APPLICATION OF HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY DENSITOMETRY FOR THE SIMULTANEOUS DETERMINATION OF AMLODIPINE BESYLATE AND LISINOPRIL IN BULK DRUG AND TABLET FORMULATION
K.H. Gopani, S.S. Havele, S.R. Dhaneshwar*
Department of Quality Assurance, Bharati Vidyapeeth University, Poona College of Pharmacy, Erandwane, Pune-411038, Maharashtra, India.
E-mail: sunil.dhaneshwar@gmail.com

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Abstract

Method describes an HPTLC method for the simultaneous determination of amlodipine besylate and lisinopril from tablet dosage form. This employs a precoated silica gel 60 F254 (0.2 mm thickness) on aluminium sheets and a mobile phase methanol: ethyl acetate: ammonium sulphate (0.2%) in the ratio of 3.0: 6.0: 4.0 (v/v/v), having chamber saturation for 30 min at room temperature. The mobile phase was run upto 8cm. The plate was scanned and quantified at 218 nm for both amlodipine besylate and lisinopril. The Rf values were found to be 0.30 and 0.72 for lisinopril and amlodipine besylate respectively. The linear detector response was observed between 2,000 ng.spot⁻¹ to 10,000 ng.spot⁻¹ for both amlodipine besylate and lisinopril. The method so developed was validated for its accuracy and precision. The LOD and LOQ were found to be 1 ng.spot⁻¹ and 3 ng.spot⁻¹ for amlodipine besylate and 80 ng.spot⁻¹ and 242 ng.spot⁻¹ for lisinopril respectively. The recovery was carried out by standard addition method. The average recovery was found to be 99.13 % and 99.35% for amlodipine besylate and lisinopril respectively.

Keywords: Amlodipine besylate, Antihypertensive, High performance thin layer chromatography, Lisinopril and Tablet formulation.
1. Introduction

Amlodipine besylate and lisinopril are available in tablet dosage form in the market in the ratio of 1:1. Amlodipine besylate (AML), is chemically $[3\text{-ethyl-5-methyl (4RS)-2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-methyl-1-dihydropyridine-3,5-dicarboxylate benzenesulfonate}]^{[1-3]}$ (Fig 1). AML is a calcium channel blocker, which is used as an antihypertensive agent. It is official in EP$^{[4]}$ and BP$^{[5]}$.

![Fig-1. Chemical structure of Amlodipine.](image)

Lisinopril (LIS) chemically $1\text{-[6-Amino-2-(1-carboxy-3-phenyl-propylamino)-hexanoyl]-pyrrolidine-2-carboxylic acid}$, is an angiotensin converting enzyme inhibitor used in treatment of hypertension and heart failure in prophylactic treatment after myocardial infarction and in diabetic nephropathy$^{[6]}$ (Fig 2). The official methods for determination of LIS are potentiometric titration$^{[7]}$ and HPLC$^{[8]}$.

![Fig 2. Chemical structure of Lisinopril.](image)
A number of spectrophotometric,[9-15] HPLC,[16-23] HPTLC,[24-25] LC-MS/MS,[26] LC-MS,[27] methods are reported in the literature for the estimation of AML, both individually as well as in combination with other drugs.

Reported methods for lisinopril include spectrophotometric,[29-33] flurimetric,[34-35] HPLC,[36] HPTLC,[37] and NMR.[38]

To our knowledge, no article related to HPTLC determination of AML and LIS in fixed dose combination has been reported in literature. The objective of the present work was to develop an accurate, specific and reproducible method for the simultaneous determination of AML and LIS in pharmaceutical formulations by HPTLC. The proposed method is optimized and validated as per the international conference on harmonization (ICH) guidelines[38] and its updated international convention.[39-40]

2 Experimental

2.1. Materials and Methods

Pharmaceutical grade Amlodipine besylate [AML] (batch no. PPC/10/AMB-053) was procured as generous gifts from Prudenace Pharma, Ankleshwar, Gujrat and Lisinopril [LIS] (batch no. ALS/0800150027) was procured as generous gifts from Vidhya Pharma, Mumbai, Maharashtra). Both drugs were used without further purification and certified to contain 96.5 % (w/w) and 98.05 % (w/w) of AML and LIS on dry weight basis respectively. Fixed-dose combination tablets Amlopress L (batch no. A02304) containing 5 mg of each AML and LIS were procured from CIPLA limited. All chemicals and reagents were of analytical-grade and were purchased from Merck Chemicals, Mumbai, India.

2.2 Instrumentation

The samples were spotted in the form of bands of 6 mm width with a 100 µL sample syringe (Hamilton, Bonaduz, Switzerland) on silica gel precoated aluminum 60F254 plates, (20×10 cm with 250 mm thickness; E. Merck, Darmstadt, Germany) using a CAMAG Linomat 5 (Muttenz, Switzerland) sample applicator. The plates were prewashed with methanol and activated at 110°C for 5 min prior
to chromatography. A constant application rate of 0.1 mL/s with nitrogen aspirator was used, and the space between two bands was 6 mm. The slit dimension was 5 × 0.45 mm, and the scanning speed was 10 mm/s. The monochromator bandwidth was set at 20 nm, each track was scanned three times, and baseline correction was used. The mobile phase consisted of methanol: ethyl acetate: ammonium sulphate (0.2%) in the ratio of 3.0: 6.0: 4.0 (v/v/v). Linear ascending development was carried out in an HPTLC twin-trough glass chamber (CAMAG) saturated with the mobile phase vapor. The optimized chamber saturation time was 30 min at room temperature (25 ± 2°C) at a relative humidity of 60 ± 5%. The length of each chromatogram run was 8 cm. Following the development, the HPTLC plates were dried in a current of air using an air dryer. Densitometric scanning was performed using a CAMAG TLC Scanner 3 in the reflectance-absorbance mode at 218 nm for both AML and LIS operated by WINCATS software (Version 1.4.4.6337, CAMAG). The radiation source used was the deuterium lamp emitting a continuous uv spectrum between 190 to 400 nm. Concentrations of the compound chromatographed were determined from the intensity of the diffused light. Evaluation was based on peak areas with linear regression.

2.3. Preparation of standard and sample solutions

Standard stock solutions at a concentration of 20 mg.mL⁻¹ for both AML and LIS were prepared separately using methanol. From the standard stock solution, the mixed standard solution was prepared by dilution with methanol to obtain 2,000 µg.mL⁻¹ – 10,000 µg.mL⁻¹ of both AML and LIS.

For the analysis of tablets, 20 tablets were weighed, their average weight determined and finely ground in a mortar. The portion equivalent to 5 mg of each AML and LIS was transferred in a 25 mL volumetric flask, 20 mL of diluent was then added, and sonication was done for 15 min with swirling. After sonication, the volume was made up to the mark with the diluent, and mixed well. The solution was filtered through 0.45µ Whatman filter. The analysis was performed six times.
2.4. HPTLC method optimization and chromatographic conditions

The Chromatographic conditions were optimized for simultaneous estimation of AML and LIS. For effective separation of AML and LIS, the mobile phase containing a mixture of methanol: ethyl acetate: ammonium sulphate (0.2%) in the ratio of 3.0: 6.0: 4.0 (v/v/v) was found to be optimum. After chamber saturation, the plates were developed to a distance of 80 mm and then dried in hot air. Simultaneous estimation of AML and LIS by HPTLC was very critical due to high polarity of LIS as compared to AML. Due to this the above mobile phase was found to be suitable for optimum separation of AML and LIS. Densitometric analysis was carried out using a Camag TLC Scanner 3 (Camag) in the absorbance mode at 218 nm for both AML and LIS. The chromatograms were integrated using winCATS evaluation software.

2.5 Method validation

Validation of the optimized HPTLC method was carried out with respect to the following parameters.

2.5.1. Linearity and range

Mixed standard stock solutions at a concentration of 2,000 µg.mL⁻¹ – 10,000 µg.mL⁻¹ for both AML and LIS were prepared using methanol. From the mixed standard stock solution 1 µL solution were spotted on the TLC plate to obtain a final concentration of 2,000–10,000 ng.spot⁻¹ for both AML and LIS. Each concentration was applied six times on the TLC plate. The plate was then developed using the previously described mobile phase and the peak areas were plotted against the corresponding concentrations to obtain the calibration curves.

2.5.2. Limit of Detection and Quantification

The limits of detection (LOD) and quantification (LOQ) were determined as the amounts of the analyte for which the signal-to-noise ratios (S/N) were 3 and 10, respectively. LOD and LOQ were determined by measuring the magnitude of analytical background (N), by spotting a blank, then calculating S/N for different amounts of AML and LIS after application a series of solutions (prepared by dilution of the standard stock solution) furnishing amounts in the range 10–100 ng.spot⁻¹.
2.5.3. Precision

The precision of the method was verified by intraday and interday precision studies. Intraday studies were performed by analysis of three different concentrations (2,000, 6,000, 10,000 ng.spot$^{-1}$) for both AML and LIS for six times on the same day. The interday precision of the method was checked by repeating studies on two different days.

2.5.4. Specificity

The specificity of the method was ascertained by analyzing standard drug and sample. The spot for AML and LIS in sample was confirmed by comparing the $R_f$ and spectra of the spots with that of standards. The peak purity of AML and LIS were assessed by comparing the spectra at three different levels, i.e. peak start, peak apex and peak end positions of the spot.

2.5.5. Robustness of the method

By introducing small changes in the mobile phase composition, the effects on the results were examined. Mobile phases having different composition like, methanol: ethyl acetate: ammonium sulphate (0.2%) (2.5: 6.0: 4.0 v/v/v), (3.0: 5.0: 4.0 v/v/v), (3.0: 6.0: 3.0 v/v/v) were tried and chromatograms were run. The amount of mobile phase, temperature and relative humidity was varied in the range of ±5 %. The plates were prewashed by methanol and activated at 60°C ± 5 for 2, 5, 7 min prior to chromatography. Time from spotting to chromatography and from chromatography to scanning was varied from 0, 20, 40 and 60 minutes. Robustness of the method was done at three different concentration levels 2,000, 6,000, 10,000 ng.spot$^{-1}$ for both AML and LIS.

2.5.6. Analysis of marketed formulation

The marketed formulation was assayed as described above. Fixed volumes solutions (10 µL) were spotted on plates and analyzed for AML and LIS in the same way as described earlier.
2.5.7. Accuracy

Accuracy of the method was determined by applying the method to drug sample (AML and LIS combination tablets) to which known amounts of AML and LIS standard powder corresponding to 80, 100 and 120% of label claim had been added (standard addition method), mixed and the powder was extracted and analyzed by running chromatograms in optimized mobile phase.

3. Results and Discussion

The results of validation studies on the simultaneous estimation method developed for AML and LIS in the current study involving methanol: ethyl acetate: ammonium sulphate (0.2%) in the ratio of 3.0: 6.0: 4.0 (v/v/v) as the mobile phase which gives highest resolution, minimum tailing and \( R_f \) values of 0.30 and 0.72 for LIS and AML respectively (Fig 3).

![Densitogram](image)

**Fig 3.** Densitogram of lisinopril (3000 ng.spot\(^{-1}\)); (\( R_f : 0.30, \)) and amlodipine besylate (3000 ng.spot\(^{-1}\)); ((\( R_f : 0.72 \)) in mobile phase methanol: ethyl acetate: ammonium sulphate(0.2%) (3: 6: 4) scanned at 218 nm.

UV scanning at 200-400 nm shows that 218 nm is the suitable wavelength for detection of both AML and LIS (Fig 4).
Fig. 4. Overlaid spectra of amlodipine besylate (10ug/ml) and lisinopril (10ug/ml) measured from 200nm to 400 nm.

3.1. Linearity: The AML and LIS showed a good correlation coefficient ($r^2 = 0.999$ for AML and 0.999 for LIS) in the given concentration range 2,000–10,000 ng.spot$^{-1}$ for both AML and LIS Table 1.

Table-1: Linear regression data for the calibration curves$^a$.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Linearity (ng.spot$^{-1}$)</th>
<th>$y = A + Bx$</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML</td>
<td>2000-10000</td>
<td>3237</td>
<td>0.511</td>
</tr>
<tr>
<td>LIS</td>
<td>2000-10000</td>
<td>840.3</td>
<td>0.679</td>
</tr>
</tbody>
</table>

$^a n = 6; ~ r^2$, coefficient of correlation

3.2. Precision

The intraday and interday precision RSD (%) values for AML was found to be 1.09 and 0.92 respectively, and the RSD (%) values for LIS were found to be 1.21 and 1.10, respectively. The developed method was found to be precise as the RSD values for repeatability and interday precision studies were <2%, respectively, as recommended by ICH guidelines Table 2.
Table-2: Intra and inter day precision of HPTLC method$^a$.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Interday precision</th>
<th>Intraday precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S.D of areas.</td>
<td>% R.S.D.</td>
</tr>
<tr>
<td>AML</td>
<td>63.073</td>
<td>1.09</td>
</tr>
<tr>
<td>LIS</td>
<td>22.2345</td>
<td>1.21</td>
</tr>
</tbody>
</table>

$^a$ n = 6

3.3. LOD and LOQ

Signal-to-noise ratios of 3:1 and 10:1 were obtained for the LOD and LOQ, respectively. The LOD and LOQ were found to be 1.0 ng.spot$^{-1}$ and 3.0 ng.spot$^{-1}$ for AML and 80 ng.spot$^{-1}$ and 242 ng.spot$^{-1}$ for LIS respectively.

3.4. Robustness of the method

The standard deviation peak of the peak areas was calculated for each parameter and the % RSD was found to be less than 2%. The low values of the % RSD indicated robustness of the method Table 3.

Table-3: Robustness testing$^a$.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>AML</th>
<th>LIS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SD of peak area</td>
<td>% RSD</td>
</tr>
<tr>
<td>Mobile phase composition</td>
<td>35.06</td>
<td>0.96</td>
</tr>
<tr>
<td>Amount of mobile phase</td>
<td>73.96</td>
<td>1.12</td>
</tr>
<tr>
<td>Temperature</td>
<td>46.09</td>
<td>0.07</td>
</tr>
<tr>
<td>Relative humiditys</td>
<td>10.288</td>
<td>0.28</td>
</tr>
<tr>
<td>Plate pretreatment</td>
<td>81.16</td>
<td>1.02</td>
</tr>
<tr>
<td>Time from spotting to chromatography</td>
<td>12.32</td>
<td>1.09</td>
</tr>
<tr>
<td>Time from chromatography to scanning</td>
<td>10.03</td>
<td>0.08</td>
</tr>
</tbody>
</table>

$^a$ n = 6
3.5. Specificity

The peak purity of AML and LIS was assessed by comparing their respective spectra at the peak start, apex, and peak end positions of the spot, i.e., $r_{SM} = 0.9933$ and $r_{ME} = 0.9901$ for AML and $r_{SM} = 0.9901$ and $r_{ME} = 0.9981$. A good correlation ($r^2 = 0.9991$ and $r^2 = 0.9990$ for AML and LIS respectively) was also obtained between the standard and sample spectra of AML and LIS, respectively. Also, excipients from formulation were not interfering with the assay.

3.6. Recovery studies

Good recoveries of the AML and LIS were obtained at various added concentrations for the above mentioned tablet as shown in table 4.

Table-4: Recovery studies$^a$.

<table>
<thead>
<tr>
<th>Label claim</th>
<th>Amount of drug added (%)</th>
<th>Total amount of drug present(ug)</th>
<th>Amount found</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML</td>
<td>80</td>
<td>3600</td>
<td>3589.56</td>
<td>99.71</td>
</tr>
<tr>
<td>5 mg</td>
<td>100</td>
<td>4000</td>
<td>3922</td>
<td>98.05</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>4400</td>
<td>4384.16</td>
<td>99.64</td>
</tr>
<tr>
<td>LIS</td>
<td>80</td>
<td>3600</td>
<td>3600.72</td>
<td>100.02</td>
</tr>
<tr>
<td>5 mg</td>
<td>100</td>
<td>4000</td>
<td>3966</td>
<td>99.15</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>4400</td>
<td>4351.16</td>
<td>98.89</td>
</tr>
</tbody>
</table>

$^a n = 6$

3.7. Analysis of a formulation

Experimental results of the amount of AML and LIS in tablets, expressed as a percentage of label claims were in good agreement with the label claims thereby suggesting that there is no interference from any of the excipients which are normally present in tablets. The above mentioned brand of fixed dose combination tablets was analyzed using the proposed procedures (Table 5).

The data of summary of validation parameters are listed in Table 6.
Table-5: Applicability of the HPTLC method for the analysis of the pharmaceutical formulations.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Label claim (mg)</th>
<th>Drug Content (%)</th>
<th>% R.S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML</td>
<td>5</td>
<td>98.94</td>
<td>1.10</td>
</tr>
<tr>
<td>LIS</td>
<td>5</td>
<td>99.32</td>
<td>1.21</td>
</tr>
</tbody>
</table>

Table-6: Summary of validation parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>AML</th>
<th>LIS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range (ng. spot⁻¹)</td>
<td>2000- 10000</td>
<td>2000- 10000</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.999 ± 0.09</td>
<td>0.999 ± 0.01</td>
</tr>
<tr>
<td>Limit of detection (ng. spot⁻¹)</td>
<td>1</td>
<td>80</td>
</tr>
<tr>
<td>Limit of quantitation (ng. spot⁻¹)</td>
<td>3</td>
<td>242</td>
</tr>
<tr>
<td>Recovery (n = 6)</td>
<td>99.13</td>
<td>99.35</td>
</tr>
<tr>
<td>Precision (% R.S.D.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intraday</td>
<td>1.09</td>
<td>1.21</td>
</tr>
<tr>
<td>Interday</td>
<td>0.92</td>
<td>1.10</td>
</tr>
<tr>
<td>Robustness</td>
<td>Robust</td>
<td>Robust</td>
</tr>
<tr>
<td>Specificity</td>
<td>0.9991</td>
<td>0.9996</td>
</tr>
</tbody>
</table>

4. Conclusion

Introducing HPTLC into pharmaceutical analysis represents a major step in terms of quality assurance. Today, HPTLC is rapidly becoming a routine analytical technique due to its advantages of low operating costs, high sample throughput, and the need for minimum sample preparation. The major advantage of HPTLC is that several samples can be run simultaneously using a small quantity of mobile phase-unlike HPLC; thus reducing the analysis time and cost per analysis. The developed HPTLC technique is precise, specific, and accurate. Statistical analysis proves that the method is suitable for the analysis of AML and LIS as a bulk drug and in pharmaceutical formulation without any interference from the excipients. It may be extended to study the degradation kinetics of AML and LIS and also for its estimation in plasma and other biological fluids.
5. References


Corresponding Author:
S.R. Dhaneshwar*
E-mail: sunil.dhaneshwar@gmail.com