INTRODUCTION

Lynestrenol is a derivative of progesterone, which is an endogenous steroid hormone produced by the ovaries, and cortex. Lynestrenol is used for women contraception and as a treatment for menstrual bleeding, maturation of the follicles in the ovaries and ovulation. For menstruation delay, doses of lynestrenol is 1 mg/day, which plasma levels exceeded 0.7 ng/ml [1, 2].

Lynestrenol in the digestive tract is absorbed rapidly with bioavailability, about 64%. Lynestrenol is metabolized in the liver through norethindrone through hydroxylation reaction on C-3 and 3β hydroxy-lynestrenol with CYP2CP, CYP2C19, and CYP3A4 enzymes [3, 4].

Determination of the concentration of drugs in plasma through bioavailability studies that can measure the speed and amount of drugs absorbed by the body is a study of drug products that illustrates the effectiveness of drug preparations [5].

High-performance liquid chromatography (HPLC) methods are commonly used in analyzing drug concentrations in plasma. HPLC can separate substances that interfere with analysis and determine the smallest concentration of drug in plasma [6, 7].

Guidelines for validation of bioanalysis methods generally refer to the Food and Drug Administration and also the European Medicines Agency. However, research that refers to EMEA is still rarely done because of relatively new in the revision of the bioanalysis validation method [8, 9].

In this research, the liquid-liquid extraction method was performed on human blood plasma obtained from the Indonesian Red Cross. Liquid-liquid extraction using pentane and bioanalysis validation of the extraction method used refers to European Medicines Agency guidelines (EMEA). High-Performance Liquid Chromatography was used for analysis with a mobile phase of acetonitrile containing 0.1% formic acid in water (60:40) with a flow rate of 1.0 ml/min in the reverse phase of column C18 (150 mm) and detected at wavelength 204 nm [10].

MATERIALS AND METHODS

Instrumentation

High-Performance Liquid Chromatography (Shimadzu, Japan) equipped with a UV-VIS SPD 10A detector. LC-Solution data processor and integrator CBM 10A; microsyringe 5 µl (Hamilton, Nevada); column C-18 (Waters, SunfireTM 5 μm; 150 x 4.6 mm); ultracentrifuge (Corning); vortex mixer (VM-300 Germany Industrial Corporation, USA), sonicator (Branson 3200, USA).

Chemical

Lynestrenol (Indian Penal Code, Pirumadara), Levonorgestrel (Sigma Aldrich, Singapore), Aconitripr Pro HPLC (Merck), Methanol Pro HPLC (Merck), Formic Acid (Merck), Pentane (Merck), Potassium Dihydrogen Phosphate (Merck), Sodium Hydroxide (Merck), anticoagulant Citrate (Indonesian Red Cross), human blood (Indonesian Red Cross).

Chromatography system

This study was implementing HPLC with a UV-Vis detector, column C18 with length 150 x 4.6 mm, detected at 204.0 nm wavelength, using a mobile phase of acetonitrile containing 0.1% formic acid in water (60:40) with a flow velocity of 1.0 ml/min, injection volume was 20 µL and used levonorgestrel as the internal standard.

Preparation of standard solution

10.0 mg lynestrenol was dissolved in methanol in a 10 ml volumetric flask to obtain 1.0 mg/ml (1000 ppm) concentration. Levonorgestrel standard 10.0 mg was dissolved in the mobile phase to obtain 1.0 mg/ml (1000 ppm). The dilution was conducted to obtain solutions in certain concentrations.

Plasma sample preparations

The liquid-liquid extraction method was conducted by adding 25 µl levonorgestrel 100 µg/ml to 500 µl plasma which containing 50 µg/ml lynestrenol and 1 ml buffer phosphate 7.4 in microtube and vortexed for 30 seconds. Next, 1 ml pentane was added, and the mixtures were
vortexed for 3 min and then centrifuged at 10,000 rpm for 10 min on 25 °C. The organic phase was transferred to another tube and repeated three times and evaporated with Nitrogen gas (N\textsubscript{2}). The residue was reconstituted in 100 μl methanol. Afterward, 20.0 μl aliquot was injected into the HPLC system.

Validation of lynestrenol analysis in plasma

Refraining to EMEA Guideline for Bioanalytical Method Validation 2011, before validation running, system suitability must be apparent. Validation of the lynestrenol analytical method in plasma was conducted with selectivity carry over, LLOQ, linear calibration curve, accuracy, precision, and recovery, dilution integrity, and stability [10].

RESULTS AND DISCUSSION

The plasma concentration of lynestrenol is relatively low and shows significant inter-individual variations in uptake, storage, and metabolism. The analysis of lynestrenol in human plasma has been reported with detection limits lower than 0.7 ng/ml by HPLC. Therefore, a more sensitive analytical method is needed to monitor the precise concentrations in plasma during pharmacokinetic studies. The study was performed with low lynestrenol dosing.

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A different method for extraction of lynestrenol in plasma is described using C18 solid-phase extraction. Extraction of lynestrenol

of this research in plasma with various organic solvents. Extraction with pentane gave high recoveries regardless of the concentration of lynestrenol; therefore, pentane was chosen for the extraction.

Based on the mobile phase was used in this validation is acetonitrile, containing 0.1% formic acid in water (60:40). The addition of formic acid on the mobile phase had the desirable effect of maintaining a reasonably high capacity factor (k) resolution (Rs) and tailing factor (Tf) for the analyte. A concentration of 1 mmol formic acid in the mobile phase was large enough to achieve the reproducible elongated retention time (t\textsubscript{R}) for the analyte [11].

System suitability

According to the European Medicines Agency in 2011, system suitability tests are an integral part of any liquid chromatographic method. The parameters were performed on lynestrenol and levonorgestrel, including retention time (t\textsubscript{R}), areas (µV. s), resolutions (Rs), the number of theoretical plates (N), HETP, and tailing factor (Tf). All these parameters were found to be satisfactory and within the reported acceptance listed in the reference. As shown in table 1, the tailing factor was<2, acceptable retention time along with good resolution is indicative of good efficiency and selectivity of the method for separation of lynestrenol and levonorgestrel as an internal standard.

Table 1: HPLC system suitability parameters for determination of lynestrenol and levonorgestrel

| Analytes       | Retention time (t\textsubscript{R}) | Areas (µV. s) | resolutions (Rs) | Tailing factors (Tf) | HETP | Theoretical plates (N) |
|----------------|-------------------------------------|---------------|------------------|----------------------|------|------------------------|
| Lynestrenol    | 4.018                               | 11334         | 13.241           | 0.782                | 0.00034 | 21324.5                |
| Levonorgestrel | 5.560                               | 8498          | 9.242            | 0.837                | 0.00024 | 2435.4                 |

Lower limit of quantification (LLOQ)

The LLOQ value is related to the sensitivity of a method, smaller the LLOQ value indicating of more sensitive method that can measure the lowest concentration of analytes from the plasma matrix. In bioanalysis, the LLOQ value must cover a minimum of 1/20 of the maximum concentration (C max) of the analyte in the plasma. The LLOQ which meet the requirements giving 20% of %diff (EMEA, 2011). Based on the analysis of lynestrenol in plasma, table 2 shows that the LLOQ value of 40.0 ng/ml was applied, while that was applied at 20.0 ng/ml (half concentration), the value of %diff would rise to above the requirements.

Table 2: LLOQ value of lynestrenol

| Real concentration (ng/ml) | Measured concentration±SD (ng/ml) | % CV | %Diff      |
|---------------------------|-----------------------------------|------|------------|
| 40                        | 42.596±2.493                      | 5.853| -3.445 to 11.786 |
| 20                        | 14.545±2.918                      | 20.061| -3.4969 to 1.178 |

Selectivity

Selectivity test was conducted in the blank plasma and LLOQ concentrations, 40.0 ng/ml using six different plasmas (A to F). Each sample was analyzed and evaluated separately to determine interference. The results show, the selectivity study on the blank did not show any interference or impurities during the analysis of the analytes and the internal standard. The results show that the value of the % interference is 0.063-11.658%. Selectivity data of lynestrenol are shown in table 3.

Table 3: Selectivity value of lynestrenol

| Real concentration (ng/ml) | Plasma | Measured concentration±SD (ng/ml) | % CV | %Diff |
|---------------------------|--------|-----------------------------------|------|-------|
| 40                        | A      | 38.877±0.217                      | 6.607| 0.063-11.658 |
|                           | B      | 42.25±1.823                      | 0.000 | 0.252 |
|                           | C      | 41.365±2.002                     | 3.445| 11.786 |
|                           | D      | 38.909±0.836                     | 3.445| 11.786 |
|                           | E      | 39.961±1.027                     | 3.445| 11.786 |
|                           | F      | 35.336±1.242                     | 3.445| 11.786 |

Calibration curve and linearity

The calibration curve consists of plasma blanks (plasma without analytes and internal standards), zero samples (plasma with internal standards) and non-zero plasma (plasma with analytes and internal standards) as many as 7 concentrations, namely 40.0 ng/ml, 80.0 ng/ml, 120.0 ng/ml, 250.0 ng/ml, 500.0 ng/ml, 750.0 ng/ml, 1000.0 ng/ml. The value of % diff from the results of the measurement concentration should not deviate more than±15%, except for LLOQ not to deviate more than±20%. Based on statistical calculations, a linear regression equation is produced, namely y = 0.023x+0.252 with a correlation coefficient (r) = 0.9994 where x is the sample concentration, and y is PAR (peak area ratio) between the response area of the analyte and the internal standard. The calibration curve was linear with the correlation coefficient (r=0.9991) in the concentration range from 40.0 to 1000.0 ng/ml. LLOQ concentration of lynestrenol was 60.0 ng/ml, with the CV value of 3.6% and % diff between 3.445 to 11.786%.

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Carry-over

Carry-over must be minimized during the method development process. During the validation process, carry-over was assessed by injecting blanks, after previously being injected with a high concentration sample or calibration standard on ULOQ (1000.0 ng/ml) of five replicas and analyzed sequentially. Carry-over on the blank should not be more than 20% of LLOQ and 5% for internal standards [11]. Carry-over value after injection of high concentrations of lynestrenol range from 2.88% to 6.09% of the LLOQ response, while the standard %carry-over ranged from 0.98% to 3.35%. Based on research, carry over study results did not exceed the requirements, so it could be concluded during the analysis process that there was no meaningful participation between injections. The chromatogram of blank plasma and chromatogram of ULOQ shown in fig. 2.

Accuracy and precision

In this study, intra-day and inter-day (3 d) accuracy and precision were tested. Lynestrenol in plasma was made at LLOQ concentrations of 40.0 ng/ml, QCL low concentrations of 120.0 ng/ml, QCM moderate concentrations of 500.0 ng/ml, and QCH high concentrations of 750.0 ng/ml. Based on the results of the study, the accuracy and precision test lynestrenol values obtained %diff in the four concentrations range from -13.68% to -13.81% and the coefficient of variation in LLOQ concentrations, low, medium and high respectively was 5.62%, 7.62%, 8.53%, and 8.25%. The accuracy and precision test results have met the requirements of %diff, and CV values do not exceed ±15% for low, medium, and high concentrations; and at LLOQ concentrations do not exceed ±20% shown in table 4.

| Actual cons. (ng/ml) | Within run | Between run |
|---------------------|------------|-------------|
|                     | Measurable concentration±SD (ng/ml) | Mean accuracy (%diff) | Precision (%CV) | Measurable concentration±SD (ng/ml) | Mean accuracy (%diff) | Precision (%CV) |
| 40.0                | 40.09±2.09 | -4.44 to 6.61 | 4.48 | 40.68±3.60 | -3.51 to 8.76 | 8.86 |
| 120.0               | 125.44±6.68 | -2.18 to 7.49 | 3.84 | 116.44±4.74 | -9.61 to 6.71 | 4.07 |
| 500.0               | 468.31±42.22 | -5.42 to 8.72 | 7.17 | 518.57±30.04 | -1.28 to 7.90 | 8.53 |
| 750.0               | 726.90±38.78 | -10.81 to 1.77 | 8.12 | 785.72±48.15 | -4.24 to 9.18 | 6.12 |

Recovery

Recovery study was also carried out in the research, with the concentrations used were low, medium, and high concentrations. An absolute recovery study was obtained by comparing the measured concentration with the actual concentration. The results obtained for low, medium, and high concentrations range from 102.41% - 107.94%, 96.41% - 106.22%, and 86.32% - 104.80%, respectively. The coefficient of variation for low, medium, and high concentration analytes were 2.31%, 4.71%, and 4.55%, respectively. Next, the extraction efficiency
study was calculating by %recovery. The recovery test (%recovery) provides information about the extraction efficiency of an analytical method under conditions that can change. The results do not have to be 100% but should be consistent, precise, and reproducible. The % recovery is obtained by comparing the analyte response that was added after the plasma blank was extracted with the response of the analyte that was extracted. Based on the results of the study, shown in table 3, the coefficient of variation for low, medium, and high concentration analytes in a row were 2.81%, 3.49%, and 8.82%. Recovery data of lynestrenol are shown in table 5.

Dilution integrity

The effect of dilution was explored to validate that human plasma samples more significant than the highest concentration of the calibration range could be diluted with blank plasma and quantified without losing reproducibility. This parameter serves to assess the dilution process during the bioanalysis process to be accurate, precise, and reliable. The test results obtained must be within the established criteria, namely ±15%. The dilution integrity test must include the dilution used in the study sample [12]. The accuracy and precision values of the dilution still fulfill the strict and precise criteria so that the dilution process carried out during the bioanalysis process can be trusted.

Stability

The storage stability of lynestrenol was investigated to establish the probability of degradation occurred during long term storage in each plasma. Stability was analyzed using QCL and QCH samples, each with three replications. For the short-term stability, samples were stored at room temperature, and the stability was observed in 0, 6, and 24 h. The obtained results showed that lynestrenol samples were stable to be stored in the room temperature minimum of 24 h, shown in table 6. The obtained results showed %diff for the short-term stability of lynestrenol was from -1.95% to 1.99%, and %diff for lynestrenol short stability was from -1.85 to -1.64%. The results showed that the lynestrenol solutions and levonorgestrel solutions were stable when stored at room temperature for a minimum of 24 h.

For long-term stability, samples were stored in the freezer in -4 °C for 7 and 14 d. The obtained results showed that lynestrenol samples were stable to be stored in the freezer in -4 °C minimum 14 d, shown in table 7. For long-term stability, the obtained results of %diff lynestrenol were between -2.06% to -1.96%, and the %diff of lynestrenol solutions were between -1.81% to -1.57% for 14 d in -4 °C. Therefore, lynestrenol and levonorgestrel stock solutions could be used for 14 d.

The freeze-thaw stability test was also conducted. Lynestrenol in plasma was found stable after freeze-thaw test in three cycles minimum. The results are shown in Table 8. The short-term stability of standard solutions of lynestrenol and the internal standard were tested at room temperature for 24 h. The long-term stability of the standard solutions was stored in 4 °C for 1, 10, and 18 d.

Table 5: Recovery values of analyte and internal standard

| Actual cons. (ng/ml) | Analit | Internal standard |
|-------------------|--------|------------------|
|                   | % Recovery±SD (ng/ml) | CV (%) | % Recovery±SD (ng/ml) | CV (%) |
| 120.0             | 106.49±2.95          | 2.31   | 102.82±5.73          | 2.81   |
| 500.0             | 99.81±23.51          | 4.71   | 98.91±4.82           | 3.49   |
| 750.0             | 90.27±33.57          | 4.55   | 105.82±6.49          | 8.82   |

Table 6: Lynestrenol short-term stability in the plasma in room temperature

| Time       | QCL 120.0 ng/ml Measurable Concentration±SD (ng/ml) | CV (%) | QCH 750.0 ng/ml Measurable concentration±SD (ng/ml) | CV (%) |
|------------|-----------------------------------------------|--------|-----------------------------------------------|--------|
| 0 hour     | 126.2±6±0.20                                   | 4.91   | 740.7±28±3.22                                  | 5.17   |
| 6 h        | 128.0±7±10.26                                  | 8.01   | 740.6±51±3.86                                  | 8.35   |
| 24 h       | 129.7±5±15.96                                  | 12.3   | 749.5±3±4.63                                   | 84.62  |

Table 7: Lynestrenol long-term stability in the plasma in -4 °C

| Day        | QCL 120.0 ng/ml Measurable Concentration±SD (ng/ml) | CV (%) | QCH 750.0 ng/ml Measurable concentration±SD (ng/ml) | CV (%) |
|------------|-----------------------------------------------|--------|-----------------------------------------------|--------|
| 0          | 126.5±4±8.02                                   | 3.80   | 724.1±41±1.70                                  | 5.76   |
| 8          | 120.4±4±6.3                                    | 3.84   | 710.6±36±6.2                                   | 5.15   |
| 18         | 123.6±4±8.2                                    | 3.94   | 729.8±56±17                                    | 7.69   |

Table 8: Lynestrenol freeze-thaw stability in the plasma

| Cycles     | QCL 120.0 ng/ml Measurable concentration±SD (ng/ml) | CV (%) | QCH 750.0 ng/ml Measurable concentration±SD (ng/ml) | CV (%) |
|------------|-----------------------------------------------|--------|-----------------------------------------------|--------|
| 0          | 132.6±4±1.68                                   | 1.27   | 734.2±15±8.88                                  | 2.16   |
| 3          | 120.0±4±2.21                                   | 1.72   | 788.4±30±12                                    | 3.82   |

CONCLUSION

The method has been validated and can be used to analyze lynestrenol in blood plasma. The simplicity of the assay, with a simple pretreatment procedure using pentane, makes it an attractive procedure for the high-throughput bioanalysis of lynestrenol.

AUTHORS CONTRIBUTIONS

All authors participated in the practical work and writing of the manuscript. Iskandaryah acted as the corresponding author, and Nurfitriyana acted as the first author, while others as co-authors.

CONFLICT OF INTERESTS

The authors declared that they have no conflict of interest.

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