Synthesis and establishment of Amlodipine impurity G reference standard

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ABSTRACT

Dimethyl-4-(2-chlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate which is impurity of amlodipine besilate was synthesized. Hantzsch condensation of methyl acetoacetate with 2-chlorobenzadehyde in 2-propanol at heated under reflux temperature at 85 ^{o}C for about 10 hours yielded the yellow powder. This product was purified by column chromatography and the structure was identified by spectroscopic methods (IR, MS, NMR), that was characterized amlodipine impurity G (dimethyl -4-(2-chlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate). The quality of the impurity was determined by high performance liquid chromatography HPLC method and validated by guideline of The International Conference on Harmonization 2003 Then this impurity was determined homogeneity and assigned value to establish reference standard as Appendix 3 of World Health Organization (WHO technical series 943, 2006) and Statistical methods for use in proficiency testing by inter-laboratory comparison, International Organization for Standardization (ISO 13528), 2005.

INTRODUCTION

The regulatory agencies and pharmaceutical industries are of increasing concern to the impurities affecting safety, efficacy, and quality of pharmaceutical products. The impurities were to be formed from starting with raw materials, intermediate stages or impurity process. Quality and Safety of The International Conference on Harmonisation 2003, requires that impurities which are present in a drug substance although a quantity exceeding a certain limit, should be characterized, identified and needs to be evaluated both qualitatively and quantitatively. Amlodipine (*RS*)-3-ethyl-5-methyl-2-[(2-amino ethoxy)methyl]-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine -3,5-dicarboxylate is a medication used to treat high blood pressure and coronary artery disease (Burges and Dodd, 1990).

Chava *et al*., 2006 reported that 1,4-dihydropyridine derivative were yielded during in the formative stage of the amlodipine, one of degradative products was dimethyl-4-(2-chlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate. Harishkumar *et al*., 2009 published the synthesis of diethyl-2,6-dimethyl-4-substituted phenyl-1,4-dihydropyridine-3,5-dicarboxylate by Knoevenagel and Hantzsch condensation. Litvie and co-workers 2007 described Hantzsch condensation reaction of two equivalents of methyl-1,3-aminocrotonate with (*m* and *p*)-methoxybenzaldehyde which resulted in formation of the expected products 2,6-dimethyl-3,5-dimethoxycarbonyl-1-4-(*m*-methoxyphenyl)-1,4-dihydropyridine and 2,6- dimethyl-3,5-dimethoxycarbonyl-1-4-(*p*-methoxyphenyl)-1,4-dihydropyridine.

However, the synthesis of dimethyl-4-(2-chlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate (amlodipine impurity G) has not been reported yet, while the monograph of amlodipine salt in Pharmacopoeia (Bristist Pharmacopoeia, 2016 or European Pharmacopoeia 8.8) required for controlling amlodipine impurity G as related substance.

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The paper reports a synthesis route and establishment of dimethyl-4-(2-chlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate as impurity reference substance of drug substance of amlodipine in order to quality control of the impurity before manufacturing process.

MATERIALS AND METHODS

Materials

Chemicals (AR- Analytical Grade reagents) methyl acetoacetate, 2-propanol 2-chlorobenzaldehyde, ammonium acetate were purchased from Merck Chemicals. Acetonitrile (HPLC grade), methanol (HPLC grade) were purchased from JT. Baker. For column chromatography silica gel (Sigma-Aldrich, 63-200 μm) was used. Thin layer chromatography was carried out using silica gel 60 F254 aluminum sheets from Merck.

Equipments

The IR spectrum was recorded by using FT-IR Spectrophotometer Nicolet iS50, Thermo Scientific (KBr dishs). Mass spectrum was recorded on Shimadzu LC/MS IT-TOF. Melting point and purity was determined on Q20 DSC. NMR was performed by using Bruker Mercury 500 MHz spectrometer. TMS was used as an internal reference standard for the proton experiment (δ=0.00 ppm). The HPLC method was developed on Prominence Liquid Chromatography SPD20-A, Shimadzu, Japan. Gemini-NX C_{18} (150×4,6 mm; 5 μm) column was used.

Methods

Synthesis of amlodipine impurity G (dimethyl-4-(2-chlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate).

Dissolve 3.9 g (0.05 mol) ammonium acetate in 20 ml 2-propanol. Add gradually drop by drop in round bottom flask 12.9 ml (0.1 mol, 13.13 g) methyl acetoacetate and 5.7 ml (0.05 mol, 7.1 g) 2-chlorobenzaldehyde. Stir vigorously and headed under reflux temperature at 85 °C for about 10 hours with stirring. The precipitate was filtered and washed with cool methanol (3 x 30mL) and dried in the vacuum at 40 °C to constant weight. The residue transfered to chromatography column (10 x 30 cm, silica gel 40-60 μm), 1- 99 % CH_2Cl_2-EtOAc and 5- 95 % CH_2Cl_2 - EtOAc as eluant to give two fraction AmG1-2. The AmG2 was evaporated to dryness and recrystallized from cool methanol to obtain while powder (15,04 g).

Method validation and establishment of impurity Reference standard

The purity of compound AmG2 was checked by TLC and high performance liquid chromatography equipped with Auto Sampler and PDA detector. Mobile phase: 2.3 g/L solution of amonium acetate - methanol (30:70 v/v), Column: Gemini-NX (octadecylsilyl silica gel for chromatography)-C_{18} (150 x 4.6 mm, 5 μm) or equivalent; Flow rate: 1.0 mL / min, Wavelength set up at 273 nm; Injection volume: 20 μl; Column oven: Ambient; Run time: 3 times of RT of amlodipine impurity G (British Pharmacopoeia, 2016).

The analyse method of synthesized impurity and the various parameters for development and validation according to ICH guidelines (ICHQ2A) were performed. Selectivity and sensitivity was determined by analyzing control samples in replicates (n = 6) spiked with the analyte at the lowest level of the calibration standard. Accuracy of the samples was calculated using the calibration curve.

The establishing impurity reference standard was based on the homogeneity; assigned value from two Lab conformed to Good Laboratory Practice (GLP) or ISO/IEC 17025 main principles.

RESULTS AND DISCUSSION

Synthesis of amlodipine impurity G (dimethyl-4-(2-chlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate).

The reaction Hantzsch condensation of *o, m, p*-methoxylbenzaldehyde isomers with methyl-3-aminocrotonate in propanol showed that the *o*-methoxylbenzaldehyde was compiled much slower during 48 hours, but the mainly obtained product was not 1,4-DHP as the hypothesis of mechanism involves to form derivative 1,4-DHPs of Litvie and co-workers had proved. Addition, Angeles *et al* (2001) reported that Hantzsch condensation of symmetric 1,4-DHPs are formed as impurites other than to yield 1,4-DHP ring, such as observation of reaction of 2-nitrobenzaldehyde with ammonium hydroxyde and acetoacetic acid ester by Hantzsch, isolated the different compounds including 1,2-dihydropyridine, cyclic amide and substituted hydroxamic acid without the 1,4-DHP product. In the case of our paper, the steric hindrance and electron drawing ability of the substituted *2*-chloro on benzaldehyde of amlodipine impurity G which has not been mentioned, as expected reaction would be Michael addition of the second molecule of methyl acetoacetate rather than to proton transfer and dehydration lead to formation of the imino-Knoevenagel intermediate (Fig.1). An interesting observation the spot trace of imino-knoevenagel intermediate was noted.

By TLC was detected only synthesized impurity in comparison with authentic sample of amlodipine impurity G EPCRS. Thus, the reaction of methyl acetoacetate with *2*-chlorobenzaldehyde as starting material in propanol gave the Hantzsch condensation was convenient for synthesis amlodipine impurity G which was higher yield than was expected (yield 78 %).

AmG2 compound was obtained as while off powder, Rf (CH_2Cl_2 – EtOAc, 98:2) = 0,313; mp 194.10 °C, and purity test on DSC Q20 and HPLC was about 99.85 %. The IR absorption bands IR $\nu_{max}(cm^{-1})$: 3306, 2947, 1702, 1651, 1493, 1231, 1020, 1118 and 755 cm^{-1}. HR-MS revealed the molecular formula to be $C_{28}H_{27}ClN_2O_7$ *m/z* [M-H]$^-$: 334.0829 (Cal. 335.0814).
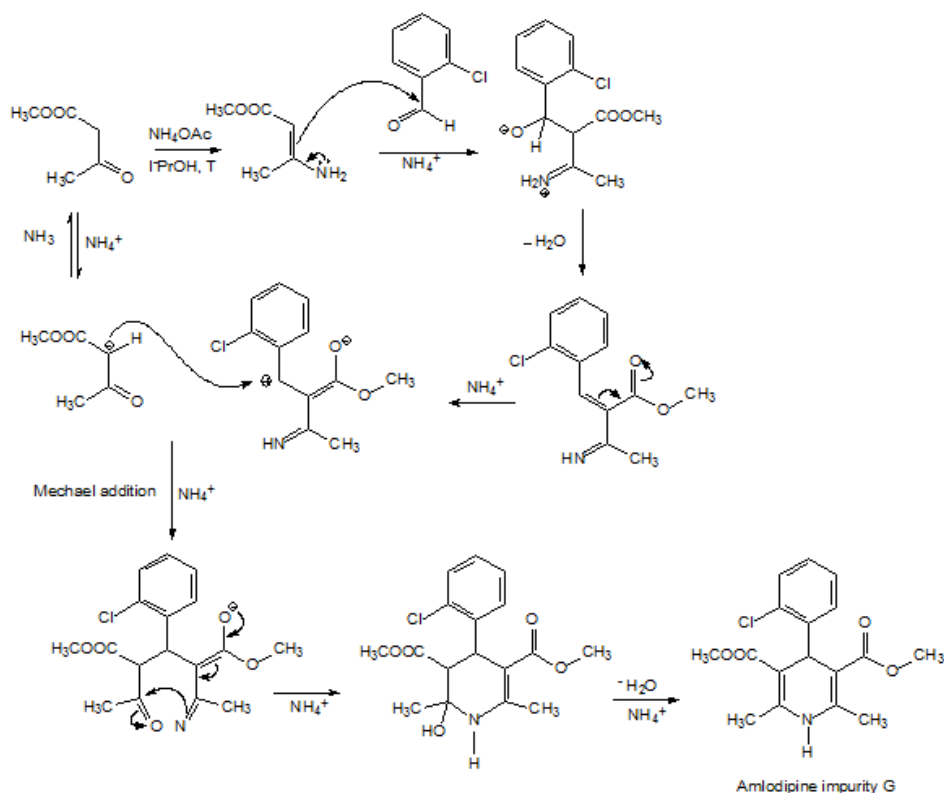
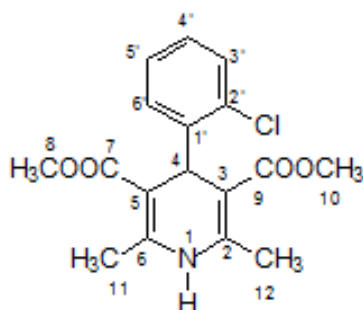**Fig. 1:** The pathway of synthesis of amlodipine impurity G.

Fig. 2: Structure of amlodipine impurity G.

^{1}H-NMR (500 MHz, CDCl$_3$) δ_H 2.31 (6H, s, CH$_3$), 3,61 (6H, s, COOCH$_3$), 5.40 (1H, s, CH), 5.67 (1H, s, NH), 7.03 (1H, dt, arom., *J*=7.5 &1.0 Hz, 7.12 (1H, t, arom., *J* =7.5 Hz), 7.24 (1H, d, arom., *J* =7.5 Hz), 7.36 (1H, d, arom., *J*=7.5 Hz); ^{13}C NMR (125 MHz, CDCl$_3$): δ_C 19.4, 37.3; 50.8, 104.0, 126.9, 127.3, 129.3, 131.2, 144.0, 145.9, 168.0.

Method validation and establishment of impurity Reference standard

Method validation
System suitability
Reference solution: Dissolve 4 mg of *amlodipine impurity G EPCRS* in acetonitril and dilute to 20.0 mL with the mobile phase.
Test solution: Dissolve 10.0 mg of the substance to be examined in

acetonitril and dilute to 10.0 mL with the mobile phase. Dilute 2.0 mL of this solution to 10.0 mL with the mobile phase.

The % RSD of areas from repeated injects of samples not be more than 2.0 %. The SST is suitable for analysis (Table 1 & Fig 3).

Table 1: The parameters of System Suitability Testing.

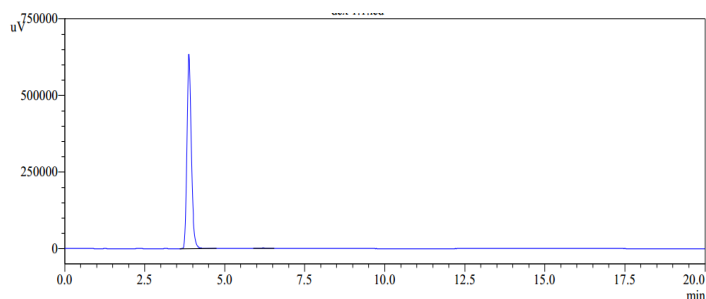| No Injects	RT (min)	S (µV x second)	A$_S$ asymetric	N Theory places
1	3.872	6023226	1,272	3521
2	3.879	6033593	1.269	3530
3	3.881	6028266	1.273	3532
4	3.883	6037178	1.274	3544
5	3.877	6030399	1.271	3526
6	3.875	6032021	1.272	3523
Average	3.878	6030781	1.272	3529
RSD	**0.104**	**0.079**	**0.114**	**0.233**

Fig. 3: HPLC Chromatography of amlodipine impurity G.

Specificity Study

       The sample was analyzed in Specific conditions mobile phase Study. Chromatogram of mobile phase doesn't show interference at the retention time of amlodipine impurity G. Therefore this method is specific for determination of amlodipine impurity G.

Linearity study

       Preparation of sample concentrations were about 100 µg/mL, 160 µg/mL, 200 µg/mL, 240 µg/mL, 300 µg/mL respectively. Correlation coefficient should not be less than 0.999 (Table 2 & Fig 4)

       The calibration curves were linear over the range of 100.3 to 307.6 µg/mL resulted in the regression equation $\hat{y} = 30110\,x + 15733$ ($r^2 > 0.9999$). The LOD and LOQ of the calibration graph were 0.97 µg/ml and 3.23 µg/mL, respectively.

Table 2: Linearity Results of compound amlodipin impurity G

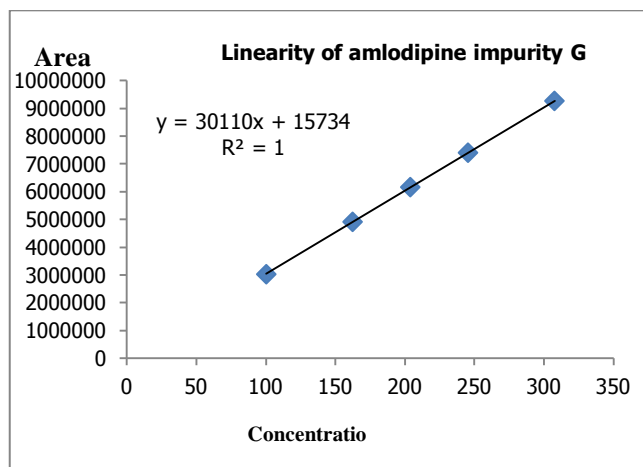| Concentration (µg/ml)	100.3	162.4	203.9	245.4	307.6
Average Areas	3025914	4913021	6164405	7404504	9270727

Fig. 4: Linearity of amlodipine impurity G.

Precision (Repeatability):

       The same procedure is repeated to remaining six preparations about 200 µg/ml concentration. % RSD for the RT and area are tabulated as below in Table 3.

Table 3: Results of Repeatability of amlodipine impurity G by HPLC method.

Reference solution amlodipine impurity G EPCRS		Synthesis of amlodipine impurity G		
Concentration (µg/ml)	Area of amlodipine impurity G peak	Concentration (µg/ml)	Area of amlodipine impurity G peak	Content %
202.0	6059302	205.4	6062882	99.34
	6061920	213.8	6314527	99.40
	6011289	229.0	6780092	99.65
	6014870	199.2	5887082	99.46
	6041440	204.8	6027511	99.05
	6036902	206.6	6095237	99.29
Number of samples				6
Average mean (%)				99.37
SD				0.20
RSD (%)				0.20

Table 4: Results of Intra- and inter-day precision of amlodipine impurity G by HPLC method.

Number of samples	Intra-day 1 Content (%)	Intra-day 2 Content (%)
1	99.34	99.00
2	99.40	99.26
3	99.65	99.15
4	99.46	99.41
5	99.05	99.25
6	99.29	99.60
Average	99.37	99.28
SD	0.20	0.21
% RSD	0.20	0.21

Table 5: Results of accuracy of amlodipine impurity G by HPLC method.

% Level concentration	Area of amlodipine impurity G peak	Concentration (µg/ml)	Concentration from regression equation (µg/ml)	% Recovery ± SD
50	3103426	104.2	102.5	98.6 ± 0.287
	2995989	100.5	99.0	
	3048975	101.8	100.7	
100	6194965	205.8	205.2	99.8 ± 0.114
	5986070	198.9	198.3	
	6094431	202.1	201.9	
150	9249577	305.3	306.7	99.0 ± 1.125
	8934276	301.6	296.2	
	9101464	306.8	301.8	

Intra- and inter-day precision

       Determination of amlodipine impurity G concentration in samples ($n = 6$ at each concentration for intra-day and $n = 6$ days for interday precision). The % RSD of areas from six preparations precision level is not more than 2.0 %. These results indicate high precision of the proposed methods (table 4).

Accuracy

       The accuracy of the test method was demonstrated by preparing recovery samples of at the level of 50 %, 100 % and 150 % of target concentration). The recovery of amlodipine impurity G by HPLC method estimated 99.0 % with RSD < 2 % which comply to accuracy method (table 5).

Robustness

The robustness of test method was demonstrated by carrying out Mobile phase variation ± 2.0 % (76: 24 → 64: 36), flow variation ± 10 % (0.9 mL to 1.1 mL/min) the results are tabulated as below in Table 6. Careful validation proved advantages of the method: high sensitivity, accuracy, selectivity and suitability for quality control laboratories.

Table 6: Robustness Study.

Parameters	Flow rate (mL/min)		Mobile phase (v/v)	
	0.9	1.1	76: 24	64: 36
RT	4.14	3.48	5.12	3.02
Area of amlodipine impurity G peak	6162882 ± 0.14*	5897082 ± 0.31*	6262582 ± 0.22*	5597102 ± 0.61*
% Content	99.05	99.31	99.11	99.41

SD of n=3.

Establishment of impurity Reference standard
Homogeneity test

For a homogeneity check, 12/100 vial of the samples of amlodipine impurity G packaged in glove-box with nitrogene were selected at random, and the contents of two test portions from each sample were determined. The data are shown in Table.7, together with sample averages and between-test-portion ranges.

The homogeneity was tested by Data analysis-tools of Microsoft excel 2010, Anova-single factor, comparison with values F and F_{crit}, showed that F< F_{crit}, so it may be concluded that the samples are adequately homogeneous in the batch.

For the stability check, three of the samples were tested by the same laboratory one month later, and gave an average result of X_1 = 99.23 %. The difference $X_1 - X_o$ = 0.04 %.

Standard deviation for method assessment has been set at 0.211 (Table 9). Assessment criterion for a stability check 0.3.s* = 0.3∗ 0.211 = 0.063, so it has to be concluded that the samples are adequately stable.

Table 7: Measurement results for homogeneity check.

Sample number	Test portion 1 (%)	Test portion 2 (%))	Descriptive Statistics
1	99.28	99.03	
2	99.36	99.44	
3	99.30	99.39	
4	99.10	99.39	Sample average = 99.27%;
5	99.66	98.92	Standard deviation of sample averages SD= 0.22
6	98.76	99.48	RSD = 0.22 %
7	99.06	99.20	Between-samples standard deviation Ss = 0.0004
8	99.55	99.39	
9	99.35	99.42	
10	99.52	99.07	
11	99.01	99.42	
12	99.32	99.17	

Table 8: The parameter of SST from two Lab for assay of amlodipin impurity G.

Parameters	Lab1	Lab 2
N > 12000	3877	3854
A_s: 0.8 - 1.5	1.315	1.320
RSD ≤ 2 %	0.11%	0.16%

Lab1: Physical Instrument department, Institute of drug Quality Control HCMC. Lab 2: Reference substances department, Institute of drug Quality Control HCMC.

Table 9: Determination of assigned value of amlodipine impurity G by two laboratories, together with values for the expanded uncertainties (*U*) of the results as reported by the laboratories.

Iteration	x^*_o	x^*_1	x^*_2	x^*_3
x* - 1.5.s*	-	99.04	99.02	99.02
x* + 1.5.s*	-	99.61	99.65	99.65
Average	99.343			
Standard deviation	0.194			
New x*	**99.325**	**99.337**	**99.337**	**99.337**
New s*	**0.193**	**0.211**	**0.211**	**0.211**

Assigned value

After checking the suitability system of two lab (Table 8), the assigned value was determined. The result showed that there is no change the values of *x** and *s** from one iteration to the third modified data. So convergence may be assumed and the assigned value was 99.34 %, with the uncertainty

$$U_x = 1.25s^*/\sqrt{p} = 0.076.$$

CONCLUSION

In conclusion, a facile synthetic route to amlodipine impurity G has been developed with overall 78 % yield starting from *2*-chlorobenzaldehyde, methyl acetoacetate and amonium salt in propanol and heated under reflux temperature at 85 °C for about 10 hours with stirring. The product was purified and structural elucidation by spectroscopic techniques to confirm its of dimethyl-4-(2-chlorophenyl)-2, 6-dimethyl-1,4- dihydropyridine-3,5-dicarboxylate.

The simple, accurate and precise RP-HPLC method for the determination of amlodipine impurity G as BP 2016 was verified. The establishment of authentic sample was carried out, it is concluded that the amlodipine impurity G (99.34 % as is) may be recommended as reference standard for routine and quality-control analysis in the drug substances and formulations.

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