Concurrent analysis of Simvastatin and citicoline using a Reversed-phase High Performance Liquid Chromatography-Ultra Violet Method

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\section*{Abstract}
To develop and evaluate a sensitive, accurate, rapid and reproducible high performance liquid chromatography analytical method for concurrent assay of simvastatin, a hyperlipidemia controlling agent, and citicoline, a psychostimulant agent, a C18 column (Eurosphar 100-5, 150 mm \times 4.6 mm) used as a reversed stationary phase and mobile phase was water (previously adjusted with phosphoric acid to a pH of 5.5), methanol and acetonitrile (20:20:60) with the flow rate 1.0 ml/min. The ultraviolet detector was set at 247 nm. A linear correlation between each concentration and its own AUC within concentration ranges of 15 to 100 μg/ml for citicoline and 7.5 to 50 μg/ml for simvastatin with a correlation coefficient 0.9969 for citicoline and 0.994 for simvastatin were produced. The within and between-day precision and accuracy were both in acceptable ranges. The outcomes of these tests show an accurate, rapid and robust HPLC-UV method for successful analysis of both simvastatin and citicoline simultaneously.

\textit{Keywords:} Citicoline, HPLC, Simvastatin.

\section*{1. Introduction}
The most effective drugs for hyperlipidemia are statins. They inhibit a rate-limiting enzyme in the cholesterol biosynthesis, called 3-hydroxy-3-methyl-glutaryl-CoA reductase (1). Citicoline or Cytidine 5’(triadhydrogen diphosphate) P’[2-trimethylammonio) ethyl] ester is a cerebral vasodilator agent which used for central nerves system disorders. A role of statins, in neurological disorders like Alzheimer’s disease has been demonstrated in several studies (2-7). As well as, the positive effect of citicoline on Alzheimer’s disease in elderly patients has been demonstrated (8). Hence, it can be realized that the concurrent use of simvastatin and citicoline have beneficial effect on Alzheimer’s disease. For pharmacokinetic evaluating, a reliable analysis method to simultaneous assay both simvastatin and citicoline, is a critical step. In the present study, a selective, accurate, sensitive and reproducible high performance liquid chromatography with ultraviolet detector (HPLC-UV) method has been developed for simultaneous detection of simvastatin and citicoline.

\section*{2. Materials and methods}

\subsection*{2.1. Materials}
Citicoline was purchased from Alborz Darou pharmaceutical company, Iran. Simvastatin was purchased from Artemis biotech Ltd (a group company), India. All solvents used in this study, were HPLC grade. Water which used in present study, was filtered and deionized by deionized water filtration system (Millipore, Germany).
2.2. HPLC apparatus and conditions

The high performance liquid chromatography system used in this work had an ultraviolet detector (Knauer, model k-2600, Berlin, Germany), a pump-controller unit (Knauer, Wellchrom®, k-1001, Berlin, Germany) and a Rheodyne injector which equipped with a 20 μl loop (Rheodyne, Model 7725, USA). A C18 column (Eurosphere 100-5 C18, 150 mm×4.6 mm with precolumn, Germany) was used as a stationary phase and mobile phase was water (previously adjusted with phosphoric acid to a pH of 5.5), methanol and acetonitrile (20:20:60), delivered at a flow rate 1.0 ml/min. the detector was set at a 247 nm. The analysis of chromatograms was performed by compatible software (EZChrom, Elite®, Germany). The assay was validated through a complete series of validation tests.

2.3. Standard preparation

Concentrations of standard solutions are given in Table 1.

2.4. System suitability tests

To verify the acceptable performance of current method, system suitability tests should be used. System suitability test parameters are given as follow (Eq. 1):

\[
N = 5.54 \left( \frac{T_R}{W_{h/2}} \right)^2
\]

(Eq. 1)

Where \(N\) is the number of theoretical plates represented column efficiency, \(T_R\) is the peak retention time and \(W_{h/2}\) is the peak width at 0.5 peak height in (Eq. 2).

\[
ps = \frac{W}{2f'}
\]

(Eq. 2)

Where \(ps\) belongs to peak symmetry or tailing factor, \(W\) stands for the peak width at 0.05 peak height and \(f\) is the front half-width of the peak at 0.05 peak height (Eq. 3).

\[
K' = \left( \frac{Rt}{Ta} \right) - 1
\]

(Eq. 3)

Where \(K'\) indicates the retainability or capacity factor, \(T_R\) is the peak retention time and \(T_a\) is the retention time of solvent peak (9).

2.5. Analysis validation tests

To achieve a high degree assurance of method, characteristics like selectivity, linearity, accuracy and precision should be evaluated during the method development (10).

2.5.1. Accuracy and selectivity

To determine the accuracy of the method, absolute recoveries of samples were obtained by measuring the ratio of the concentration obtained from standard curve to nominal concentration.

For calculating the selectivity of method, analyzing different samples included both citicoline and simvastatin in aim to find out probable interferences with possible degraded as well as investigate the power of method in both analytes separation was performed.

2.5.2. Linearity

In order to prepare standard solutions (part 2.3), three samples were prepared for each concentrations. For each drug, based on its peak AUC versus its own related concentration, linear regression were analyzed.

2.5.3. Precision

2.5.3.1. Within-day variations

Each concentration was prepared triplicate and each of them was injected to HPLC in

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Citicoline Concentration(μg/ml)</th>
<th>Simvastatin Concentration(μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>75</td>
<td>37.5</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>25</td>
</tr>
<tr>
<td>4</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>30</td>
<td>15</td>
</tr>
</tbody>
</table>
same day. Coefficient of variations (CV%) for all cases were measured.

2.5.3.2. Between-day variations

For between day variations analysis, each standard solutions were analyzed by HPLC in three different days. For each case, the CV% was calculated.

3. Results

3.1. Drug assay

In this present study, HPLC was used to analyze citicoline and simvastatin simultaneously. The HPLC system used for this purpose was isocratic with water (with pH of 5.5), methanol and acetonitrile (20:20:60) as a mobile phase and C18 column as the stationary phase. Retention time was 2 and 9 minutes at 247 nm for citicoline and simvastatin respectively (Figure 1).

Table 2. Parameters of system suitability for citicoline.

<table>
<thead>
<tr>
<th>Retainability</th>
<th>Tailing factor</th>
<th>Number of theoretical plates</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.08</td>
<td>0.92</td>
<td>120.65</td>
</tr>
</tbody>
</table>

Table 3. Parameters of system suitability for simvastatin.

<table>
<thead>
<tr>
<th>Retainability</th>
<th>Tailing factor</th>
<th>Number of theoretical plates</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.85</td>
<td>1.89</td>
<td>1734.12</td>
</tr>
</tbody>
</table>

3.1.1. System suitability tests

The chromatographic suitability was demonstrated by system suitability tests. Its parameters (tailing factor(ps), retainability(K´) and number of theoretical plates(N)) for each citicoline and simvastatin are given in table 2 and 3.

3.1.2. Analysis validation tests

In order to prove the validation of the current method for further works, evaluation of validation tests by precision, accuracy, selectivity, and linearity (Table 4 and 5) is essential (11). The acceptance of current assay was proved in between and within day variations tests.

3.1.2.1. Accuracy and selectivity

The accuracy of current method obtained during the within and between variations are given in table 6 and 7.

To discover the power of method in analyte separation, the selectivity of the method was
Figure 2. Calibration curves for citicoline (n=3).

determined by injecting different samples containing both citicoline and simvastatin to HPLC system (12). As shown in figure 1, this analytical method has an enough selectivity for analysis of both analytes and has no interaction with each other.

3.1.2.2. Linearity

Figure 2 and 3 demonstrated a linear correlation concentration-AUC in concentrations 15 to 100 μg/ml and 7.5 to 50 μg/ml for citicoline and simvastatin respectively. The linear regression was

Table 4. Values of standard curves for citicoline.

<table>
<thead>
<tr>
<th>R square</th>
<th>Adjusted R square</th>
<th>F value of regression</th>
<th>P value of intercept</th>
<th>P value of X variable</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9969</td>
<td>0.9958</td>
<td>956.4338</td>
<td>0.133402</td>
<td>7.43E-05</td>
</tr>
</tbody>
</table>

Table 5. Values of standard curves for simvastatin.

<table>
<thead>
<tr>
<th>R square</th>
<th>Adjusted R square</th>
<th>F value of regression</th>
<th>P value of intercept</th>
<th>P value of X variable</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9944</td>
<td>0.9925</td>
<td>529.84860</td>
<td>0.08009</td>
<td>0.00018</td>
</tr>
</tbody>
</table>

Table 6. Within and between day variations of the assay method for quantitation of citicoline (n=3).

<table>
<thead>
<tr>
<th>Citicoline concentration (μg/ml)</th>
<th>Within day variations</th>
<th>Between day variations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CV%</td>
<td>Accuracy%</td>
</tr>
<tr>
<td>15</td>
<td>9.19</td>
<td>92.66±1.28</td>
</tr>
<tr>
<td>20</td>
<td>5.89</td>
<td>107.92±1.27</td>
</tr>
<tr>
<td>50</td>
<td>7.30</td>
<td>103.88±3.79</td>
</tr>
<tr>
<td>75</td>
<td>1.93</td>
<td>103.60±1.50</td>
</tr>
<tr>
<td>100</td>
<td>1.99</td>
<td>92.16±1.84</td>
</tr>
</tbody>
</table>
Concurrent analysis of Simvastatin and citicoline

0.9969 for citicoline and 0.994 for simvastatin.

3.1.2.3. Precision

Table 6 and 7 show values obtained during within and between day variations.

4. Conclusion

Results show that the method used in this work has an enough selectivity for concurrent determination of both citicoline and simvastatin in short time. As given in table 3 and 4, the differences between adjusted R squared (0.9958 and 0.9925 for citicoline and simvastatin respectively) and R squared (0.9969 and 0.9944 for citicoline and simvastatin respectively) are negligible. As expected, P value of intercept is more than 0.05 and P value of X variable is less than 0.05 for both citicoline and simvastatin (Table 3 and 4) that are significant and pointless respectively. Precision and accuracy have been proven by between and within day variation tests (Table 5 and 6), as the amount of accuracy for all concentrations is within 80% to 120% which prove the accuracy and reliability of current analysis.

In conclusion, we can claim that the described analysis method has enough optimum potency and can be used in further pharmaceutical

<table>
<thead>
<tr>
<th>Simvastatin concentration (μg/ml)</th>
<th>Within day variations</th>
<th>Between day variations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CV%</td>
<td>Accuracy%</td>
</tr>
<tr>
<td></td>
<td>Mean ± SD</td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>3.45</td>
<td>96.17±0.25</td>
</tr>
<tr>
<td>9.9</td>
<td>3.43</td>
<td>99.60±0.34</td>
</tr>
<tr>
<td>24.9</td>
<td>3.94</td>
<td>106.30±1.04</td>
</tr>
<tr>
<td>37.5</td>
<td>0.90</td>
<td>94.52±0.32</td>
</tr>
<tr>
<td>50</td>
<td>7.24</td>
<td>105.80±3.83</td>
</tr>
</tbody>
</table>
Acknowledgement

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Conflict of Interest

None declared.

5. References


