrochlorothiazide Proposed by RP-HPLC Method

S. No	Concentration level	% Recovery		Mean % Recovery ± SD*		% RSD	
		AML	HCT	AML	HCT	AML	HCT
1	50%	89.36	99.11	91.12 ± 2.01	97.56 ± 1.34	0.552	0.137
		90.68	96.72				
		93.31	96.84				
2	100%	97.16	89.83	96.60 ± 0.8	95.54 ± 4.98	0.12	0.417
		95.99	97.84				
		96.66	98.97				
3	150%	99.72	98.69	98.93 ± 0.5	98.96 ± 0.32	0.008	0.003
		99	99.4				
		98.06	98.79				

*SD is standard deviation # % RSD is percentage of relative standard deviation.

Table 7: Robustness Results of Amlodipine and Hydrochlorothiazide

S.No	Parameter	Optimized	Used	Peak asymmetry		Remark
				s	HCT	
1	Flow rate (± 0.2 mL.min^{-1})	1.0 mL.min^{-1}	0.8 ml / min	0.77	0.424	Robust
			1.2 mL / min	0.96	0.008	

Table 8: Limit of Detection (LOD) and Limit of Quantitation (LOQ)

Drugs	Limit of Detection (LOD)	Limit of Quantitation (LOQ)
Amlodipine	0.34 µg / mL	1.05 µg / mL
Hydrochlorothiazide	0.85µg/mL	2.82 µg / mL

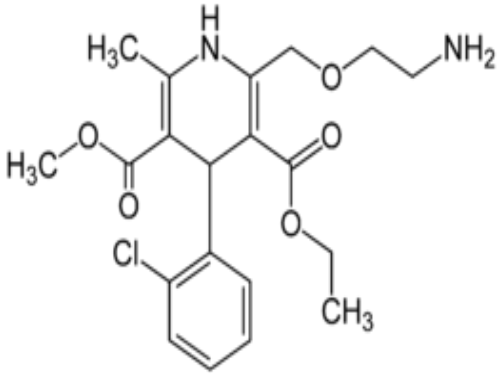

Figure 1: Structure of Amlodipine

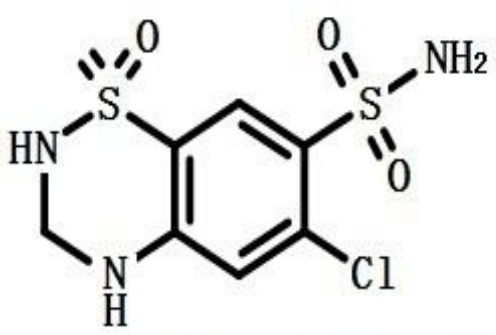

Figure 2: Structure of Hydrochlorothiazide

Precision

System and Method precision study of HCTZ and AML was carried out. For system precision Mixed Standard solution (100µg/ml of HCTZ and 40µg/ml of AML) was injected six times and for method precision Sample solution of (100µg/ml of HCTZ and 40µg/ml of AML) was injected six times. The percent relative standard deviation (% RSD) was calculated which is within the acceptable criteria of not more than 2.0. The results for System and Method precision were presented in Table 5.

Accuracy

Accuracy of the method was evaluated by performing recovery experiments. Drug Assay was performed in triplicate as per test method with equivalent amount of HCTZ and AML into each volumetric flask for each spike level to get the concentration of HCTZ and AML equivalent to 50%, 100%, and 150% of the labelled amount as per the test method and the percentage recoveries were calculated using the fresh calibration curve. The mean percentage recovery of HCTZ and AML at each level was not less than 99 % and not more than 101%. The results were presented in Table 6.

Robustness

The Robustness was evaluated by the analysis of HCTZ and AML under different experimental conditions such as making small changes in flow rate (± 0.2 ml / min).The results were presented in Table 7

Sensitivity

The sensitivity of the method was determined by calculating the limit of detection (LOD) and limit of quantification (LOQ). The standard deviation of intercept (σ) and slope of calibration curve (s) were used for the calculation of LOD (3.3 σ s^{-1}) and LOQ (10 σ s^{-1}) for both the drugs. The results were presented in [Table 8]

RESULTS AND DISCUSSIONS

The mobile phase consisting of 10 mM phosphate buffer (pH-3.4):Methanol (55 : 45 % v/v at 1 mL.min^{-1} flow rate was optimized which gave sharp peak, minimum tailing factor with short runtime for HCTZ and AML. The retention time for HCTZ and AML were found to be 2.79 minutes and 3.48 minutes respectively. System suitability parameters and optimized chromatographic conditions are shown in Table1. The calibration curve for AML was found to be linear over the range of 20-80 µg / mL with

correlation coefficient 0.9999 and HCTZ was found to be linear over the range of 50-200 µg / ml with correlation coefficient 0.9998. The data of the calibration is shown in Table 2. The developed method was applied to the assay of HCTZ and AML tablets. The experimental results are given in Table 3. The results were very close to labeled value of commercial tablets. The representative standard and sample chromatograms of HCTZ and AML are shown in Figure 4 and 5 respectively. The regression equation for HCTZ was found to be y = 31282x + 11218 with correlation coefficient is R^2 = 0.9999 and for AML was y = 20193x + 1902 with correlation coefficient is r^2 = 0.9998 which indicates this method has good linearity. The linearity of the graphs is shown in Figure 6 and Figure 7. The specificity was studied for the examination of the presence of interfering components, while the comparison of chromatograms there was no interference from Standard (Figure 3) with sample

They do not disturb the elution or quantification of AML and HCTZ furthermore the well-shaped peaks also indicate the specificity of the method. Therefore, it was concluded that the method is specific. The specificity results are summarized in Table 4. Precision was studied to find out system and method variations in the test methods of HCTZ and AML. The % RSD (< 2.0) indicates that the proposed method is quite precise and reproducible. The method precision was done and the low % RSD values indicates that the proposed method which was in good agreement with precision and results are shown in Table 5. Recovery studies of the drug were carried out for the accuracy parameter at three different concentrations levels i.e. multiple level recovery studies. A known amount of standard was added into pre-analyzed sample and subjected them to the proposed HPLC method. The % recovery was found to be within the limits as listed in Table 6. Generally the mean percentage recovery of HCTZ and AML at each level was not less than 99 % and not more than 101 %. Robustness was done by small changes in the chromatographic conditions like mobile phase flow rate. It was observed that there were no marked changes in the chromatograms. In fact the parameters are within the limit which indicates that the method has robustness and suitable for routine use. The Robustness results are presented in Table 7. The limit of detection (LOD) and limit of quantitation (LOQ) was calculated based on the standard deviation (SD) of the response and the slope (S) of the calibration curve at levels approximating the LOD and LOQ. The limit of detection (LOD) of HCTZ and AML was 0.85µg / mL and 0.34µg / mL respectively and the limit of quantitation (LOQ) of HCTZ and AML 2.82µg / mL and 1.05 µg / mL respectively which shows that this method is very sensitive.

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