Development and validation of HPLC method for the determination of S (-) Amlodipine Besylate and its related substance in tablet formulation by using chiral separation.

Khalid A. Ansari1*, Kunal P. Pagar1, Pradeep R. Vavia1

1Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology, Nathalal Parikh Marg, Matunga Mumbai-400019, India.

ABSTRACT

A simple, specific and sensitive high-performance liquid chromatographic (HPLC) method for determination of S (-) Amlodipine Besylate (SAB) and its related substance i.e. R (+) Amlodipine Besylate (RAB) in tablet formulation was developed and validated. The isomer separation was carried out using simple isocratic reversed-phase HPLC method using a chiral column. Isocratic elution at a flow rate of 1.0 mL/min was employed on chiral column Ultron-ES-OVM, (250 x 4.6 mm, 5 μm). The mobile phase consisted of (Buffer: ACN) (78:22, v/v) in which buffer is mixture of 0.01M disodium hydrogen phosphate dihydrate and 0.01 M potassium dihydrogen phosphate. The UV detection wavelength was 237 nm and 20 µL of sample was injected. Method was validated with respect to specificity, linearity, precision accuracy and robustness. The method was found to be linear for the concentration range of 0.04 – 0.06 mg/mL of SAB with correlation coefficient r = 0.9998. The proposed method was applied successfully for quantification of SAB and RAB in tablet formulation. The method was found to be simple, accurate, sensitive and reproducible. It can be successfully utilized for routine analysis in quality control laboratory.

KEYWORDS: S (-) Amlodipine Besylate, R (-) Amlodipine Besylate, dihydropyridine, HPLC, validation.

1. INTRODUCTION

Amlodipine is a calcium channel antagonist belonging to the dihydropyridine class. It is chemically 3-ethyl 5-methyl-2-[(2-aminoethoxymethyl)-4-(2-chlorophenyl)-l,4-dihydro-6-methy1-3,5-pyridinedicarboxylate. Amlodipine is widely used for the treatment of hypertension as well as stable and variant angina and known to be life saving drug.2 Amlodipine is therapeutically used as racemic mixture similar to the other calcium channel blocking agents of dihydropyridine type.3 The S (-) isomer of amlodipine is found to possess greater pharmacological effects than R (+) amlodipine. S (-) amlodipine is 1000 times more potent than the R (+) isomer in binding to the dihydropyridine receptor. In humans, the dominant effects of amlodipine are consequent to vasodilation. S (-) amlodipine lowers peripheral vascular resistance without causing a reflex tachycardia. It is effective as a once daily dosage in the control of hypertension.4,6 The efficacy and tolerability of 2.5 mg of SAB is almost similar with that of 5 mg of amlodipine besylate in the treatment of mild to moderate hypertension. Pharmacokinetic behaviour of RAB and SAB after single enantiomer administration is comparable to that of each enantiomer after administration of the racemate, no racemization occurs in vivo in human plasma after single enantiomer administration.5,7,8

Due to the therapeutic importance of amlodipine, several analytical methods have been reported for its quantitative determination both in pure form as well as in pharmaceutical formulations. Different analytical methods reported for the determination of amlodipine are liquid chromatography 9-14, reversed phase high performance liquid chromatography15-18, gas chromatography19,20, high performance thin layer chromatography21-23, liquid chromatography with tandem mass spectrometry24 and fluorimetry25.

In this research work, the objective is to develop and validate HPLC method for determination of SAB and its
related substance i.e. RAB in tablet formulation by using chiral column separation technique.

**MATERIALS AND METHODS**

**MATERIALS:**

S (-) Amlodipine Besylate (SAB) and R (+) Amlodipine Besylate (RAB) working standards were purchased from Glochem industries limited (Hyderabad, AP, India). Acetonitrile HPLC grade, Methanol HPLC grade, Potassium dihydrogen orthophosphate AR and disodium hydrogen orthophosphate dihydrate AR were purchased from Merck limited (Mumbai, India). High purity deionised water was obtained from Millipore, Milli-Q (Bedford, MA, USA) purification system.

**METHODS:**

**Instrumentation:**

HPLC system (Waters 717 plus auto sampler HPLC system USA) consisting of pump 515, Injector and 2487 dual wavelength absorbance UV detector were employed for analysis. Chromatographic data was acquired using Empower software.

**Chromatographic conditions:**

Ultron ES-OVM, (250 mm x 4.6 mm, 5 μm) chiral column was used as a stationary phase. The isocratic mobile phase consists of mixture of buffer and acetonitrile in the ratio of 78:22 (v/v). The buffer is mixture of 0.01M disodium hydrogen phosphate dihydrate and 0.01M potassium dihydrogen phosphate. The flow rate of the mobile phase was 1.0 mL/min and the injection volume was 20 μL. Detector signal was monitored at a wavelength of 237 nm.

**Solution preparation:**

**Standard solution:**

Accurately weighed quantity of powder (37.02 mg of Amlodipine Besylate) equivalent to 25 mg of S (-) Amlodipine was taken in 50mL volumetric flask. Thirty millilitres of mobile phase was added into the flask followed by sonication of 5 minute in order to obtain clear solution. Volume was made up to 50 ml with mobile phase. The solution was filtered by 0.45 μm membrane filter.

**Sample solution:**

Tablets (n=20) were finely grinded in a porcelain mortar. The average mass of the 20 tablets taken was determined. In a 50 ml volumetric flask, accurately weighed quantity of powder equivalent to about 25 mg of S (-) amlodipine was taken. Thirty millilitres of mobile phase was added into the flask and it was sonicated to obtain clear solution. Volume was made up to 50 ml with mobile phase. The solution was filtered by 0.45 μm membrane filter.

**Validation procedure:**

The specificity of the method was determined by injecting the sample solution containing excipients with and without drugs having concentration same as that of the standard. Five standard solutions were prepared for the linearity test of SAB in the range of 0.04-0.06 mg/ml and for the linearity test of RAB in the range of 0.03-0.07 mg/ml in mobile phase. Each solution was injected in three replicates and linear regression analysis for SAB and RAB was performed. The limit of detection (LOD) was determined from the regression data of calibration curve by using following formula (Equation 1).

$$\text{LOD} = \frac{3.3 \times \sigma}{S}$$  \hspace{1cm} (1)

Where, \(\sigma\) is the average residual standard deviation and \(S\) is slope of the calibration line.

Limit of quantification (LOQ) was calculated using following formula (Equation 2).

$$\text{LOQ} = 10 \times \frac{\sigma}{S}$$  \hspace{1cm} (2)

The accuracy of the method was carried out by adding known amount of each drug corresponding to three concentration levels 50%, 100% and 150% of the label claim along with the excipients in triplicate. The samples were given the same treatment as mentioned for the sample solution preparation. Recovery of SAB was determined in presence of excipients.

Precision of the method was checked by carrying out six independent assays of test samples against standard. Intermediate precision was performed by analyzing the samples by different analyst on different day.

Robustness was performed by deliberately changing the chromatographic conditions. The flow rate of the mobile phase was changed from 1.0 mL/min to 0.8 mL/min and 1.2 mL/min. The organic strength was varied by ±2 %. Standard solution was injected six times in replicate for each change.

Respective peak areas, dilution factors, sample and standard weights were taken into account to quantitate the amounts of SAB in milligram per tablet.

**Market sample evaluation:**

Three commercial lots of the S-Numlo 2.5 mg Emcure Pharma limited (Pune, Maharashtra, India) tablets were analyzed for content of RAB.

**RESULT AND DISCUSSION**

**Selection of column and sample preparation:**

Preliminary experiments have been performed based on various HPLC methods available in the literature for the analysis of amlodipine racemate. But the separation of isomers on normal silica gel analytical column is very difficult, so we have selected chiral analytical column, Ultron-ES-OVM, (250 x 4.6 mm, 5 μm) in order to get good resolution between SAB and RAB. The sample preparation and concentration selection for analysis was very critical. It was found that at higher concentration both isomers were found to be merged into single peak during the analysis.
Therefore, we have taken 0.05 mg/ml as a standard concentration for analysis.

**Optimization of the chromatographic conditions:**
The effect of composition of the mobile phase on the retention time of SAB and RAB was thoroughly investigated. Mobile phase composition of buffer: Acetonitrile (78:22 v/v) was found to be optimum to separate the SAB and its related compound peaks i.e. RAB peak. The optimum pH of mobile phase is 6.98 to 7.02, which was adjusted by any one the salt used in buffer. Mobile phase flow rate of 1 mL/min was found to be optimum for separation of SAB and RAB with satisfactory resolution. The chromatogram was recorded at 237 nm. The elution order was found to be for related substance i.e. RAB (RT = 6.12 min), SAB (RT= 6.93 min, resolution = 1.43) at optimized chromatographic condition as shown in Figure 1.

**METHOD VALIDATION:**

**Specificity:**
The HPLC chromatogram recorded for the mixture of the excipients revealed no peak (Figure 2). The chromatogram recorded for mixture of SAB, RAB and excipients shows distinguishing peaks for the two isomers as shown in Figure 1. None of the excipients interfered with the analyte of interest and hence method was found to be specific.

**Linearity, Limit of detection (LOD) and Limit of quantitation (LOQ):**
Calibration curve with five points were constructed covering a concentration range 0.04-0.06 mg/mL for SAB and 0.03- 0.07mg/mL for RAB (Figure 3a & Figure 3b). Three independent determinations were performed at each concentration. Linearity equation and correlation coefficient were found to be: \( y = 62271.185x - 232827.161; r=0.9998 \) for SAB. Similarly linearity equation and correlation coefficient for RAB were \( y= 50564x-312; r = 0.9999 \). LOD and LOQ for RAB were found to be 0.5 μg/mL and 1.5 μg/mL respectively.

**Accuracy:**
The data for accuracy were expressed in terms of percentage recoveries of SAB from tablet formulation. The results are summarized in Table 1. The mean recovery data of SAB in real sample were within the range of 98.34% and 104.60 %. Mean percent relative standard deviation (RSD) was 2.45 %, satisfying the acceptance criteria for the study. Assay results of formulation are shown in Table 2.

<table>
<thead>
<tr>
<th>SAB concentration (mg/ml)</th>
<th>Mean recovery (%)</th>
<th>RSD (%)</th>
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</thead>
<tbody>
<tr>
<td>12.5</td>
<td>98.35</td>
<td>1.14</td>
</tr>
<tr>
<td>25</td>
<td>98.91</td>
<td>1.10</td>
</tr>
<tr>
<td>50</td>
<td>104.60</td>
<td>0.90</td>
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<tr>
<td>62.5</td>
<td>100.76</td>
<td>0.60</td>
</tr>
<tr>
<td>75</td>
<td>100.25</td>
<td>0.45</td>
</tr>
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Table 1: Accuracy study for SAB (n = 15)
CONCLUSION
A validated HPLC method has been developed for quantification of SAB and RAB in tablet formulation. The developed method is simple, specific, accurate, precise and robust. The proposed method can be utilized for routine analysis and quality control of SAB and RAB in tablet formulation.

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