DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF AMLODIPINE AND HYDROCHLOROTHIAZIDE IN TABLETS: APPLICATION TO DISSOLUTION STUDY

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ABSTRACT

A simple, specific, accurate, rapid, inexpensive isocratic Reversed Phase-High Performance Liquid Chromatography (RP-HPLC) method was developed and validated for the quantitative determination of Amlodipine besylate and Hydrochlorothiazide in pharmaceutical tablet dosage forms and HPLC method was used for an *in vitro* dissolution study of tablets containing the above drugs. RP-HPLC method was developed by using Welchrom C₁₈ Column (4.6 X 250 mm, 5 µm), Shimadzu LC-20AT Prominence Liquid Chromatograph. The mobile phase composed of 10 mM Phosphate buffer (pH-3.0, adjusted with triethylamine): acetonitrile (50 : 50 v/v). The flow rate was set to 1.0 mL.min⁻¹ with the responses measured at 230 nm using Shimadzu SPD-20A Prominence UV-Vis detector. The dissolution test was performed using 900 mL of distilled water as the dissolution media at 50 rpm using an USP Apparatus II at 37°C. The retention times of Hydrochlorothiazide and Amlodipine besylate were found to be 3.373 minutes and 3.753 minutes respectively. Linearity was established for Amlodipine besylate in the range of 1-5 µg / mL with correlation coefficient 0.9995 and Hydrochlorothiazide in the range of 3-15 µg / mL with correlation coefficient 0.9996. The validation of the developed method was carried out for specificity, linearity, precision, accuracy, robustness, limit of detection, limit of quantitation. The developed method can be used for routine quality control analysis of Amlodipine besylate and Hydrochlorothiazide in pharmaceutical tablet dosage form.

Keywords: Amlodipine besylate, Hydrochlorothiazide, Isocratic RP-HPLC, UV-Vis detector, Method Validation.

INTRODUCTION

Amlodipine (as besylate), chemically is 3-Ethyl 5-methyl (4RS)-2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate benzenesulphonate¹ (Figure 1). Amlodipine is a dihydro pyridine derivative with calcium antagonist activity². It is used in the management of hypertension, angina pectoris and Prinzmetal variant angina³. Amlodipine acts by inhibiting the transmembrane influx of calcium ions into vascular smooth muscle and cardiac muscle and also acts directly on vascular smooth muscle to cause a reduction in peripheral vascular resistance and reduction in blood pressure. It is official in BP⁴. Hydrochlorothiazide, chemically is 6-Chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulphonamide 1,1-dioxide (Figure 2), is a diuretic, which inhibits active chloride re absorption at the early distal tubule via the NaCl co-transporter, resulting in an increase in the excretion of sodium, chloride and water. Literature survey reveals few analytical methods for the determination of Amlodipine alone and in combination with other drugs in pharmaceutical preparations and biological fluids, viz. Spectrophotometry⁵⁻⁸, HPTLC⁹⁻¹³ and HPLC¹⁴⁻¹⁸. Also there are some analytical methods reported for determination of HCT alone and in combination. No method has been reported for the estimation of Amlodipine (AMLO) and Hydrochlorothiazide (HCT) in combined dosage form with its application to *in vitro* dissolution. The reported HPLC methods so far in the literature are considered to be uneconomical, time consuming and have poor symmetry. In fact there is a need for the development of a novel, simple, rapid, efficient RP-HPLC analytical method with reproducibility for determination of Amlodipine Besylate and Hydrochlorothiazide in bulk and pharmaceutical dosage forms. The present study illustrates development and validation of a novel, simple, rapid and efficient RP-HPLC analytical method with reproducibility

for determination of Amlodipine Besylate and Hydrochlorothiazide in bulk and pharmaceutical tablet dosage form. The established method was validated with respect to specificity, linearity, precision, accuracy, robustness, LOD and LOQ according to ICH guidelines.

MATERIALS AND METHODS

Chemicals and Reagents

The reference samples of Amlodipine besylate and Hydrochlorothiazide standards were kindly supplied as gift samples by Hetero Drugs Ltd., Hyderabad, Andhra Pradesh, India. All the chemicals were analytical grade. Potassium dihydrogen orthophosphate and phosphoric acid from Rankem Ltd., Mumbai, India, while acetonitrile (HPLC grade) and triethylamine (HPLC grade) from Merck Pharmaceuticals Private Ltd., Mumbai, India. Ortho phosphoric acid used was of HPLC grade and purchased from Merck Specialties Private Ltd., Mumbai, India. Commercial tablets AMLONG-H were procured from local market.

Instruments

Quantitative HPLC was performed on a isocratic high performance liquid chromatograph (Shimadzu LC-20AT Prominence Liquid Chromatograph) with a LC-20AT VP pump, manual injector with loop volume of 20 µL (Rheodyne), programmable variable wavelength Shimadzu SPD-20A Prominence UV-Vis detector and Welchrom C₁₈ Column (4.6 X 250 mm, 5 µm particle size). The HPLC system was equipped with "Spinchrome" software. In addition an electronic balance (Shimadzu TX223L), digital pH meter (Systronics model 802), a sonicator (spectra lab, model UCB 40), UV-Visible Spectrophotometer (Systronics model-2203) were used in this study.

Chromatographic Conditions

Amlodipine Besylate and Hydrochlorothiazide were analyzed by various reversed phase columns like C₈ and C₁₈ columns. Among C₈ and C₁₈ columns, C₁₈ (250 mm X 4.6 mm, 5 µm) column was selected. Various combinations of acetonitrile, phosphate buffer and methanol with triethylamine as column modifier were tested. The mixture of 10 mM Phosphate buffer (pH adjusted to 3.0 using triethylamine) and Acetonitrile in ratio of 50 : 50 v/v was selected as mobile phase and UV detection wavelength was 230 nm with a flow rate of 1 mL.min⁻¹. Injection volume was 20 µL, with ambient temperature, run time was 6 minutes.

Preparation of Mobile Phase

A 10 mM Phosphate buffer was prepared by dissolving 6.056 g of potassium dihydrogen orthophosphate in 445 mL of HPLC grade water. To this 55 mL of 0.1 M phosphoric acid was added and pH was adjusted to 3.0 with triethylamine. The above prepared buffer and acetonitrile were mixed in the proportion of 50 : 50 v/v and was filtered through 0.45 µm nylon membrane filter and degassed by sonication.

Preparation of Standard Solution

About 100 mg of pure Amlodipine besylate and Hydrochlorothiazide were accurately weighed and separately dissolved in 100 mL of mobile phase to get 1 mg.mL⁻¹ stock solution. Working standard solutions were prepared with mobile phase. The final volume was made with the mobile phase. The standard solutions were filtered through 0.45 µm nylon membrane filter and degassed by sonication.

Preparation of Sample Solution

The content of 20 tablets of Amlong-H were accurately weighed and transferred into a mortar and ground to a fine powder. From this, tablet powder which is equivalent to one tablet was taken and the drug was extracted in 10 mL of mobile phase. The resulting solution was filtered using Whatman Grade No.1 filter paper and degassed by sonication. This solution was further suitably diluted for chromatography.

Calibration Curve for Amlodipine Besylate and Hydrochlorothiazide

Replicates of each calibration standard solutions (1-5 µg / mL for Amlodipine and 3-15 µg / mL for Hydrochlorothiazide) were injected using a 20 µL fixed loop system and the chromatograms were recorded. Calibration curves were constructed by plotting concentration on X-axis and peak areas on Y-axis and regression equations were computed. The calibration data is presented in Table 2.

In vitro Dissolution Studies

In vitro dissolution of six tablets containing AML and HCT was performed using 900 mL volume distilled water as the dissolution media at 50 rpm using an USP Apparatus II. The dissolution study was carried out in a 900 mL volume of distilled water as the dissolution media at 37°C (± 0.5) using the paddle method. 5 mL sample aliquots were withdrawn at 10, 20, 30, 45, 60 and 75 minutes using micropipettes and immediately replaced with equal volumes of fresh medium at the same temperature to

maintain constant total volume during the test. All samples were filtered through 0.45 µm membrane filters. The concentrations of AML and HCT in samples were determined by the proposed HPLC method.

Validation of the Proposed Method

The developed method of analysis was validated as per the ICH guidelines for the parameters like system suitability, specificity, linearity, precision, accuracy, robustness and system suitability, limit of detection (LOD) and limit of quantitation (LOQ).

System Suitability

System suitability tests are an integral part of chromatographic method which was used to verify reproducibility of the chromatographic system. To ascertain its effectiveness, certain system suitability test parameters were checked by repetitively injecting the drug solution at the concentration level 10 µg mL⁻¹ for Amlodipine besylate and Hydrochlorothiazide to check the reproducibility of the system. At first the HPLC system was stabilized for 40 minutes. One blank followed by six replicates of a single calibration standard solutions of Amlodipine besylate and Hydrochlorothiazide were injected to check the system suitability. To ascertain the systems suitability for the proposed method, the parameters such as theoretical plates, peak asymmetry, retention time and parameters were taken and results were presented in Table 1.

Specificity

The effect of wide range of excipients and other additives usually present in the formulation of Amlodipine Besylate and Hydrochlorothiazide in the determinations under optimum conditions was investigated. The specificity of the RP-HPLC method was established by injecting the mobile phase and placebo solution in triplicate and recording the chromatograms. The common excipients such as lactose anhydrous, microcrystalline cellulose and magnesium stearate have been added to the placebo solution and injected and tested. The representative chromatogram of placebo was shown in Figure 3. The specificity results were presented in Table 4.

Linearity

The linearity graphs for the proposed assay methods were obtained over the concentration range of 1-5 µg / mL for Amlodipine besylate and 3-15 µg / mL for Hydrochlorothiazide. A calibration curve was plotted between concentration and area response and statistical analysis of the calibration curve is shown in Figure 6 and 7.

Precision

Intraday and interday precision study of Amlodipine besylate and Hydrochlorothiazide was carried out by estimating corresponding responses 3 times on the same day and on 3 different days for the concentration of 10 µg. The percent relative standard deviation (% RSD) was calculated which is within the acceptable criteria of not more than 2.0. The results for intraday and interday precision were presented in Table 5.

Accuracy (Recovery Studies)

The accuracy of the method was determined by calculating recovery of Amlodipine besylate and Hydrochlorothiazide by the method of addition. Known amount of Amlodipine

besylate and Hydrochlorothiazide at 80 %, 100 % and 120 % was added to a pre quantified sample solution. The recovery studies were carried out in the tablet in triplicate each in the presence of placebo. The mean percentage recovery of Amlodipine besylate and Hydrochlorothiazide 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 Amlodipine besylate and Hydrochlorothiazide under different experimental conditions such as making small changes in flow rate (± 0.2 ml / min), detection wavelength (± 5 nm), Mobile phase composition (± 5 %). The results were presented in Table 7.

LOD and LOQ

Limit of Detection is the lowest concentration in a sample that can be detected, but not necessarily quantified under the stated experimental conditions. The limit of quantitation is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy.

Limit of Detection and Limit of Quantitation were calculated using following formula

$$\text{LOD} = 3.3(\text{SD})/S \text{ and } \text{LOQ} = 10 (\text{SD})/S,$$

Where SD = standard deviation of response (peak area) and
S = slope of the calibration curve.

The LOD and LOQ values are presented in Table 8.

In vitro Dissolution Studies

The average percentage drugs released within 75 minutes as detected by the proposed HPLC method after *in vitro* dissolution of tablets containing combination drug product are depicted in Figure 14. The dissolution pattern complies with the FDA standards, indicating suitability of the proposed method for the dissolution study of the two drugs. According to the FDA Guidance (Qui, Xu 2007) no less than 85 % of the active ingredients of the labeled claim should be dissolved within 30 minutes. Dissolution values are presented in Table 9. The representative chromatograms of dissolution are shown in Figure 8 to Figure 13.

Table 1: Optimized Chromatographic Conditions and System Suitability Parameters for Proposed HPLC Method for Amlodipine Besylate and Hydrochlorothiazide

Parameter	Chromatographic conditions	
Instrument	SHIMADZU LC-20AT prominence liquid chromatograph	
Column	WELCHROM C ₁₈ Column (4.6 X 250 mm, 5 μ m)	
Detector	SHIMADZU SPD-20A prominence UV-Vis detector	
Diluents	10mM Phosphate Buffer (pH-3): Acetonitrile (50 : 50 v/v)	
Mobile phase	10mM Phosphate Buffer (pH-3): Acetonitrile (50 : 50 v/v)	
Flow rate	1 mL.min ⁻¹ .	
Detection wave length	By UV at 230 nm.	
Run time	6 minutes	
Column back pressure	155-158 kgf	
Temperature	Ambient temperature (25°C)	
Volume of injection loop	20 μ L	
Retention time (R _t)	Amlodipine Besylate	Hydrochlorothiazide
	3.790 minutes	3.373 minutes
Theoretical plates [th.pl] (Efficiency)	10595	9850
Theoretical plates per meter [t.p / m]	211892	197005
Tailing factor (asymmetry factor)	1.182	1.150
Parameter	Chromatographic condition	
Instrument	SHIMADZU LC-20AT Prominence liquid chromatograph	
Column	WELCHROM C ₁₈ Column (4.6 X 250 mm, 5 μ m)	

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

S. No	Concentration, $\mu\text{g.mL}^{-1}$		Peak area, mV.s.	
	AML	HCT	AML	HCT
1	0	0	0	0
2.	1	3	675.912	94.581
3.	2	6	1278.224	176.527
4.	3	9	1896.011	258.80
5.	4	12	2492.378	343.79
6.	5	15	3091.202	425.495

Table 3: Assay Results of Amlodipine Besylate and Hydrochlorothiazide Formulation

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

* Average of 6 determinations.

Table 4: Specificity Study

S. No	Name of the solution	Retention time (R _t) minutes
1	Mobile phase	No peaks
2	Placebo	No peaks
3	Solution containing Hydrochlorothiazide (15 μ g) and Amlodipine (5 μ g)	3.373 minutes and 3.753 minutes for Hydrochlorothiazide and Amlodipine respectively.

Table 5: Results of Precision Study

S. No	Validation parameter	% RSD (acceptance criteria < 2.0)	
		Amlodipine besylate	Hydrochlorothiazide
1	Intraday precision	1.4957	1.4914
2	Interday precision	1.6024	1.2584

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

S. No	Concentration level	% Recovery		Mean % Recovery $\pm$ SD*		% RSD #	
		AML	HCT	AML	HCT	AML	HCT
1	80 %	89.36	99.11	91.12 $\pm$ 2.01	97.56 $\pm$ 1.34	0.552	0.137
		90.68	96.72				
		93.31	96.84				
2	100 %	97.16	89.83	96.60 $\pm$ 0.8	95.54 $\pm$ 4.98	0.120	0.417
		95.99	97.84				
		96.66	98.97				
3	120 %	99.72	98.69	98.93 $\pm$ 0.5	98.96 $\pm$ 0.32	0.008	0.003
		99.00	99.40				
		98.06	98.79				

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

Table 7: Robustness Results of Amlodipine Besylate and Hydrochlorothiazide

S. No	Parameter	Optimized	Used	Retention time (R _t), (minutes)		Peak asymmetry		Remark
				AML	HCT	AML	HCT	
1.	Flow rate ($\pm$ 0.2 mL.min ⁻¹)	1.0 mL.min ⁻¹	0.8 ml / min	3.843	3.576	1.263	1.250	Robust
			1.2 mL / min	3.586	3.268	1.176	1.217	
2.	Detection wavelength ($\pm$ 5 nm)	230 nm	225 nm	3.690	3.273	1.263	1.227	Robust
			235 nm	3.584	3.163	1.227	1.217	
3.	Mobile phase composition ($\pm$ 5 %)	50 : 50 v/v	55 : 45 v/v	3.843	3.498	1.288	1.238	Robust
			45 : 55 v/v	3.686	3.201	1.250	1.199	

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

	Limit of Detection (LOD)	Limit of Quantitation (LOQ)
Amlodipine besylate	0.2011 μ g / mL	0.6095 μ g / mL
Hydrochlorothiazide	0.6476 μ g / mL	1.9626 μ g / mL

Table 9: Dissolution Data for Amlodipine Besylate and Hydrochlorothiazide

S. No	Time (minutes)	% Dissolved	
		AML	HCT
1	10	66.39	44.71
2	20	79.85	72.96
3	30	85.42	86.83
4	45	90.89	95.15
5	60	96.56	97.43
6	75	97.62	98.16

Figure 1: Structure of Amlodipine Besylate

Figure 2: Structure of Hydrochlorothiazide

Figure 3: Chromatogram of Placebo

Figure 4: A typical Chromatogram of Amlodipine Besylate and Hydrochlorothiazide Standard

Figure 5: Chromatogram of Market Formulation (AMLONG-H Tablets) of Amlodipine Besylate and Hydrochlorothiazide

Figure 6: Calibration Plot of Amlodipine Besylate

Figure 7: Calibration Plot of Hydrochlorothiazide

Figure 8: Chromatogram for In Vitro Dissolution of Amlodipine Besylate and Hydrochlorothiazide at 10 minutes

Figure 9: Chromatogram for In Vitro Dissolution of Amlodipine Besylate and Hydrochlorothiazide at 20 minutes

Figure 10: Chromatogram for In Vitro Dissolution of Amlodipine Besylate and Hydrochlorothiazide at 30 minutes

Figure 11: Chromatogram for In Vitro Dissolution of Amlodipine Besylate and Hydrochlorothiazide at 45 minutes

Figure 12: Chromatogram for In Vitro Dissolution of Amlodipine Besylate and Hydrochlorothiazide at 60 minutes

Figure 13: Chromatogram for In Vitro Dissolution of Amlodipine Besylate and Hydrochlorothiazide at 75 minutes

Figure 14: In vitro Dissolution Profiles of Amlodipine and Hydrochlorothiazide

RESULTS AND DISCUSSION

The mobile phase consisting of 10 mM phosphate buffer (pH-3.0): acetonitrile (50 : 50 % v/v at 1 mL.min⁻¹ flow rate) was optimized which gave sharp peak, minimum tailing factor with short runtime for Amlodipine besylate and Hydrochlorothiazide. The retention time for Hydrochlorothiazide and Amlodipine besylate were found to be 3.373 minutes and 3.753 minutes respectively. System suitability parameters and optimized chromatographic conditions are shown in Table 1. The calibration curve for Amlodipine besylate was found to be linear over the range of 1-5 µg / mL with correlation coefficient 0.9995 and Hydrochlorothiazide was found to be linear over the range of 3-15 µg / mL with correlation coefficient 0.9996. The data of the calibration is shown in Table 2. The developed method was applied to the assay of Amlodipine besylate and Hydrochlorothiazide 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 Amlodipine besylate and Hydrochlorothiazide are shown in Figure 4 and 5 respectively. The regression equation for Hydrochlorothiazide was found to be $Y = 204.98 X + 34.917$ with correlation coefficient is $r^2 = 0.9996$ and for Amlodipine besylate was $Y = 28.165 X + 5.2911$ with correlation coefficient is $r^2 = 0.9995$ which indicates this method has good linearity. The linearity of the graphs are 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 placebo (Figure 3) with sample

peak. They do not disturb the elution or quantification of Amlodipine besylate and Hydrochlorothiazide 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 intra and inter day variations in the test methods of Amlodipine besylate and Hydrochlorothiazide for three times on the same day and different day. The intra-day and inter-day precision obtained was % RSD (< 2.0) indicates that the proposed method is quite precise and reproducible 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 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 Amlodipine besylate and Hydrochlorothiazide at each level was not less than 99 % and not more than 101 %. The method precision was done and the low % RSD values indicates that the proposed method which was in good agreement with precision. Robustness was done by small changes in the chromatographic conditions like mobile phase flow rate, wave length, mobile phase composition etc. 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 Hydrochlorothiazide and Amlodipine was 0.6476 µg / mL and 0.2011 µg / mL respectively and the limit of quantitation (LOQ) of Hydrochlorothiazide and Amlodipine 1.9626 µg / mL and 0.6095 µg / mL respectively which shows that this method is very sensitive. The results are presented in Table 8. *In vitro* dissolution was performed on tablets containing Amlodipine besylate and Hydrochlorothiazide and the results are presented in Table 9.

CONCLUSION

A New validated RP-HPLC method has been developed for the quantitative determination of Amlodipine besylate and Hydrochlorothiazide in bulk and pharmaceutical tablet dosage forms. Statistical analysis of the results shows that the proposed procedure has good precision and accuracy. The method was completely validated shows satisfactory results for all the method validation parameters tested and method was free from interference of the other active ingredients and additives used in the formulation. In fact results of the study indicate that the developed method was found to be simple, reliable, accurate, linear, sensitive, economical and reproducible and have short run time which makes the method rapid. Hence it can be concluded that this method may be employed for the routine quality control analysis of Amlodipine besylate and Hydrochlorothiazide in active pharmaceutical ingredient (API) and pharmaceutical preparations. The developed method can also be conveniently adopted for dissolution testing of tablets containing Amlodipine besylate and Hydrochlorothiazide.

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