TLC- Densitometric Method for Determination of some Cholesterol Lowering Drugs in Different Combinations

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Abstract

Sensitive, selective, precise and economic TLC-Densitometric method has been developed for determination of two binary mixtures containing the antihyperlipidemic Ezetimibe (EZ) in its combination with Atorvastatin calcium (AT) [mixture I] and with Simvastatin (SIM) [mixture II].

In the developed TLC-Densitometric method EZ, AT and SIM were quantitatively separated on 60F254 silica gel plates using ethyl acetate:hexane:glacial acetic acid (5.5:4.5:0.1 by volume) as a developing system with UV detection at 254 nm. Factors affecting the chromatographic separation have been studied, moreover the method has been validated as per ICH guidelines and it has been successfully applied for determination of the studied drugs in their different dosage forms without interference from excipients. Results obtained by the developed TLC-Densitometric method were statistically compared with those obtained by the reported spectrophotometric method and no significant difference was found between them.

Keywords: TLC-densitometry; Ezetimibe; Atorvastatin; Simvastatin

Introduction

Ezetimibe (EZ) is (3R, 4S)-1-(4-flurophenyl)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)-2-azetidinone [1]. It is the first in a new class of cholesterol absorption inhibitors that blocks the intestinal absorption of dietary and biliary cholesterol, without affecting the uptake of triglycerides or fat soluble vitamins. It reduces total cholesterol, LDL triglycerides and increases HDL in patients with hypercholesterolemia [2-7]. It is used for treatment of hypercholesterolemia and homozygous sitosterolemia [8]. Atorvastatin calcium (AT) is [R-((R*, R*))]-2-(4-flurophenyl)-5-[(1-methyl-2-phenylethyl)-3-phenyl-4-[[phenylamino]carbonyl]-1-Hpyrrole-1-heptanoic acid, calcium salt trihydrate [1]. It is a member of a class known as statins and it is a specific inhibitor of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-coA) reductase, the enzyme that catalyzes the conversion of HMG-coA to mevalonate, which is the rate limiting step in biosynthesis of cholesterol [9-13]. It is used for lowering blood cholesterol and preventing strokes thought anti-inflammatory and other mechanisms [14]. Simvastatin (SIM) is 2,2dimethyl-1,2,3,7,8a-hexahydro-3,7-dimethyl-8-naphtylphenyl-ester-[1S(1α,3α,7β,8β[2α*,4α*]-8αβ)] butanioic acid [1]. It is a member of the statins group and like Atorvastatin it acts as a specific inhibitor of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-coA) reductase [15]. It is hydrolyzed after oral administration in the liver to its active form, the β-hydroxy acid [16]. And it is used for lowering blood cholesterol. It is frequently prescribed for the treatment of hypercholesterolemia and was shown to significantly decrease the mortality associated with coronary heart diseases [17]. Combinations of either EZ/AT or EZ/SIM have the advantages of greater therapeutic effects than either drug alone [21-24]. These combinations have significant effects on reducing LDL cholesterol level in the blood compared to using each of these drugs individually.

Reviewing the literature in hand, none of the recommended pharmacopeias has been determined EZ and AT or EZ and SIM mixtures. EZ and AT have been determined by some techniques including HPLC [18], HPTLC-Densitometric [19] and spectrophotometric methods [20]. On the other hand EZ and SIM have been determined in their association by HPLC [21-23], HPTLC-Densitometric [24] and spectrophotometric methods [25].

Only one method [26] has been published for determination of EZ/AT and EZ/SIM combinations which depend on using first derivative of ratio spectra spectrophotometric method (1DD) for EZ /AT and EZ / SIM mixtures and measuring each of the two mixtures in separate steps using different instrumental conditions. From the previous literature review, no TLC-Densitometric method has been developed for simultaneous determination of the studied mixtures and no methods have been used for their determination without preliminary separation steps.

This work aims to develop selective, sensitive and accurate TLC-Densitometric method for simultaneous determination of the three studied drugs using the same solvent system, scanning wavelength and the same run hence it is time and cost effective and it can be used as alternative method to the high cost RP-HPLC method in quality control laboratories. The developed TLC-Densitometric method has the advantage of being more selective than the published spectrophotometric and HPTLC methods because it is able to separate the three components without interference from each other or from tablets excipients, moreover it does not need any sophisticated apparatus or high cost solvents compared to the published RP-HPLC methods.
Experimental Instruments

- CAMAG TLC scanner 3 S/N 130319 with win CATS software
- Sonix TV ss-series ultrasonicator (USA)
- Linomat 5 autosampler (Switzerland)
- CAMAG microsyringe (100 µl)
- Precoated silica gel aluminum plates 60 F254, ALLUGRAM® SIL G/UV 254 (Macherey-Nagel, Germany) 20 × 20 cm with 0.2 mm thickness
- CAMAG TLC scanner 3 S/N 130319 with win CATS software
- Source of radiation: deuterium lamp
- Scan mode: absorbance mode
- Slit dimension: 3 mm × 0.45 mm
- Scanning speed: 20 mm s⁻¹, and
- Output: chromatogram and integrated peak area
- Linomat 5 autosampler (Switzerland)
- CAMAG microsyringe (100 µl)
- Precoated silica gel aluminum plates 60 F254, ALLUGRAM® SIL G/UV 254 (Macherey-Nagel, Germany) 20 × 20 cm with 0.2 mm thickness
- Sonix TV ss-series ultrasonicator (USA)

Samples

- Atoreza® tablets (10/10) (B.N.1031061) labeled to contain Ezetimibe equivalent to 10 mg and atorvastatin calcium equivalent to 10 mg of atorvastatin, were manufactured by Marcyrl Pharmaceutical Industries, El-Obour City, Egypt.
- Pure AT was kindly supplied by Marcyrl Pharmaceutical Industries, El-Obour City, Egypt.
- Simvastatin (SIM) was kindly supplied by Chemipharm Pharmaceutical Industries, 6th October City, Egypt.
- Pure SIM was kindly supplied by Chemipharm Pharmaceutical Industries, 6th October City, Egypt.
- Pure EZ was kindly supplied by Egyptian Co. for Chemicals and Pharmaceuticals, ADWIA CO, 10th of Ramadan City, Egypt.
- Pure EZ was kindly supplied by Egyptian Co. for Chemicals and Pharmaceuticals, ADWIA CO, 10th of Ramadan City, Egypt.
- Pure AT was kindly supplied by Marcyrl Pharmaceutical Industries, El-Obour City, Egypt.

Pharmaceutical dosage forms

1. Atoreza® tablets (10/10) (B.N.1031061) labeled to contain Ezetimibe equivalent to 10 mg and atorvastatin calcium equivalent to 10 mg of atorvastatin, were manufactured by Marcyrl Pharmaceutical Industries, El-Obour City, Egypt.
2. Zocozet® tablets (10/10) (B.N.1031118) labeled to contain 10 mg each of Ezetimibe and Simvastatin, were manufactured by Marcyrl Pharmaceutical Industries, El-Obour City, Egypt.
3. Lipitrin® tablets (10/10) (B.N.90478A) labeled to contain 10 mg each of Ezetimibe and Simvastatin, were manufactured by Chemipharm Pharmaceutical Industries 6th-October City, Egypt.
4. Lipitrin® tablets (10/20) (B.N.80330B) labeled to contain 10 and 20 mg each of Ezetimibe and Simvastatin, respectively, were manufactured by Chemipharm Pharmaceutical Industries 6th-October City, Egypt.
5. Alkorplus® tablets (10/20) (B.N.008) labeled to contain 10 and 20 mg each of Ezetimibe and Simvastatin, respectively, were manufactured by Hikma Pharm for Pharmaceutical Industries 6th October City, Egypt.
6. Alkorplus® tablets (10/40) (B.N.012) labeled to contain 10 and 40 mg each of Ezetimibe and Simvastatin, respectively, were manufactured by Hikma Pharm for Pharmaceutical Industries 6th October City, Egypt.

Chemicals and Solvents

- All chemicals and solvents used throughout this work were of analytical grade and were used without purification. Ethyl acetate, hexane, glacial acetic acid and methanol (El-Nasr Pharmaceutical Chemicals Co. Abu- Zabaal, Cairo, Egypt)
- Sonix TV ss-series ultrasonicator (USA)
- CAMAG TLC scanner 3 S/N 130319 with win CATS software
- Source of radiation: deuterium lamp
- Scan mode: absorbance mode
- Slit dimension: 3 mm × 0.45 mm
- Scanning speed: 20 mm s⁻¹, and
- Output: chromatogram and integrated peak area

Results and Discussion

Ezetimibe is a cholesterol lowering drug that is co-formulated with both AT and SIM which are used in cases of high cholesterol level. Hence, they play an important role in the treatment of some serious diseases such as heart disease [26]. Instrumental planar chromatography with precise application of the samples, and computer controlled evaluation and quantification of the developed chromatograms has been considered as reliable for quality control and quantitative drug determination.
The main task of this work is to establish sensitive and selective TLC-Densitometric method for determination of EZ in its binary mixture with either AT or SIM in their bulk powder and in their combined pharmaceutical dosage forms using one and the same developing system and scanning wavelength with satisfactory precision for Good Analytical Practice (GAP).

**Method optimization**

Experimental conditions such as developing system composition, band dimensions, scanning wavelength and slit dimension were optimized in order to provide accurate, precise and reproducible chromatographic separation. The first step is to test all the published TLC-Densitometric developing systems [19,24] [in the first method the mobile phase was composed of chloroform: benzene: acetic acid (6:3:1:0.1 by volume) and the detection of the developed spots was carried out at 250 nm while in the second method the mobile phase was composed of n-hexane: acetone (6:4 v/v) and the detection of the developed spots was carried out at 234 nm]. Unfortunately, none of them was able to separate all the EZ/AT and EZ/SIM mixtures together using one developing system.

Different developing systems of different compositions were tried such as chloroform: methanol [(7:3 v/v) and (5:5 v/v)], chloroform: ethyl acetate [(7:3 v/v) and (5:5 v/v)], ethyl acetate: hexane (5:5:4.5:0.1 v/v) and ethyl acetate: hexane: glacial acetic acid (5:5:4.5:0.1 v/v). Using the first and second systems resulted in very bad resolution between the studied drugs. Replacing chloroform with hexane in the third one slightly enhanced the resolution but with tailed asymmetric peaks. On the other hand addition of glacial acetic acid in the last system enhanced both the chromatographic resolution and the peaks symmetry. So developing system consisted of ethyl acetate: hexane: glacial acetic acid (5:5:4.5:0.1 by volume) was used as the developing system.

Different band dimensions were tested in order to obtain sharp and symmetric peaks. The optimum band width chosen was 4 mm and inter-space between bands was 8.9 mm. Moreover Different scanning wavelengths were tried such as 210, 220 and 254 nm where scanning at 254 nm gave the best sensitivity for all the separated components. After method optimization the Rf values of AT, SIM and EZ are 0.2, 0.4 and 0.59 respectively as shown in Figure 1. The slit dimensions of scanning system enhanced both the chromatographic resolution and the peaks symmetry. So developing system consisted of ethyl acetate: hexane: glacial acetic acid (5:5:4.5:0.1 by volume) was used as the developing system.

**Method validation**

Validation was performed according to ICH guidelines [28].

**Linearity and range:** Linearity of the proposed method was evaluated and it was evident in the range of 0.4-4 µg/band, 0.4-3.1 µg/band and 0.5-2.9 µg/band for EZ, AT and SIM, respectively. Figure 2. The regression equations for EZ, AT and SIM were calculated and found to be:

\[
Y_1 = 0.2740C_1 + 0.3190 \quad r_1 = 0.9996, \text{ for EZ}
\]

\[
Y_2 = 0.4090C_2 + 0.1060 \quad r_2 = 0.9996, \text{ for AT}
\]

\[
Y_3 = 0.0271C_3 + 0.0951 \quad r_3 = 0.9995, \text{ for SIM}
\]

Where \(Y_1, Y_2\) and \(Y_3\) are integrated peak area \(\times 10^4\), \(C_1, C_2\) and \(C_3\) are the corresponding concentrations of EZ, AT and SIM in µg/band, respectively, \(r_1, r_2\) and \(r_3\) are the corresponding correlation coefficients.
Good linearity is evident from the high value of correlation coefficient and low value of intercept as shown in Table 1.

Accuracy: Accuracy of the method was checked by applying the proposed method for determination of different blind samples of pure EZ, AT and SIM. The concentrations were calculated from the corresponding regression equations and the results are presented in Table 1. Accuracy of the method was further assured by applying the standard addition technique on different pharmaceutical dosage forms where good recoveries were obtained revealing no interference from excipients, Tables 5, 6.

Precision

A) Repeatability: Three concentrations of EZ, AT and SIM (0.6, 1.2 and 1.8 µg/band) were analyzed three times intra-daily using the proposed method. Good % RSD was obtained, confirming the repeatability of the method as shown in Table 1.

B) Intermediate precision: The previous procedure was repeated inter-daily on three different days for the analysis of the three chosen concentrations. Acceptable % RSD was obtained and given in Table 1.

Specificity: Specificity of the method was tested by how accurately and specifically the analytes of interest are determined in the presence of other components (e.g.: co-formulated drugs, excipients, impurities, degradation products, etc). This is evident from TLC-Densitograms in Figure 1.

The good recovery percentages obtained by applying the proposed method on pharmaceutical dosage forms, Tables 2, 3 also proved the specificity of the proposed method as shown in Figure 3.

**Table 1:** Regression and analytical parameters of the proposed method for determination of EZ, AT and SIM

| Parameters | TLC- Densitometric Method | Reported Method [26] |
|------------|---------------------------|----------------------|
| EZ         | AT                        | EZ                   |
| Atoreza®   | 101.05±1.302              | 99.86±1.695          |
| (B.N. 1031061) |                        | 101.93±0.740         |
| Standard Addition | 100.26±2.072 | 99.78±1.049 |
| F-test c  | 3.022 (9.272)             | 2.584 (6.591)        |
| Student’s t-test | 1.165 (2.446) | 0.125 (2.364) |

*Average of 5 determinations.

*Average of 3 determinations.

*The values in the parenthesis are corresponding theoretical value at p= 0.05 [degree of freedom in dosage form =6 for EZ and 7 for AT].

[26] Spectrophotometric determination of EZ and ATR using 1DD at 299.5 and 288 nm for EZ and AT, respectively and methanol as solvent.

**Table 2:** Determination of the EZ and AT in Atoreza® tablets by the proposed method and statistical comparison with the reported method.

| Parameters | TLC- Densitometric Method | Reported Method [26] |
|------------|---------------------------|----------------------|
| Substance 1 | EZ | SIM | EZ | SIM |
| Zocozet®   | 98.29±2.656               | 101.25±1.553         |
| (B.N.1031118) |          | 101.1±1.341            | 100.86±1.470 |
| F-test (6.388)* | 4.534 | 1.115 |
| Student’s t-test (2.306)* | 1.279 | 0.603 |
| Liplint®  | 101.7±1.281               | 100.59±1.967        |
| (B.N.90478A) |          | 102.68±0.858           | 102.74 0.960 |
| F-test (6.388)* | 2.227 | 4.193 |
| Student’s t-test (2.306)* | 1.304 | 2.193 |
| Alkorplus® | 100.6±1.516               | 102.46±2.123         |
| (B.N.008)   |          | 102.08±0.725            | 101.82±1.273 |
| F-test (6.388)* | 4.364 | 2.778 |
| Student’s t-test (2.306)* | 1.968 | 0.583 |

*Average of 5 determinations

*The values in the parenthesis are corresponding theoretical value at p= 0.05 [degree of freedom in dosage form =8].

[26] Spectrophotometric determination of EZ and SIM using 1DD at 299.5 and 242.5 nm for EZ and AT, respectively and methanol as solvent.

**Table 3:** Results of analysis of EZ and SIM in different dosage forms by proposed method and results of statistical comparison with the reported one.

**Figure 3:** TLC-chromatograms of (a) Atoreza® and Zocozet® tablets

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Robustness: The robustness meaning is the capacity of the method to remain unchanged upon intended small change in method parameters e.g.: changing pH ± 0.1, changing mobile phase composition, changing saturation time ± 5 min and changing the scaling wavelength ± 1 nm. The low value of % RSD shows that the method is robust and that deliberate small changes in the studied factors do not lead to significant changes in \( R_f \) values, area or symmetry of the peaks.

System suitability: In order to validate the suggested TLC-Densitometric method, an overall system suitability testing was done to determine if the operating system are performed properly. Parameters including resolution (\( R_s \)), peak symmetry, and selectivity (\( \alpha \)) were calculated where good results were obtained and the peak information is given in Table 4.

After method optimization and validation it has been applied for the determination of EZ, AT and SIM in different pharmaceutical dosage forms where acceptable percentage recoveries have been obtained and shown in Tables 2,3. Furthermore, its validity was assessed by applying the standard addition technique which showed that tablet excipients did not interfere (Table 5,6).

On the other hand, statistical comparison of the results obtained by the developed method with those obtained by the reported spectrophotometric one [26] using F and Student’s t-tests showed no significant difference. The developed method has the advantages of being economic, reproducible, and accurate and can be easily applied in quality control laboratories.

| Parameters                  | EZ  | SIM | AT  |
|-----------------------------|-----|-----|-----|
| Symmetry factor             | 1   | 1.25| 1   |
| Resolution (Rs)             | 3   | 2.72| 7   |
| Selectivity (\( \alpha \))  | 1.75| 1.42|     |

Table 4: System suitability testing parameters of TLC-Densitometric method.

| Parameters                  | Recovery [%] | Pure added [\( \mu g/band \)] | Pure Found\(^a\) [\( \mu g/band \)] | Recovery [%] | Pure added [\( \mu g/band \)] | Pure Found\(^a\) [\( \mu g/band \)] | Recovery [%] |
|-----------------------------|--------------|-------------------------------|--------------------------------|--------------|-------------------------------|--------------------------------|--------------|
| EZ                          |              |                               |                                |              |                               |                                |              |
| Atoreza\(^a\)\(\) (B.N. 1031061) |              |                               |                                |              |                               |                                |              |
| 6                           | 6.08         | 101.33                       | 6                              | 6.08         | 101.40                       |
| 8                           | 8.21         | 102.62                       | 8                              | 7.77         | 97.16                        |
| 10                          | 9.95         | 99.58                        | 10                             | 10.13        | 101.33                       |
| 12                          | 1.18         | 98.55                        | 12                             | 12.14        | 101.17                       |
| Mean±SD                     | 101.05±1.302 | 100.52±1.81                  | 99.86±1.695                   | 100.26±2.072 |                             |

\(^a\)Average of 5 determinations

Table 5: Determination of EZ and AT Atoreza\(^a\) tablets by the proposed method and application of standard addition techniq

| Parameters                  | Recovery [%] | Pure added [\( \mu g/band \)] | Pure Found\(^a\) [\( \mu g/band \)] | Recovery [%] | Pure added [\( \mu g/band \)] | Pure Found\(^a\) [\( \mu g/band \)] | Recovery [%] |
|-----------------------------|--------------|-------------------------------|--------------------------------|--------------|-------------------------------|--------------------------------|--------------|
| EZ                          |              |                               |                                |              |                               |                                |              |
| Zocozet\(^a\)\(\) (B.N. 1031118) |              |                               |                                |              |                               |                                |              |
| 6                           | 6.13         | 102.23                       | 6                              | 6.09         | 101.58                       |
| 8                           | 7.85         | 98.23                        | 8                              | 8.01         | 100.12                       |
| 10                          | 10.03        | 100.30                       | 10                             | 9.97         | 99.70                        |
| Mean±SD                     | 98.29±2.856  | 100.25±2.00                  | 101.25±1.553                  | 99.46±0.986  |                             |
| Liprin\(^a\)\(\) (B.N.90478A) |              |                               |                                |              |                               |                                |              |
| 6                           | 6.16         | 102.66                       | 6                              | 6.02         | 100.42                       |
| 8                           | 8.22         | 102.75                       | 8                              | 7.92         | 99.06                        |
| Mean±SD                     | 101.76±1.281 | 102.70±0.063                 | 100.59±1.967                  | 99.74±0.961  |                             |
| Aikorplus\(^a\)\(\) (B.N. 008) |              |                               |                                |              |                               |                                |              |
| 4                           | 3.99         | 99.75                        | 6                              | 6.12         | 102                           |
| 6                           | 6.04         | 100.66                       | 8                              | 8.23         | 102.87                       |
| 8                           | 8.12         | 101.50                       | 10                             | 10.22        | 102.2                         |
| Mean±SD                     | 100.6±1.516  | 100.63±0.875                 | 102.46±2.123                  | 102.35±0.455 |                             |

\(^a\)Average of 5 determinations

Table 6: Determination of EZ and SIM in different tablets by the proposed method and application of standard addition technique.
Conclusion

In the present work sensitive and selective TLC-Densitometric method for the determination of EZ, AT and SIM in their pure form and in different dosage forms has been developed and validated.

The developed TLC-Densitometric method is considered superior to the reported spectrophotometric methods of being more selective and sensitive. On the other hand it can be used as alternative method to the published HPLC methods in laboratories lacking the facilities of HPLC. The developed TLC-Densitometric method is time effective since several samples can be run simultaneously using a small quantity of the developing system, which lowers the analysis time and cost. Finally we can conclude that the suggested method can be used in routine analysis of the studied drugs without any preliminary separation step.

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