Validation of High Performance of Liquid Chromatography Method for the Determination of Levonorgestrel and Ethinyl Estradiol in Tablets

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Abstract-
The objective of this research was to develop and validate a simple, specific and accurate reverse phase of high performance of liquid chromatographic method for the determination of levonorgestrel (LVG) and ethinylestradiol (EE) in tablets. The chromatographic system included column SunFire ODS (150 mm × 4.6 mm i.d., particle size at 5 µm), mobile phase consisting of acetonitrile: methanol: aquabidest (60:15 :25) with the flow rate of 1 mL/minute and effluents monitored at 230 nm. The validation of RP HPLC method for the simultaneous determination of LVG and EE was determined by accuracy, precision, linearity, and limit of detection (LOD) as well as the limit of quantitation (LOQ) parameters. The linearity range of both drugs was 1-70 µg/mL and 2-14 µg/mL for LVG and EE, respectively. The recoveries of LVG and EE were at 101.78% and 102.44% with the coefficients of variation of 0.94% and 1.92%, successively. The LOD of LVG and EE value were of 0.84 µg/mL and 0.03 µg/mL, and LOQ value were of 2.79 and 0.09µg/mL, respectively.

Conclusion:
RP-HPLC method for the simultaneous determination of LVG and EE was simple, rapid and valid.

Keywords: Levonorgestrel, ethinylestradiol, validation.

INTRODUCTION
Levonorgestrel is a synthetic progestogen [1] used both as an effective, safe and emergency contraception and as an alternative or a combination of hormonal replacement therapy (HRT) [2]. Oral levonorgestrel is perfectly absorbed and does not undergo hepatic metabolism (first pass effect). Serum levels are achieved within 1.1 ± 0.4 hours depending upon the dose and individual variation [3]. Other studies have mentioned that after the administration of levonorgestrel, the levels per oral 64 nmol/L per vaginal and 10.7 nmol/L will be achieved much faster time after oral administration of 1.4 hour to 6.6 hours per vaginal [4].

Research on the determination of levonorgestrel has reported such as radioimmunoassay [5], chemiluminescent immunoassay [6], spectrophotometry [7], voltammetry [8], HPTLC [9]; [10], capillary electrophoresis [11], HPLC [12,13], LC-MS/MS [14-17], GC / MS [18], and GC-MS/MS [19] in human plasma, HPLC-UV in rat plasma [20] and HPLC in pharmaceutical preparations [21]. It also reported the determination of levonorgestrel levels in water samples from the environment or municipal waste stream with GC-MS method, LC-ESI/MS, LC-APCI/MS and LC-MS/MS [22-24], HPLC with a cloud point extraction [25].

Through playing an important role in contraception, levonorgestrel has some serious side effects such as irregular period menstruation, changes in libido, and breast cancer [26]. Pharmacologic and toxic effect depends upon the doses of the regiment. It is necessary to determine the active substances in the product to ensure the quality and safety for consumer.

No research has been found that conducted the assay of levonorgestrel and ethinylestradiol in tablets by chromatographic techniques using a detector PDA (Photo Diode Array) so far. The advantages of the PDA detector are the capabilities in detecting multiple wavelengths simultaneously, analyzing the peak purity, and possibility to be used for kinetic studies [27]. Previous research can be developed to obtain a better separation with several modifications such as the composition and kinds of solvent and flow rates. Therefore, it is necessary to develop and validate a procedure to simultaneously determine levonorgestrel and ethinylestradiol in tablet preparations by reversed-phase HPLC.

MATERIALS AND METHODS
Materials
HPLC Waters Alliance 2965 with PDA 2998 detector, software Empower, stationary phase SunFire C18 (150 x 4.6 mm, particle size 5 µm), sonificator (Branson), Millipore filter (Acrodisc 25 mm PVDF 0.45 µm), vortex (MaxiMax II), analytical balance (AND with sensitivity 0.01 mg), Levonorgestrel standard (pharmaceutical grade from PT. Triyas Naga Mas Farma), ethinyl estradiol 95% of purity (Beijing Zizhu Tiangong Science And Technology Co., LTD), tablet contraception (factory A, B, and C), Methanol (E. Merck), acetonitrile (E. Merck), Aquabidest (PT. Ika Pharmindo Putramas).
Instrumentation

HPLC with automatic sampler, Stationary phase SunFire C18 (150 x 4.6 mm, particle size 5 µm), mobile phase of acetonitrile: methanol: water (60:15:25), and detection with PDA at λ = 230 nm.

PROCEDURE

Preparation procedure

The uniformity of dosage units of contraception tablets was performed based upon USP [32]. It was started by accurately weighing 10 tablets individually and calculating the content, as expressed by % of label claim. The procedure of uniformity of contraception tablet weight was performed individually until reaching 10 weighed tablets. Then the average concentration of tablet and standard deviation was calculated. The tablets were crushed and sieved in which the powder obtained was weighed approximately at 80.0 mg. The samples were diluted by adding methanol to 5.0 mL and sonificated for 10 minutes. The solution then was filtered by Millipore and injected to HPLC.

Validation

Accuracy and precision. To study the reliability and suitability of the developed method, tablet samples were added with three concentration levels of levonorgestrel and ethinylestradiol. Intra-day precision was determined by analysing one concentration level of levonorgestrel and ethinylestradiol in one day, while inter-day precision was determined within three consecutive days. Each sample was determined in 6 replicates. The result was then compared with the standard of requirement [28]

Linearity. To study the linearity range of levonorgestrel and ethinylestradiol, seven serial dilutions were prepared by mixing standard stock solutions in the range of 1-70 µg/mL for levonorgestrel and 2-14 µg/mL for ethinylestradiol, respectively. The peak areas of samples were recorded. The slope and intercept of the calibration graph were obtained by the linear regression of peak area versus concentration: y = bx + a where x refers to the concentration and y refers to the peak area. The linearity was assessed by linear regression analysis, which was calculated by the least square method. The coefficient regression was compared by the standard of requirement [29].

Sensitivity. The sensitivity of the method was determined by means of the limit of detection (LOD) and limit of quantitation (LOQ); both of which were calculated based on a statistical method through a linear regression. LOD was calculated based on the standard deviation of the calibration curve (s) and the slope of curve (S), using the equation LOD = 3 x SD/b. LOQ meanwhile was calculated by the equation LOQ = 10 x SD/b.

RESULTS AND DISCUSSION

Determination of levonorgestrel and ethinylestradiol in tablets

Research on the determination of levonorgestrel and ethinylestradiol in tablet dosage was previously carried out by Prasad et al., [21] using the reversed-phase HPLC method with mobile phase acetonitrile and water (42:58), flow rate of 2.2 mL/min and effluent monitored by UV detector at λ 210 nm. The value of separation resolution was 6 and the retention time of ethinylestradiol and levonorgestrel was within 3.34 minutes and 4.89 minutes, respectively. Modification chromatographic system from Prasad et al. [21] was the changes of composition and flow rates of mobile phase and the use of PDA detector. Optimum mobile phase was decided based on the resolution value and number of theoretical plates. The results of the optimization phase were acetonitrile: methanol: aquabidest (60:15:25) with the flow rates at 1.0 mL/minute. In addition to perform the optimization of mobile phase, the optimization of wave length was also performed to obtain an appropriate wave length (Figure 1).

![Figure 1. Chromatographic of separation of levonorgestrel and ethinylestradiol](image)

In the reversed-phase HPLC system ethinylestradiol and levonorgestrel mixture would be separated in order. Water of levonorgestrel and ethinylestradiol contained the solubility of 2.05 mg/mL and 11.30 mg/mL, respectively [30]. In this case ethinylestradiol appeared faster due to its weaker relative solubility to the mobile phase and intermolecular forces with stationary phase. Levonorgestrel here emerged in the second time in view of the stronger intermolecular forces on the non-polar stationary phase. The type of inter-molecular interactions between levonorgestrel and stationary phase (ODS) is called Van der Waals forces. Retention time for ethinylestradiol and levonorgestrel were achieved in 2.320 and 2.991 minutes, respectively.

Validation Parameters

Accuracy

The determination of accuracy is very important parameters in the method of quantitative analysis as this parameter shows if systematic or random errors occur. There are several techniques to determine accuracy such as spike method, addition method, and comparison method.
with standard method. In this research, accuracy parameters were obtained by addition method. The accuracy (Table 1) showed that good recoveries and RSD that agree with acceptance criteria. Acceptance criteria for accuracy with a concentration level of 10-100 µg/mL were 90-107% and RSD value of 5.3% [28].

<table>
<thead>
<tr>
<th>Concentration added (%)</th>
<th>Recovery EE (%)</th>
<th>Recovery LVG (%)</th>
<th>RSD of Recovery EE (%)</th>
<th>RSD of Recovery LVG (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>104.33</td>
<td>105.00</td>
<td>0.90</td>
<td>1.29</td>
</tr>
<tr>
<td>100</td>
<td>99.33</td>
<td>98.33</td>
<td>2.11</td>
<td>0.88</td>
</tr>
<tr>
<td>120</td>
<td>103.67</td>
<td>101.67</td>
<td>2.76</td>
<td>0.65</td>
</tr>
<tr>
<td>Average (%)</td>
<td>102.44</td>
<td>101.78</td>
<td>1.92</td>
<td>0.94</td>
</tr>
</tbody>
</table>

Precision

Precision was described by intra and inter-day precision. The results of determination of intra-day precision (Table 2) showed the RSD value of ethinylestradiol and levonorgestrel were 6.42% and 5.64% respectively. Meanwhile, inter-day precision (Table 2) showed the RSD value of ethinylestradiol and levonorgestrel on different days were at 4.65% and 2.28% respectively. The RSD values of ethinylestradiol and levonorgestrel were in line with the acceptance criteria [28,31].

<table>
<thead>
<tr>
<th>Precision</th>
<th>%RSD (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVG</td>
<td>EE</td>
</tr>
<tr>
<td>Intra-day</td>
<td>5.64</td>
</tr>
<tr>
<td>Inter-day</td>
<td>2.28</td>
</tr>
</tbody>
</table>

Linearity Range

Linearity is a parameter that provides a linear response of the method to the changes of sample concentrations. The results of the determination of this parameter are r or r² (r square). Acceptance criteria for linearity parameter are the value of the correlation coefficient > 0.98 for sample and > 0.99 for standard measurements [29]. The correlation coefficients (r²) of ethinylestradiol and levonorgestrel were at 0.992 and 0.997, respectively. Linearity range of ethinylestradiol and levonorgestrel were at 2-14 µg/mL and 1-70 µg/mL. These results were same with the ones obtained by Prasad et al. [21]. This research did not determine the concentration still providing a linear response.

Calculation of LOD and LOQ

The determination of the parameters in this study used the calculation method based on the standard deviation of the response (peak area) and the slope of the calibration curve. The results of the limit of detection and limit of quantification of ethinylestradiol and levonorgestrel were at 0.838 µg/mL; 2.792 µg/mL, and 0.028 µg/mL; 0.092 µg/mL, respectively. LOD and LOQ were obtained by calculation tested by comparing the values with the smallest analytes. A correct method for recording a determination result depending on the quantity of analytical signals is presented in Table 3 [32].

<table>
<thead>
<tr>
<th>Result, x</th>
<th>Recording of result</th>
</tr>
</thead>
<tbody>
<tr>
<td>X &lt; LOD</td>
<td>Not determined</td>
</tr>
<tr>
<td>LOD ≤ x &lt; LOQ</td>
<td>Not quantified</td>
</tr>
<tr>
<td>X ≥ LOQ</td>
<td>Value of concentration</td>
</tr>
</tbody>
</table>

Uniformity of Content Evaluation

Content uniformity is required by the USP 32 [33] for a product with the ratio of active substance to the additional material is <25% active substance or content of <25 mg. The acceptance criteria of content uniformity are the result of measurement of 10 tablets that should not be more than L1% (the maximum value allowed). If it is larger than L1%, the measurements will be repeated for the other 20 tablets. Claims on the label for ethinylestradiol and levonorgestrel products A, B and C respectively are at 0.03 mg and 0.15 mg. RSD values of measurements of 10 tablets from three different products were 3.73 and 5.29%; 5.50 and 4.10%; 2.72 and 2.16% for ethinylestradiol and levonorgestrel, product A, B, and C, respectively.

The acceptance of content uniformity values for L1% was at 15.0% of the average value of content compared with the SD value measurements. Calculation results (Table 4) found that all products have met the requirements of the content uniformity.

<table>
<thead>
<tr>
<th>product</th>
<th>Average of concentration in tablet</th>
<th>SD</th>
<th>L1%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EE (mg)</td>
<td>LVG (mg)</td>
<td>EE</td>
</tr>
<tr>
<td>A</td>
<td>0.030</td>
<td>0.149</td>
<td>0.002</td>
</tr>
<tr>
<td>B</td>
<td>0.029</td>
<td>0.143</td>
<td>0.001</td>
</tr>
<tr>
<td>C</td>
<td>0.029</td>
<td>0.143</td>
<td>0.001</td>
</tr>
</tbody>
</table>
CONCLUSION
1. The development and validation of reverse-phase HPLC method for the determination of levonorgestrel and ethinylestradiol have been valid. The results of accuracy parameters for levonorgestrel and ethinylestradiol were at 101.78% and 102.44% with RSD, respectively 0.94% and 1.92%. Intra-day and inter-day precision of levonorgestrel and ethinylestradiol were at 5.64% and 6.42%, 2.28% and 4.65%, respectively. Linearity parameters was in the range 1-70 µg/ mL levonorgestrel and ethinylestradiol was in the range 2-14 µg/ mL. the value of $r^2 = 0.997$ and $r^2 = 0.992$, respectively. LOD value of levonorgestrel and ethinylestradiol is 0.092 µg/ mL and 2.792 µg/ mL.

2. Modification of chromatographic system had a better separation.

REFERENCES
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