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Simultaneous estimation of levonorgestrel and ethinyl estradiol by HPTLC in oral tablet formulation

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ABSTRACT

A simple and accurate High Performance Thin Layer Chromatography (HPTLC) method has been developed for the simultaneous estimation of Levonorgestrel (LNG) and Ethinylestradiol (EED) in Tablet formulation. The chromatographic separation was achieved on aluminium plates precoated with silica gel 60GF-254 with mobile phase Dichloromethane: Methanol in ratio of9:1 %v/v measured at the wavelength of 254nm. The R_f values was found to be 0.47 and0.42 for LNG andEEDrespectively. The method was validated according to ICH guidelines and the reliability of the method was assessed by evaluation of Linearity range from1000-4000 ng/spot for LNG and 300-2100 ng/spot for EED. Recovery studiesinclude 92.43% (± 0.82%)w/w for LNG and 103.53% (±2.16%)w/w for EED.This method is simple, accurate, precise and sensitive. This method can be used for the routine analysis of these drugs in pharmaceutical formulation.

Keywords: HPTLC, Levonorgestrel, Ethinylestradiol

INTRODUCTION

Oral contraceptives consist of small amount of steroidal hormones, which comes in forms of pill comprising of either single hormones, or mostly comes with combination forms. However, Levonorgestrel is most commonly used orally active progestin, that prevents the pregnancy by impairment of ovulation and interferes withsperm migration [1], has formula ($C_{21}H_{28}O_2$) with mass of 312.45, is chemically 17(α)-(±)-13ethyl-17hydroxy-18,19-dinorpregn-4-en-20-yn-3-one[2](fig.1). This is combined with at least 5 times to that of estrogen. Ethinylestradiolissynthetic derivative of endogenous estrogen, as estradiol with high oral estrogenic potency, which is used for treatment vasomotor symptoms associated with menstrual problem, chemically it is 17(α)-19-norpregna-1,3,5(10)-trien-20-yn-3,17-diol,has formula ($C_{20}H_{24}O_2$) with mass of 296.4 [2] (fig.2).

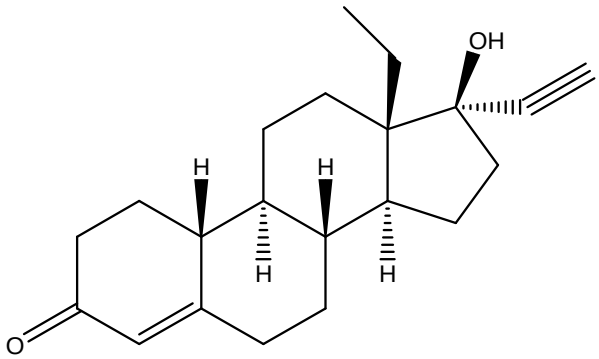

Fig.1: Chemical structure of Levonorgestrel

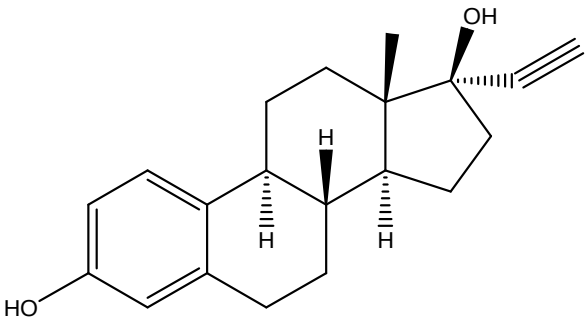

Fig. 2: Chemical Structure of Ethinylestradiol

Combination oral contraceptives act by suppression of gonadotropins primarily by inhibition of ovulation, other alterations include changes in thecervical mucus (which increase the difficulty of sperm entry into the uterus) impairing endometrial receptivity to implantation of afertilized egg.[1]

This combination is official in Indian Pharmacopoeia (IP)[3],United State of Pharmacopoeia (USP) [4], describes various chromatographic methods for their estimation.Literature survey reveals several reported [5-14] on determination of EEDand LNG, including the use of derivative spectrometry [5], High performance Thin layer Chromatography [6],high performance liquid chromatography with UV-visible detector [7],fluorescencedetection [8] Photo-diode array (PDA) detector [9], gas chromatography-mass spectrometry on thepentafluorobenzoyl derivatives through [10] and pentafluorobenzyl-trimethylsilylderivatives [11], stability indicating liquid chromatographic method [12] solidphase extraction followed by liquid chromatography-diodearray detection-mass spectrometry [13], liquid–liquid extraction and derivatization with dansyl chloride followed by ultra-performance liquid chromatography coupled with tandem mass spectrometry in human plasma [14], etc.

The aim of this research work was standardization of HPTLC methods for quantitative simultaneous estimation of Levonorgestrel (LNG) and Ethinylestradiol (EED) in commercially available oral contraceptives.

MATERIALS AND METHODS

Chemicals and Reagents

The raw materials were obtained from Aurobindo Pharma Ltd, Hyderabad as gift sample and used as reference materials throughout the experiment without any prior treatment. A commercial uncoated tablet formulation (OVRAL* L, WYETH LTD) each containing 0.15mg of Levonorgestrel and 0.03mg of Ethinyloestradiol were procured from local pharmacy.The reagents used were of analytical grade and purchased from Merck Laboratories, Mumbai.

Preparation of Stock Solutions

The standard stock solutions were prepared by dissolving 0.15mg of LNG and 0.3mg of EED in small quantity of methanol individually in 10ml volumetric flask.The mixture was sonicated for 10 min and then makes up to volume with methanol.

Preparation of Sample Solution

Commercially available uncoated 10 tablets were weighed and the average weight was taken and tablets were powdered. From the powdered mixture a weight equivalent to the label claim of LNG and EED was accurately weighed and dissolved in small quantity of methanol. Shake well and sonicate for 20 min and make up the volume to 10ml methanol. Then filter the solution and the filtrate was used to carry out for further analysis.

Selection of wavelength

UV spectra of LNG and EED were shown that λ_{max} was found at 249 nm and 280 nm respectively. Isobestic points were observed at 225 nm and 254 nm. At 254nm LNG and EED shows maximum absorption, so that wavelength is selected for determinations.

Instrumentation and Chromatographic Condition

The sample were spotted as a band of width 6mm using Camag 25µlsample (Hamilton, Bonaduz, Switzerland)syringe on a precoated silica gel 60 F_{254}aluminium plate (10 cm× 10 cm) with 200 µm thickness (E. MERCK,Mumbai) using a Camag Automatic TLC Sampler 4 (ATS4). The rate of flow from the syringe was maintained at 5µl and the distance between the spots were 14mm.

Linear ascending development was carried out in a 10 cm × 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 10-15 min at room temperature (25°C±2) at relative humidity of 60±5%. The mobile phase consisted of Dichloromethane: Methanol in ratio of 9:1 %v/v and 10 ml of mobile phase was usedper chromatography. The length of chromatogram run was 8 cm.Subsequent to the development, HPTLC plates were dried in currentof air. Densitometric scanning was performed on a Camag HPTLCscanner III in the reflectance absorbance mode at 254 nm andoperated by WINCATS software (V 1.4.6, Camag).The source ofradiation utilized was deuterium lamp emitting continuous UVspectrum between 200 and 400 nm. Concentrations of thecompound on chromatographic plate were determined from theintensity of Reflected light. Evaluation was via peak areas with linearregression.

Method Validation [15]

Linearity

The linearity of calibration curves in pure solution was checked over the ranges of 1000-4000 ng/spot for LNG and 300-2100 ng/spot for EEG. The calibration curves were linear in the studied range and equations of the regression analysis were obtained for LNG and EED. The results were tabulated in Table 2 and 3and the linearity plots were shown in Fig. 5 and 6 respectively.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ for LNG and EED by the proposed methodwere calculated using the following formula

$$\text{LOD} = \frac{3.3 \times SD}{Slope}, \qquad \text{LOQ} = \frac{10 \times SD}{Slope}$$

Where, SD – Standard Deviation of response.

Precision (Reproducibility)

Standard Linearity solution of 100% concentration wasspotted six times on the precoated TLC plates and the developmentwas carried out. The densitogram was recorded and the results were tabulated in Table 4.

Method Precision

About 5µl of the sample solution was spotted six times on the precoatedTLC plates and the development was carried out. The densitogramwas recorded and shown in (Fig.4), the % RSD was calculated whichwas found to be within the limits (<2.0) according to ICH guidelines.
The results were tabulated in Table 5.

Accuracy

An accuracy study was carried out at 80, 100 and 120% of test concentration.Toensure accuracy of the developed method, known quantity ofstandard stock solution was mixed with unknown sample and the %recovery was calculated.

Preparation of bands for Accuracy

Recovery (80%)

To prepare a spot, sample solution 1.6µl of standard EED solution,0.8µl of standard LNG solution and 2µl of sample tablet solution were directly applied.

Recovery (100%)

To prepare a spot, sample solution 2µl of standard EED solution, 1µl of standard LNG solution and 2µlof sample tablet solution were directly applied.

Recovery (120%)

To prepare a spot, sample solution 2.4µl of standard EED solution, 1.2µl of standard LNG solution and 2µl of sample tablet solution were directly applied.

Sample solution was spotted in three replicates on the precoated TLC plates and development was carried out. After development the plateswas dried and scanned at 254nm. The % recovery was calculatedand the results weretabulated in Table 6**.**

$$\% \text{ Recovery} = \frac{Peak\ Area\ of\ API\ Spiked\ in\ Tablet}{Peak\ Area\ of\ (API+Tablet)}$$

Specificity

Specificity of method was ascertain by comparing densitogram of tablet formulation with that of same concentration of standard drug of LNG and EED, mobile phase and diluent. The spots for drugs in tablet formulation was confirmed by comparing the R_f value and spectra of spots with the standards drugs.

Robustness

Robustness was studied to ensure the reliability of analytical method to remain unaffected by small, deliberate change in method parameters such as changing mobile phase composition in ratio by ±0.5ml (i.e. 9.5: 0.5 v/v and8.5: 1.5 v/v) and by spiking 2.1µl of EED and 4µl of LNG as spot. The densitogramwas recorded and the % RSD was calculatedand the results weretabulated in Table 7.

RESULTS

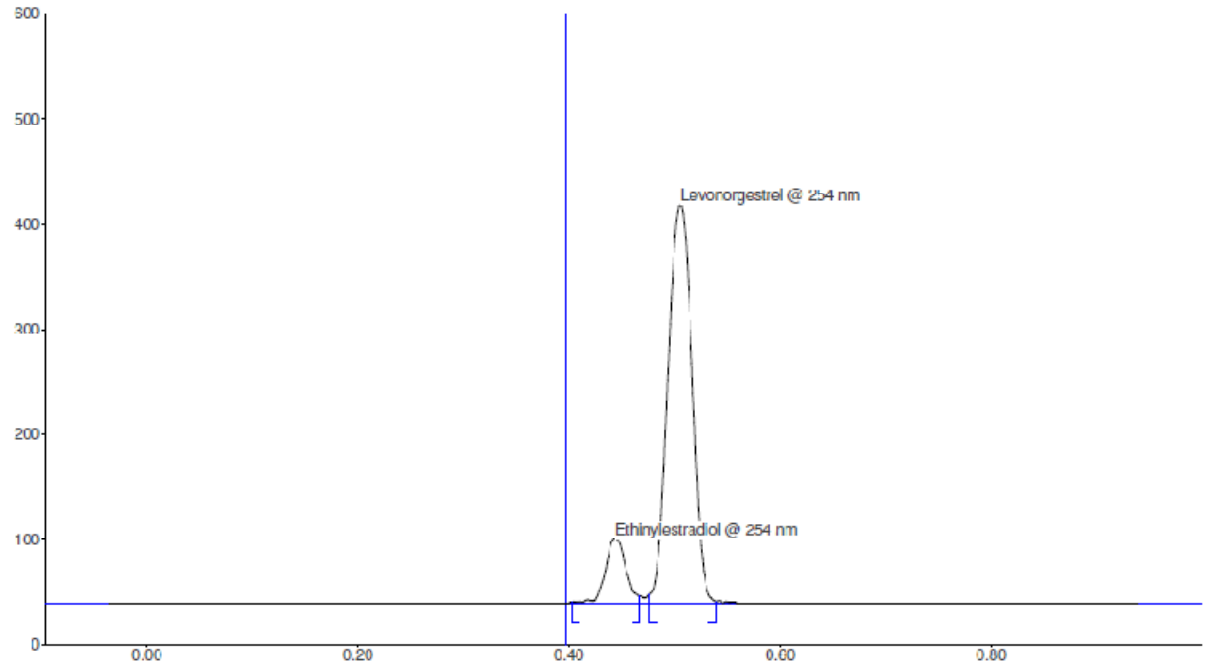

Fig. 3: A Representative Densitogram of Standards LNG and EED at 254nm

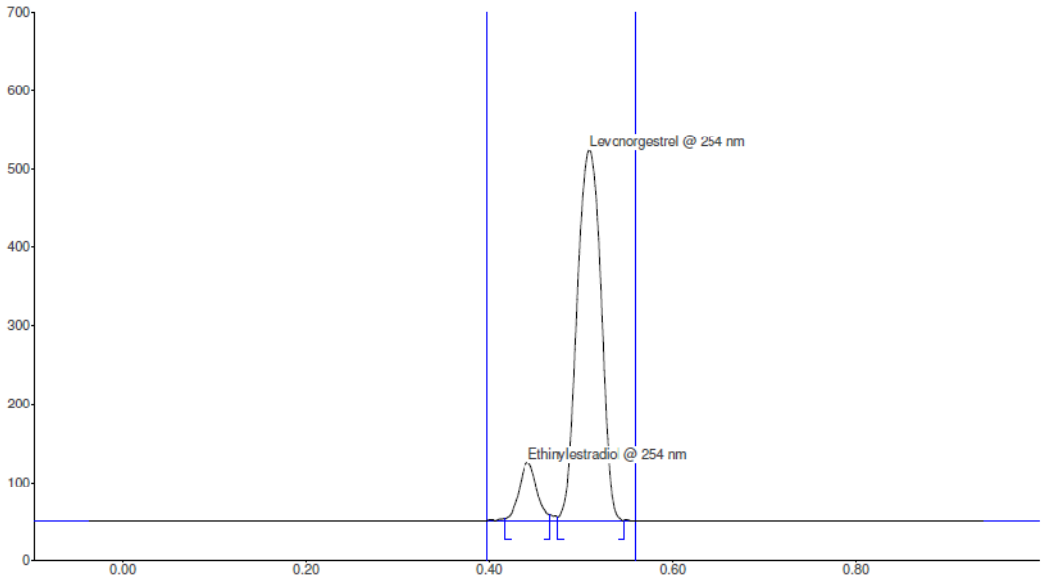

Fig. 4: The Densitogram showing Recovery of LNG and EED in Tablet at 254nm

Table 1: Data showing Retention factor

Drug	R_f Value
Levonorgestrel (LNG)	0.47
Ethinylestradiol (EED)	0.42

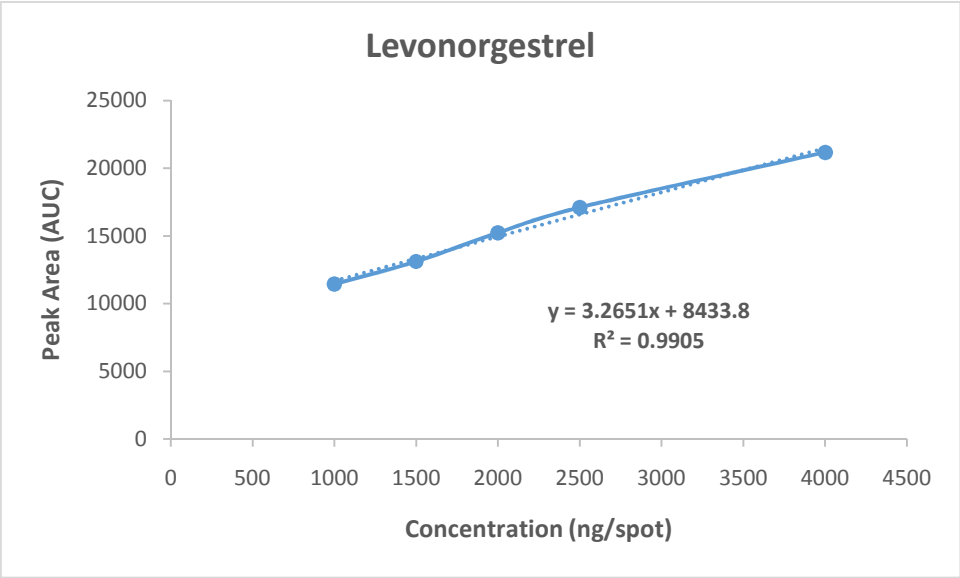

Fig. 5: Calibration graph of Levonorgestrel (LNG)

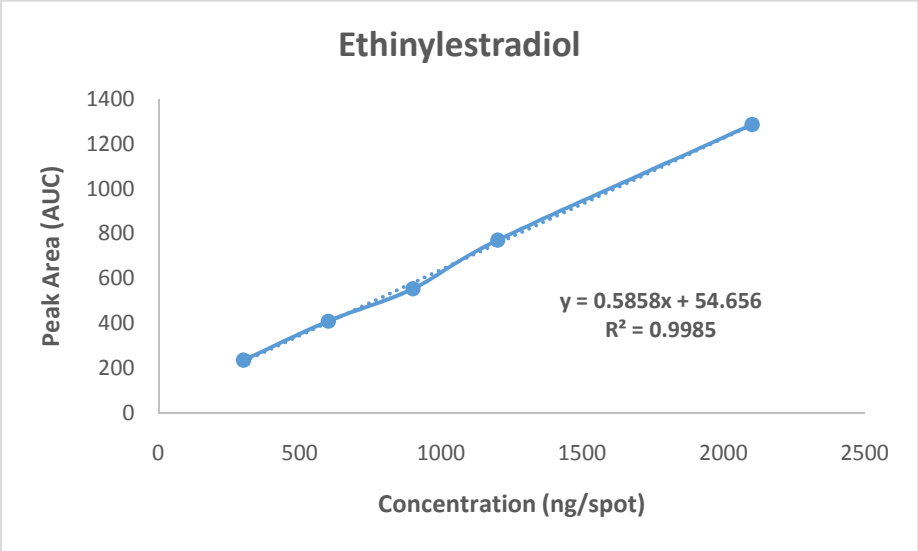

Fig. 6: Calibration graph of Ethinylestradiol (EED)

Table 2: Linearity data

S. No.	Drugs	Concentration (ng/spot)	Peak area
1	LNG	1000	11442.25
		1500	13113.80
		2000	15238.55
		2500	17106.22
		4000	21183.96
2	EED	300	237.41
		600	410.79
		900	554.96
		1200	771.83
		2100	1286.01

Table 3: Linearity parameters

Drug	Linearity range (ng/spot)	R^2	SD	Slope	Intercept	LOD (ng)	LOQ (ng)
LNG	1000-4000	0.9905	2.73	3.26508	8433.78	2.7591	8.3612
EED	300-2100	0.9985	2.80	0.585827	54.65	15.7725	47.7956

Table 4: Data showing Precision

Type	S. No.	Peak Area	
		LNG	EED
Inter-Day	1	15814.86	1603.18
	2	15974.24	1562.76
	3	15678.95	1624.71
	4	15449.05	1565.67
	5	15241.29	1566.43
	6	15674.71	1553.57
Average		**15654.10**	**1582.93**
Standard Deviation (S.D.)		**241.596**	**27.228**
%R.S.D.		**1.54**	**1.72**
Intra-Day	1	21989.94	1516.61
	2	21986.86	1552.14
	3	21643.17	1587.92
	4	21592.89	1519.7
	5	21692.8	1569.53
	6	22715.39	1538.09
Average		**21936.84**	**1547.33**
Standard Deviation (S.D.)		**418.464**	**28.128**
%R.S.D.		**1.91**	**1.82**

Table 5: Method Precision

Drug	Label claim (mg/tablet)	Amount estimated (mg)*	Drug content (%)	SD	%RSD
LNG	0.15	0.152634	101.756	0.00254	1.66
EED	0.03	0.03042	100.418	0.000331	1.08

** Values are the average of six determinations*

Table 6: Results showing Recovery of the method

Drug	Recovery Level(%)	Peak Area			Recovery (%)	Overall Recovery (%)	S.D. (±)
		Drug Present in Tablet	API	API spiked on Tablet			
LNG	80	4426.85	8236.1	11892	93.18	92.433	0.8718
	100	4426.85	10696.3	14007	92.62		
	120	4426.85	11302.3	14387	91.47		
EED	80	362.48	785.02	1214.26	105.81	103.53	2.1586
	100	362.48	1068.97	1467.1	102.49		
	120	362.48	1273.02	1664.33	101.76		

Table 7: Results showing Robustness of the method

S. No.	LNG	EED
Mobile Phase Composition in ratio of 9.5: 0.5 %v/v		
1	15239.25	1655.37
2	15372.88	1639.44
3	15231.97	1694.52
4	14923.52	1710.90
5	15176.25	1650.92
6	15231.02	1696.53
Average	**15126.731**	**1673.467**
S.D.	**138.0841**	**27.133**
%R.S.D.	**1.505**	**1.6213**
Mobile Phase Composition in ratio of 8.5: 1.5 %v/v		
1	14892.32	1598.99
2	14932.46	1625.75
3	14855	1591.31
4	14954.21	1628.56
5	14912.11	1617.12
6	14879.62	1615.22
Average	**14904.29**	**1612.8**
S.D.	**36.133**	**14.782**
% R.S.D.	**0.2424**	**0.9165**

RESULTS AND DISCUSSION

The mobile phase optimized containing Dichloromethane: Methanol, in a ratio of 9:1% v/v were showing sharp peaks withgood resolution between LNG and EED at less retention time. Detection was carried out at 254nm as both drugs showedgood response. The Retention time was found to be 0.47 and 0.42 respectively and the peak shapes of all the drugs were symmetrical. The Linearity experiments were performed and the range was found to be 1000-4000 ng/spot for LNG and 300-2100 ng/spot for EEG and the precision of the method was found to be 1.08 – 1.91which was within the limits of ICH guidelines indicating that the method was precise for the estimation of these drugs. Accuracy ofthe method was calculated by recovery studies at three levels. The recovered was found to be 92.43% (± 0.82%) for LNG and 103.53% (±2.16%) for EEDwhich was within the range of standards according to ICH guidelines.

Table 8: Summary of Validation parameters and their limits

Parameters	Results		Acceptance criteria
	LNG	EED	
Retention time	0.47	0.42	-
Linearity Range (ng/spot)	1000-4000	300-2100	-
Correlation coefficient (R^2)	0.9904	0.9984	≤ 0.9999
Slope	3.26508	0.585827	-
Intercept	8433.78	54.65	-
Method Precision (%R.S.D.)	1.66	1.08	< 2
Inter-day Precision (%R.S.D.)	1.54	1.72	< 2
Intra-day Precision (%R.S.D.)	1.91	1.82	< 2
% Recovery	92.43% (± 0.82%)	103.53% (±2.16%)	90.0%-110.0%
Robustness (9.5:0.5)(%R.S.D.)	1.505	1.621	< 2
Robustness (8.5:1.5)(%R.S.D.)	0.2424	0.9165	< 2
Specificity	No Interference		

CONCLUSION

The newly developed HPTLC technique was validated by evaluatingvarious validation parameters. The results obtained for eachparameter was tabulated and all the results were found to bewithin the prescribed limits. The method developed in the study wasfound to be simple, accurate, precise and reproducible fordetermination of LNG and EED incombined dosage formulation. Therefore, the developed method canbe recommended for routine quality control analysis of these drugsin pharmaceutical formulations.

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