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Analytical Method Validation of RP-HPLC Method for Simultaneous Estimation of Levonorgestrel and Ethinylestradiol from Combined Drug Product

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Abstract

The aim of recent study is to explore a rapid, sensitive and selective Reversed Phase HPLC method for simultaneous quantification of steroidal hormones Levonorgestrel and Ethinylestradiol from drug products. Chromatographic separation of Levonorgestrel and Ethinylestradiol were achieved by Kromasil C_8 column (4.6 mm × 150 mm, 5 µm) at 4.8 and 3.9 min by using 60:40 volume of acetonitrile and deionized water mobile phase at a flow rate of 1.0 ml/min at wavelength of 247 nm and 310 nm respectively. The injection volume was 100 µL and the column temperature was maintained at 25°C. The method was validated according to USP category I requirements which includes specificity, accuracy, precision, intermediate precision, linearity and range and robustness. The standard calibration curve were found linear (r^2>0.99) over the analytical range throughout the analysis day. Precision and intermediate precision was <3 % for Levonorgestrel and Ethinylestradiol at QC standard range. The method was found to be accurate in the range of 50 to 150% from QC standard. This method was also found robust in accordance with variation in the flow rate (± 0.5 ml/min.), organic phase ratio (± 10%), change column oven temperature (± 5°C), auto sampler temperature (5°C and 25°C) and changing column brand. The HPLC method was successfully applied to the analysis of dissolution samples of marketed Levonorgestrel and Ethinylesradiol combined tablets.

Keywords: ***Reversed phase HPLC; Accuracy; Linearity; Robustness; Dissolution***

Introduction

Oral contraceptives are three types on the basis of administration in the single steroidal hormones or combined form. In the sequential type, estrogen is administered alone for the first week, followed by a lower dosage of the estrogen in conjunction with a progestogen for the remainder of the course. In the second, type, commonly used, both an estrogen and a progestogen are present in the tablets (as either a single dose or in three different doses). Thirdly in the progestogen type, a progestogen alone is administered (FIG. 1).

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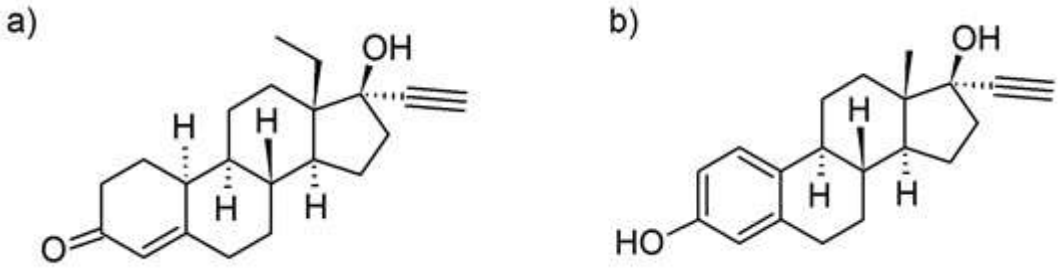

FIG. 1. **Structures of (a) Levonorgestrel and (b) Ethinylestradiol**.

Estrogen are responsible for preventing producing follicle stimulating hormone (FSH) and luteinizing hormone (LH) from pituitary gland in the brain and prevent development of the egg and supporting the uterine lining to prevent mid-cycle breakthrough bleeding. On the other side progestin are responsible for the Stopping LH production from occurring in the pituitary gland so no egg is released, causing changes to the uterine lining which make it harder for an egg to implant, limit the ability of an egg to be fertilized by sperm and causing cervical mucus to thicken, hindering the ability of the sperm to travel, so that no fertilization occur [1,2]. Combined oral contraception (COC) (both estrogen and progestin) formulations and pill improved contraceptive safety and tolerability [3]. There are several types of combinations of estrogen and progestin are now available. Some of them are Ethinylestradiol/Norgestimate [4-8], Ethinylestradiol/Norethindrone [9], Ethinylestradiol/Norethindrone acetate [10], Ethinylestradiol/Norgestrel [11], Ethinylestradiol/Levonorgestrel [12-15], Ethinylestradiol/Drospirenone [13,16-18], Ethinylestradiol/Desogestrel [19-21].

The accurate determination of Levonorgestrel along with Ethinylestadiol in complex media such as dissolution requires selective and sensitive analytical methodologies. There are various analytical methods cited in the literature used for the quantitative determination for pharmaceutical purposes. Examples include isotope dilution tandem mass spectroscopy [1], capillary electrophoresis [22], high performance liquid chromatography [23,24], Ultra high performance liquid chromatography [25], solid-phase extraction coupled with high-performance liquid chromatography–tandem mass spectrometry [26,27], ultra-high performance liquid chromatography coupled with tandem mass spectrometry [28,29], gas chromatography coupled with mass spectroscopy [27]. However some of those methods suffer from disadvantages such as a complicated and time consuming sample preparation procedures and quantification procedure.

The main objective of this study was to develop a fast, sensitive and robust method to quantify Levonorgestrel and Ethinylestradiol simultaneously from the dissolution media in accordance with USP and ICH guideline [30-32].

Materials and Method

Materials

Levonorgestrel and Ethinylestradiol chemical reference standard was purchased from the Excella GmbH & Co (Germany). Chromafil® Xtra PTFE 0.45 µm syringe filters were purchased from the Pall Corporation (Ann Arbor, MI, USA). HPLC grade acetonitrile, Sodium acetate trihydrate and glacial acetic acid were purchased from Fisher Scientific (Fairlawn, NJ, USA). HPLC ready deionized 18 Milli-Q water was obtained, in-house, from a Milli-Q Gradient A-10 water purification system, Millipore, (Bedford, MA, USA).

Dissolution

A calibrated dissolution apparatus (USP II) was used with paddles at 50 rpm and bath temperature was maintained at 37 ± 0.5°C. Dissolution media was prepared by dissolving 11.96 gm of sodium acetate trihydrate in 3 liter Milli-Q water, add 56 ml 2 M glacial acetic acid then volume 4 liter with Milli-Q water. Adjust pH to 4.5 with dilute acetic acid. Five hundred milliliters of freshly prepared dissolution media was added in each vessel and sample (6 tablet) were evaluated and dissolution samples were collected after 45 min by using Chromafil® Xtra PTFE 0.45µm syringe filters. Four milliliter was removed and next 1.5 ml was transferred to the HPLC vial, and analyzed by HPLC.

The amount of Levonorgestrel and Ethinylestradiol in test samples was calculated quantitatively and percent dissolution, from the measured peak area response for the test samples (A_U) and compared to the peak area response (A_S) for the standard Levonorgestrel and Ethinylestradiol by using following formula:

$$\text{Quantity} = (\text{Au})/\text{As} \times \text{C} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

$$\%\ \text{Dissolution} = \frac{\text{Amount Recovery}}{\text{Amount Decleared}} \times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

Here, C in the concentration in ppm.

Instrumentation and chromatographic conditions

Prominence I HPLC (Shimadzu Corporation, Japan) consisted of a quaternary pump, an automatic injector, variable wavelength detector, and a column oven. Data were processed by using Lab solution 6.82-ST1 software. Separation was achieved on a Kromasil C8 column (4.6 mm × 150 mm, 5 µm) and ProntoSIL C8 column (4.6 mm × 150 mm, 5 µm) was used for intermediate precision analysis. The flow rate was 1.0 mL/min and run time was set 12 min. The column temperature was controlled at 30°C and the injection volume was 100 µL. The detection wavelength were 247 nm for

Levonorgestrel analysis and a spectrofluorometric detector with an excitation wavelength of 285nm and emission wavelength of 310nm for Ethinylestradiol. Filtered and degassed acetonitrile and deionized water in the ratio of 60:40 was used as mobile phase.

Preparation of standard (Stock) solutions

Levonorgestrel (6 ppm) stock solution: Levonorgestrel stock solution was prepared by dissolving 12 mg of Levonorgestrel chemical reference standard in 100 ml ethanol. 5 ml of above solution was transferred in 100 ml volumetric flask and volume to the mark with dissolution media.

Ethinylestradiol (1.2 ppm) stock solution: Ethinylestradiol stock solution was prepared by dissolving 12 mg of Ethinylestradiol chemical reference standard in 100 ml ethanol. 1ml of above solution was transferred in 100 ml volumetric flask and volume to the mark with dissolution media.

Standard solution preparation

5 ml of Levonorgestrel and 5 ml of Ethinylestradiol stock solution were transferred in to 100 ml volumetric flask and volume to the mark with dissolution media.

Preparation of calibration standard solution

Levonorgestrel and Ethinylestradiol stock solution were used to prepare calibration standard solution in daily basis. Calibrated Levonorgestrel solutions were prepared at 5 concentrations by diluting stock solution to the concentrations of 0.15, 0.24, 0.30, 0.36 and 0.45 ppm. On the other side calibrated Ethinylestradiol solution were prepared at 5 concentrations by diluting Ethinylestradiol stock solution to the concentration of 0.03, 0.048, 0.06, 0.072 and 0.09 ppm. These solutions were then transferred to the HPLC vial for HPLC analysis.

Results and discussion

Method validation

The method was validated according to the ICH and United States Pharmacopeia Category I requirements. The following validation characteristics were addressed: specificity, accuracy, precision, linearity and range and robustness.

System suitability standard: System suitability standard solution was prepared daily using stock solution, for that purpose 5 ml of each stock solution (Levonorgestrel and Ethinylestradiol) were transferred in to 100 ml amber volumetric flasks and dilute up to the mark with dissolution media. System suitability was determined from five replicate injections of the system suitability solution before sample analysis. The acceptance criteria were less than 2% relative standard deviation (RSD) for peak area, greater than 2000 theoretical plates, USP tailing factor less than 2 (FIG. 2). All critical parameters were tested before sample run and it was found that all parameter met the acceptable criteria throughout all days which is shown in the (TABLE 1)

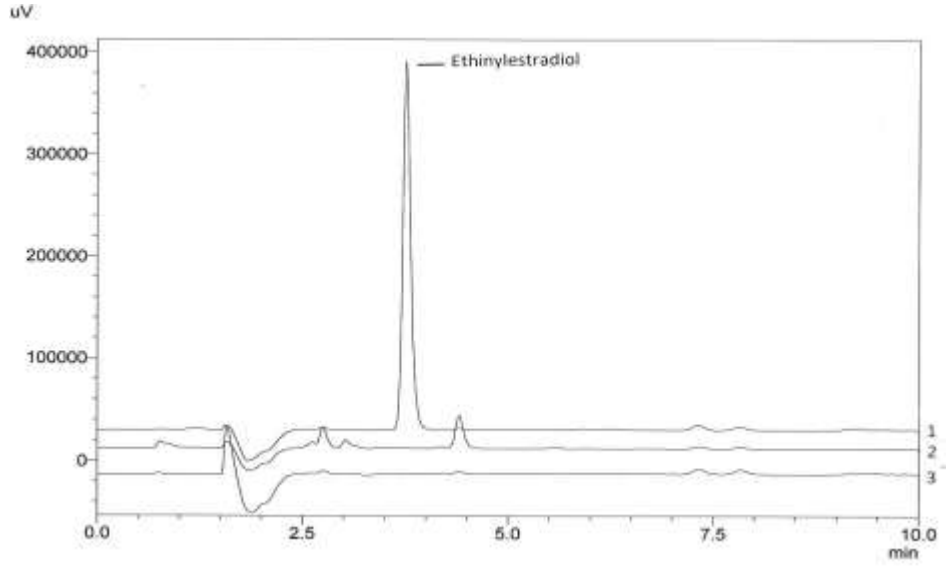

FIG. 2. **Peak purity of Levonorgestrel 1) Standard Sample, 2) Placebo and 3) Diluent.**

Parameter	Specifications	Day 01		Day 02		Day 03	
		Lev	EE	Lev	EE	Lev	EE
Retention Time (% RSD)	≤ 2.0	0.04	0.04	0.13	0.08	0.07	0.03
Area (% RSD)	≤ 2.0	0.24	0.42	0.53	0.76	0.15	0.49
Tailing Factor	≤ 2.0	1.17	1.20	0.96	0.98	1.15	1.18
Theoretical plates	≥ 2000	6374 ± 64	5694 ± 44	6853 ± 76	6287 ± 34	6084 ± 55	5538 ± 23
n: number of replicates per concentration levels and per series.							

TABLE 1. **System Suitability Test Results (n=5).**

Specificity: Specificity of the method was determined by comparing the system suitability standard and drug product. All chromatograms were examined to determine active components were specifically and accurately measured without any interference of dissolution media (diluent) and placebo. The acceptance criteria were peaks of active should be pure that means diluent and placebo does not show any interfere at the retention time of active components. It was found from the

chromatogram that there were no interference at 4.9 min retention time of Levonorgestrel at 247 nm whereas diluent peak were found at 3.4 and placebo peak was at 3.1 min. Etinylestradiol peak was detected at 3.8 min at 310 nm which is completely segregated from diluent peaks at 4.4, 7.3 and 7.8 min and placebo peak at 5.6 min.

Precision and intermediate precision: System precision and intermediate precision were determined for Levonorgestrel and Ethinylestradiol by analyzing the stock solution at concentration of Levonorgestrel 0.3 ppm and Ethinylestradiol at 0.06 ppm. The method precision and intermediate precision were established by six injection of the standard drug samples containing 0.3 ppm of Levonorgestrel and 0.06 ppm Ethinylestradiol (FIG. 3). Precision and intermediate precision were expressed as a relative standard deviation (RSD %) of the analyte peak and absolute difference of average result of precision and intermediate precision. Results for precision and intermediate precision were summarized in (TABLE 2).

Parameter	Specifications	Observed results			
		Precision		Intermediate precision	
		Lev	EE	Lev	EE
Area of Sample	-	82016	2582988	83716	2589381
Amount Recovered	95-100%	99.67	101.0	100.2	99.9
Recovery (% RSD)	≤ 5.0	1.73	2.69	1.68	2.21
Area (% RSD)	≤ 5.0	1.82	2.82	1.77	2.21
n: number of replicates per concentration levels and per series.					

TABLE 2. **Precision and intermediate precision Results (n=6).**

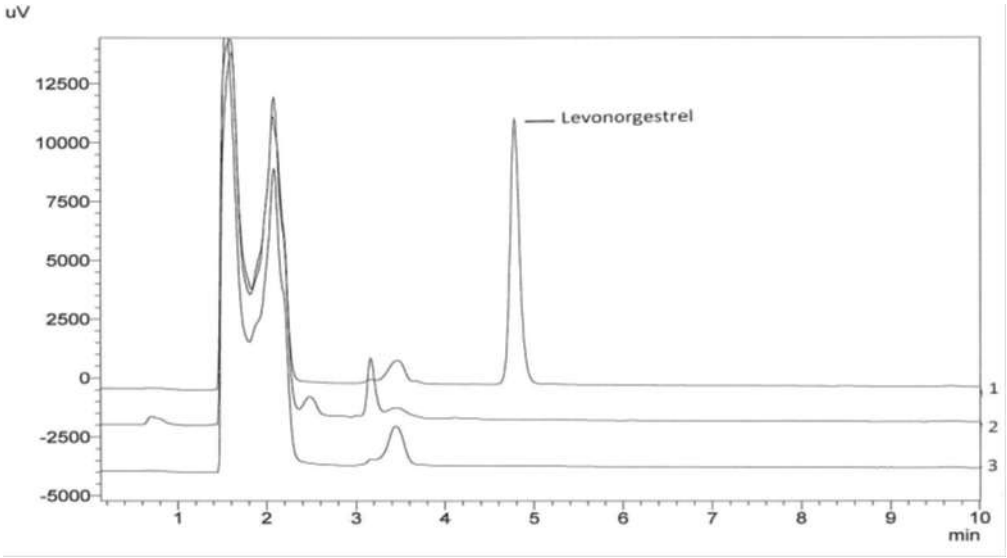

FIG. 3. **Peak purity of Ethinylestradiol 1) Standard Sample, 2) Placebo and 3) Diluent.**

Accuracy: Accuracy expresses the closeness of agreement between the measured value and the value that is accepted as either a true value or a reference value [33]. Accuracy of the method were determined by analyzing three different concentrations of Levonorgestrel (0.15, 0.30 and 0.45 ppm) and Ethinylestradiol (0.03, 0.06 and 0.09 ppm) that were prepared from stock solutions. According to USP guideline accuracy of dissolution samples should be within 95.0 to 105.0% [34]. Recovery from 98.5 to 99.6% of Levonorgestrel and 98.9 to 99.9% of Ethinylestradiol were obtained for the three concentration levels which is summarized in the (TABLE 3).

Parameter	Specifications	Levonorgestrel			Ethinylestrediol		
		0.075 ppm	0.15 ppm	0.225 ppm	0.015 ppm	0.03 ppm	0.045 ppm
Recovery (%)	95-105	98.5	99.6	98.8	98.9	99.9	99.4
Retention Time (% RSD)	≤ 2.0	0.11	0.11	0.07	0.10	0.05	0.07
Area (% RSD)	≤ 5.0	0.80	0.86	0.42	0.76	0.65	0.81
n: number of replicates per concentration levels and per series.							

TABLE 3. **Accuracy Results (n=6).**

Linearity and range: Standard calibration curves were prepared with five calibrators over a concentration range from 0.15 to 0.45 ppm for Levonorgestrel and 0.03 to 0.09 ppm for Ethinylestradiol. Correlation between analyte peak area and concentration (ppm and percentage) of the samples were observed with $r^2 \geq 0.999$ for all days throughout the analysis which is shown in the (TABLE 4). Range was set from 50 to 150% of the active component present in the drug product which was 0.15 to 0.45 for Levonorgestrel and 0.03 to 0.09 for Ethinylestradiol.

Standard Curve	Levonorgestrel				Ethinylestradiol			
	Analytical Range (ppm)	Slope	y-intercept	r^2 value	Analytical Range (ppm)	Slope	y-intercept	r^2 value
Validation day 1	0.075-0.225	277705	336	0.9997	0.015-0.045	4.26×10^7	10875	0.9997
Validation day 2	0.075-0.225	277065	1557	0.9996	0.015-0.045	4.61×10^7	-92093	0.9998
Validation day 3	0.075-0.225	277705	4436	0.9997	0.015-0.045	4.26×10^7	112053	0.9997
m: number of concentration levels or calibrator; *n*: number of replicates per concentration levels and per series.								

TABLE 4. **Linearity results (m=5; n=3).**

Robustness: Robustness of the method was carried out by deliberately making variation in the flow rate (± 0.5 ml/min.), organic phase ratio (± 10%), change column oven temperature (± 5°C), Auto sampler temperature (5°C and 25°C) and changing column brand. During performing robustness test standard stock solution at concentration of 0.3 ppm for Levonorgestrel and 0.06 ppm for Ethinylestradiol were used and it was found that all the criteria for system suitability was satisfactory. So that it can be concluded that this method was robust at that changing parameter. The results is summarized in the (TABLE 5).

Parameter	Value	Levonorgestrel					Ethinylestradiol				
		Retention Time	Tailing Factor	Theoretical Plates	% Area RSD	Area of Std	Retention Time	Tailing Factor	Theoretical Plates	% Area RSD	Area of Std
Acceptance Criteria	-	-	≤ 2.0	≥ 2000	≤ 2.0	-	-	≤ 2.0	≥ 2000	≤ 2.0	-
Control	As per method	4.90	1.12	7168	0.56	84551	3.74	1.16	6354	0.63	2682081
Flow rate (1 mL/min)	0.5	9.83	1.12	8206	0.30	169027	7.48	1.16	7485	0.76	4849411
	1.5	3.34	1.16	5408	0.30	56672	2.54	1.17	4948	0.18	1760469
Mobile phase (Acetonitrile: Water)	70:30	2.82	1.22	4863	0.26	82363	2.32	1.18	3784	0.46	2760169
	50:50	6.20	1.15	6873	0.53	85104	4.61	1.16	6354	0.34	2548914
Column oven temperature	20°C	5.31	1.14	7372	0.43	84555	4.03	1.17	6586	0.48	2579855
	40°C	4.62	1.15	6819	0.28	85061	4.66	1.15	6819	0.28	2556283
Auto sampler temperature	5°C	4.97	1.12	7220	0.35	84539	3.78	1.16	6431	0.18	2590844
	25°C	4.96	1.12	7248	0.26	84502	3.77	1.16	6462	0.15	2581375
Column variation	C_{18},150 mm x 4.6, 3 µm	7.25	1.06	16344	0.33	83405	8.25	1.05	16292	0.85	2503824
n: number of replicates per concentration levels and per series.											

TABLE 5. **Robustness results (n=3).**

Drug product evaluation: The above validation method was successfully applied for the evaluation of five different marketed Levonorgestrel/Ethinylestradiol 0.15 mg/0.03 mg drug products from different manufacturers in oral dosage form. Dissolution profiles of each products were performed from 5 to 45 min time period and total 6 batch from each manufacturer products were analyzed and summery of the results are shown in the FIG. 4 and 5. All products maintained a dissolution rate of > 80% at the 45 min. However, Product B showed the maximum dissolution performances for Levonorgestrel and Product D for Ethinylestradiol. On the other side, Product E showed minimum dissolution for Levonorgestrel and Product E for Ethinylestradiol. An independent t-test with equal variances for maximum and minimum release at 45 min were analyzed and it is found that for Levonorgestrel and Ethinylestradiol at a 95% confidence level p values are 0.00000015 and 0.000000071, respectively which means to fail to reject H_0 and there is no significant differences between maximum and minimum values. An one way ANOVA test also conducted for 5 drug products. It was also found that p values were 1.5×10^{-9} and 6.5×10^{-12} for Levonorgestrel and Ethinylestradiol, respectively at 95% confidence interval which also confirmed that there is no significant different between all 5 drug product after 45 min. However, all the drug products meet the FDA, BP and USP specification at 45 min time, which Q>80 %.

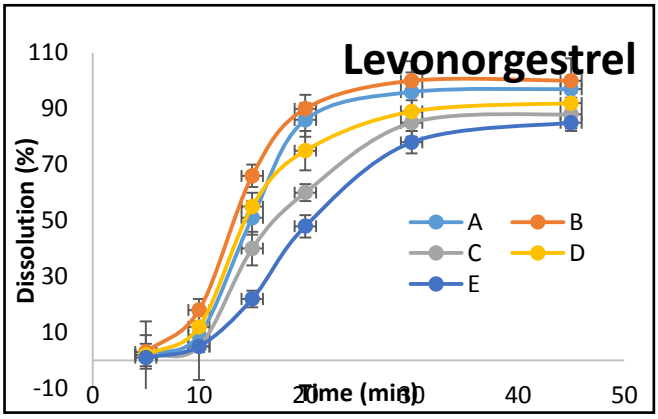

FIG. 4. **Dissolution profile of Levonorgestrel from 5 to 45 min.**

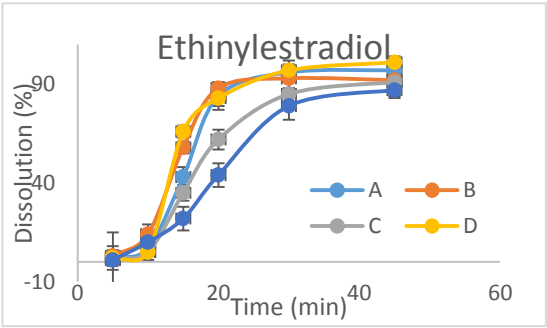

FIG. 5. **Dissolution profile of Ethinylestradiol from 5 to 45 min.**

Conclusion

This RP-HPLC method can be successfully be used for the estimation of Levonorgestrel and Ethinylestradiol in their combined dosage tablet form due to its simplicity, short analytical time (run time). The method addressed each of the analytical validation characteristics such as specificity, system suitability, precision, intermediate precision, accuracy, linearity and range and robustness met the USP acceptance criteria. The method was successfully applied to determine Levonorgestrel and Ethinylestradiol concentration from combined drug in tablet form collected from five different manufacturers and it was found that this method is found to be satisfactory.

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