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Liquid Chromatographic And Spectrophotometric Determination Of Ethinyl Estradiol And Levonorgestrel In Pharmaceutical Preparations

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ABSTRACT

The present work describes a new HPLC method and a new spectrophotometric method for the simultaneous determination of ethinyl estradiol (EE) and levonorgestrel (LNG) in their binary combination. In LC method, Nucleosil C8 column with a mobile phase composed of acetonitrile-water (90:10, v/v) with UV detection at 225 nm were used for the analysis of ethinyl estradiol-levonorgestrel combination. As spectrophotometric method, ratio spectra second derivative spectrophotometry was used. In this method, analytical signals were measured at the wavelengths corresponding to either maximums or minimums for these drugs in the second derivative spectra of the ratio spectra obtained by using each others zero-order spectra as divisor in their solution in methanol in 200-320 nm range. The spectrophotometric procedure does not require any separation step. Linearity range was found as 0.5-60 μg ml^{-1} for EE and 0.6-60 μg ml^{-1} for LNG in HPLC method and 1.6-100 μg ml^{-1} for EE and 2-40 μg ml^{-1} for LNG in spectrophotometric method. The methods have been validated by analyzing synthetic mixtures containing title drugs and were successfully applied to 2 pharmaceutical formulations, sugar-coated tablets, marketed in Turkey. The results were compared with each other and official method.

KEYWORDS

Ethinyl estradiol;
Levonorgestrel;
Ratio spectra derivative Spectrophotometry;
HPLC;
Pharmaceutical preparation.

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INTRODUCTION

Binary combination of ethinyl estradiol (EE)-levonorgestrel (LNG) are frequently prescribed in medicine as oral contraceptive. Various methods including spectrophotometry[1,2], chemometric methods in spectrophotometry[3], HPLC[4-6], RIA[7], TLC[8] and MEKC[9] have been used for the simultaneous determination of EE and LNG in their binary mixture and in pharmaceutical preparations containing this combinations.

S.Tatar, et al.[1] and J.J.Berzas et al.[2] used first derivative spectrophotometry for the simultaneous analysis of EE+LNG in solid dosage forms. In derivative spectrophotometry, it can be study only at the zero-crossing points, so the presence of other interfering compounds such as other drugs and excipients limit the methods and sometimes it is impossible to work or find any suitable wavelengths in derivative spectra for the analysis of commercial preparations. In ratio spectra derivative spectrophotometry, having more than one suitable wavelength for measurements is an advantage for the determination of drugs in the presence of other interfering compounds. So, we used this method in this work for the resolution of EE + LNG combination.

J.J.Berzas et al.[3] used PCR and PLS techniques in spectrophotometry for the simultaneous analysis of EE + LNG combination. These techniques quite complex and need stepwise time consuming mathematical calculations.

Q.G.Li and B.Nieuweboer[7] used RIA method and J.J.Berzas et al.[9] used MEKC method for the simultaneous analysis of EE+ LNG. These methods are very sophisticated methods and can not be realized in classical analytic laboratories easily.

EXPERIMENTAL

Apparatus

Shimadzu 1601 PC double beam spectrophotometer with a fixed slit width (2 nm) connected to a computer loaded with Shimadzu UVPC was used for all the spectrophotometric measurements.

In spectrophotometric method, a zero-order spectrum of the solution of EE and LNG in methanol in 200–320 nm range was used.

For HPLC, HP 1100 model liquid chromatograph was equipped with a model series of 613 22A degasser, 613 11A quaternary pump and 613 28A injector. The chromatograms were recorded and the peaks were quantitated using its automatic integrator. The separations were carried out at ambient temperature on Nucleosil C8 Column (Machere-Nagel) of 250 x 4.6 mm (5 Berzas Berzas μm particle size). The mobile phase was acetonitrile-water (90:10, v/v). The flow rate was set at 1 ml min^{-1} with 20 μl as injection volume and the wavelength of detection was 225 nm for both combinations. Miconazol nitrate was used as internal standard in the analysis.

Materials

Ethinyl estradiol and levonorgestrel were kindly donated by Wyeth Pharm.Ind., Turkey and they were used without further purification.

All the materials used in the spectrophotometric analysis were of analytical reagent grade. HPLC grade solvents were used in LC procedures.

Standard solutions

Solutions of 50 mg/100 ml ethinylestradiol and 10 mg/100 ml levonorgestrel were prepared in methanol for spectrophotometric method and for LC. Solution of 6 Berzas μg mL^{-1} miconazol nitrate was prepared in methanol and used as internal standard (IS).

Sample preparation

a) For HPLC method: The content of 20 sugar-coated tablets were accurately weighed and powdered separately in a mortar. An amount of mass equivalent to one sugar-coated tablet was dissolved in 20 ml of solution of methanol separately. After 20 min. of mechanically shaking the solutions were filtered through 4.5 μm millipore filter to 25 ml volumetric flask. 2.5 ml of miconazol nitrate (IS) was added and the volume was completed to the mark with methanol for the analysis of EE + LNG combination. These solutions were injected separately to the column selected. The ratios of peak areas were measured for the determination of EE and LNG by using its integrator.

b) For ratio spectra second derivative spectrophoto-

metric method: The content of 20 sugar-coated tablets were accurately weighed and powdered separately in a mortar. An amount of mass equivalent to two sugar-coated tablets was dissolved in 20 ml of solution of methanol separately. After 20 min. of mechanically shaking, the solutions were filtered through 4.5 μm millipore filter in a 25 ml volumetric flask. Then the volume was completed to 25 ml with the same solvent. The method was applied directly to these solutions.

Commercial pharmaceutical preparations

LO/Ovral® (0.030 mg ethinylestradiol and 0.150 mg levonorgestrel/sugar-coated tablet) Wyeth Pharm.Ind., Turkey (batch no:44079) and Micro gynon®21 (0.030 mg ethinylestradiol and 0.150 mg levonorgestrel/sugar-coated tablet) Schering Pharm.Ind., Turkey (batch no: 38458) were assayed.

RESULTS AND DISCUSSION

HPLC method

The developed HPLC method has been applied for the simultaneous determination EE+LNG in their binary mixtures. On Nucleosil C8 Column various mobile phases were assayed and acetonitril-water (90:10, v/v) mixture was found optimum for the good separation for EE and LNG. Quantitation of EE and LNG were made with UV detection at 225 nm. Retention times for miconazol nitrate (IS), EE and LNG were found 1.494, 3.345 and 4.013 and min. respectively for ten replicates.

Typical chromatograms of the drugs and internal standard were illustrated in figure 1. Peak areas were used in the quantitation procedures. Regression equations were:

$y = 0.1450 x + 0.0490$ ($r = 0.9919$) for (LNG) and $y = 0.0920 x + 0.0076$ ($r = 0.9919$) for (EE), where x is the concentration in μg ml^{-1} and y is the ratio of the areas of drug/internal standard,

Linearity range was found 0.5-60 μg ml^{-1} for EE and 0.6 - 60 μg ml^{-1} for LNG. LOD was found 0.14 mg ml^{-1} for EE and 0.2 μg ml^{-1} for LNG (determined

TABLE 1: System suitability tests results (SST) of EE and LNG in HPLC method

	EE	LNG
Theoretical plates	4059,51	6441,66
Capacity factor	5.69	7.03
Separation factor	1.23	1.23
Resolution	1.64	1.64
Tailing factor	0.90	0.96

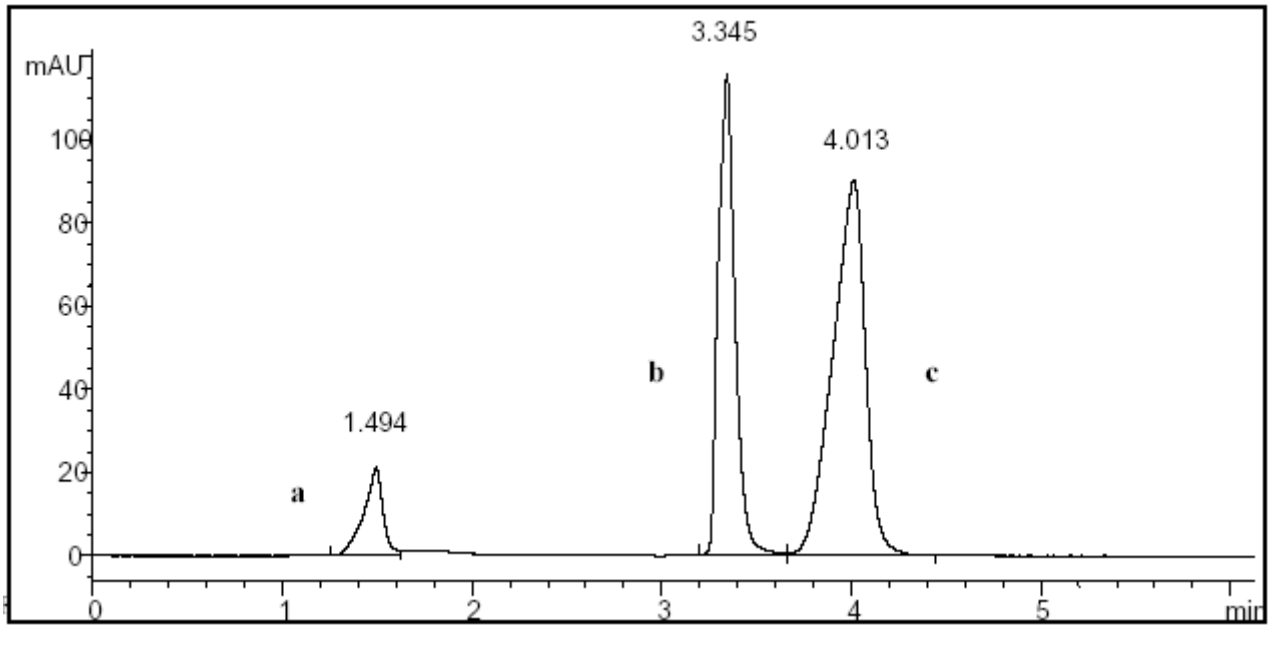

Figure 1: Typical chromatogram of a) miconazole nitrate (internal standard) b) ethinyl estradiol and, c) levonorgestrel in methanol in HPCL method

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TABLE 2: Recovery results for EE and LNG in synthetic mixtures by ratio spectra second derivative spectrophotometry and HPLC

	^{2}DD							HPLC	
	EE				LNG			EE	LNG
-	274.0	280.8	290.2	294.8 nm	242.3	247.3	254.0 nm	-	-
Mean recovery* % (± CI** for P=0.05)	100.9 (± 1.11)	101.1 (± 0.91)	102.8 (± 1.58)	98.3 (± 2.46)	101.5 (± 2.02)	99.0 (± 0.48)	100.7 (± 1.37)	100.1 (± 1.46)	97.7 (± 1.26)
RSD*** %	1.73	1.42	2.43	3.98	2.78	0,66	2.32	2.04	1.80

*mean of ten replicates **CI=CONFIDENCE INTERVAL ***RSD=RELATIVE STANDARD DEVIATION

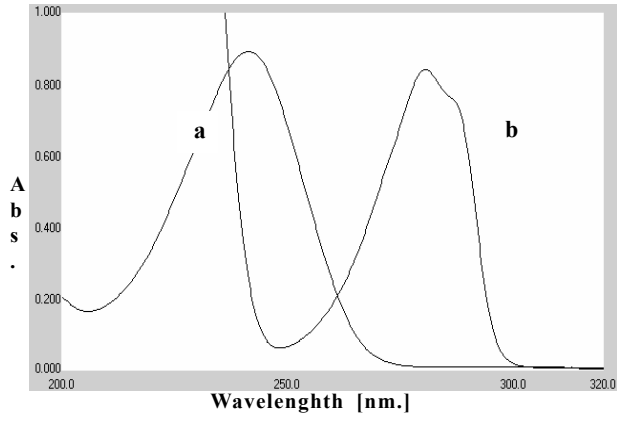

Figure 2: Zero – order absorbtion spectra of (a) 16 μg/mL solution of levonorgestrel, (b) 80 μg/mL solution of ethinyl estradiol in methanol

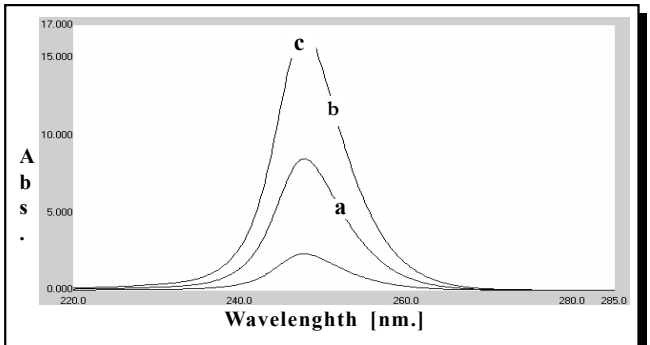

Figure 3a: Ratio spectra of (a) 2 μg ml^{-1}, (b) 8 μg ml^{-1}, (c) 16 μg ml^{-1}of levonorgestrel in methanol when 80 μg ml^{-1} of ethinyl estradiol in methanol was used as divisor (Δλ = 2 nm, scaling factor :1)

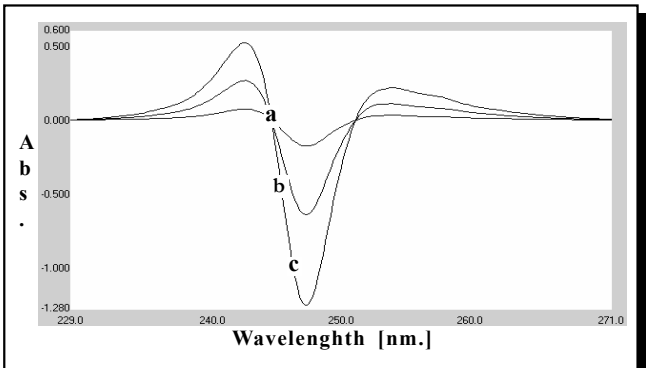

Figure 3b: Second derivative of the ratio spectra of (a) 2 μg ml^{-1}, (b) 8 μg ml^{-1}, (c) 16 μg ml^{-1} of levonorgestrel in methanol when 80 μg ml^{-1} of ethinylestradiol in methanol was used as divisor (Δλ = 2 nm, scaling factor :1)

as 3SD/m where SD=standard deviation, m=slope); LOQ was found 0.5 μg ml^{-1} for EE and 0.6 μg ml^{-1} L for LNG (determined as 10SD/m) in the method.

Mean recoveries and relative standard deviations of the methods were found as 100.1 % and 2.04 % for EE and 97.7 % and 1.80 % for LNG in the method respectively for synthetic mixtures prepared in our laboratory (TABLE 2).

Summary of the assay results for commercial preparations were shown in TABLE 4.

Ratio spectra derivative spectrophotometry:

a) For LNG: The ratio and second derivative of ratio spectra of the solutions of in different concentrations in methanol traced with the interval of Δλ = 2 nm after smoothing with Δλ = 2 nm by using the standard spectrum of EE (80 μg/mL in methanol) as divisor by computer aid was demonstrated in figure 3a and 3b, respectively. In these spectra, two maxima (242.3 and 254 nm) and one minimum (247.3 nm) were found suitable for the quantification of LNG in EE + LNG mixture.

Measured analytical signals at these wavelengths are proportional to the concentrations of the drugs. We selected 247.3 nm for the determination of in the assay of pharmaceutical preparation, capsule, due to its lower RSD value and suitable mean recovery among the wavelengths mentioned (TABLE 2).

Regression equations at these wavelengths were:

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$y = 0.032\,x + 7.99\,10^{-3}$ (r: 0.9999) at 242.3 nm,
$y = 0.014\,x + 1.28\,10^{-4}$ (r: 0.9999) at 254.0 nm,
$y = -0.076\,x - 2.50\,10^{-2}$ (r: 0.9999) at 247.3 nm

for LNG where x is the concentration in μg mL^{-1} and y is the analytical signals.

b) For EE: The ratio spectra of different EE standards at increasing concentrations in methanol obtained by dividing each with the stored spectrum of the standard solution of LNG (32 μg ml^{-1} in methanol) by computer aid are shown in figure 4a and the second derivative of these spectra (^{2}DD) traced with the interval of $\Delta\lambda$ = 2 nm after smoothing with $\Delta\lambda$ = 2 nm are illustrated in figure 4b. As seen in figure 4b, there exist two maxima (274 and 294.8 nm) and two minima (280.8 and 290.2 nm) and we found that four of them are suitable for the determination of EE in EE + LNG mixture.

Regression equations at these wavelengths were:

$y = 0.011\,x - 6.03\,10^{-3}$ (r: 0.9999) at 274 nm
$y = 0.017\,x - 4.40\,10^{-3}$ (r: 0.9999) at 294.8 nm
$y = -0.018\,x + 0.010$ (r: 0.9996) at 280.8 nm
$y = -0.019\,x + 2.01\,10^{-3}$ (r: 0.9998) at 290.2 nm

for EE where x is the concentration in μg ml^{-1} and y is the analytical signals.

We selected 280.8 nm for the determination of this compound in the assay of synthetically prepared pharmaceutical preparation, sugar-coated tablet, due to its lower RSD value and more suitable mean recovery among the wavelengths mentioned (TABLE 2).

In the method, the mean recoveries ± confidence interval and relative standard deviations calculated for synthetic mixtures prepared in our laboratory are illustrated in TABLE 2.

Divisor concentration is main instrumental parameter. The standard spectra of 80 μg ml^{-1} solution of EE and 32 mg ml^{-1} solution of LNG were considered as suitable for the determination of EE and LNG respectively as divisor. The $\Delta\lambda$ found as optimum for the second derivative of their ratio spectra was 2 nm.

Linearity range was 1.6–100 μg ml^{-1} for EE and 2-40 μg ml^{-1} for in EE + LNG mixture in the methods. LOD was found 0.6 μg ml^{-1} for EE and 0.7 μg ml^{-1} for LNG (determined as 3SD/m), LOQ was found 1.6 μg ml^{-1} for EE and 2.0 μg ml^{-1} for LNG (determined as 10SD/m) in the method

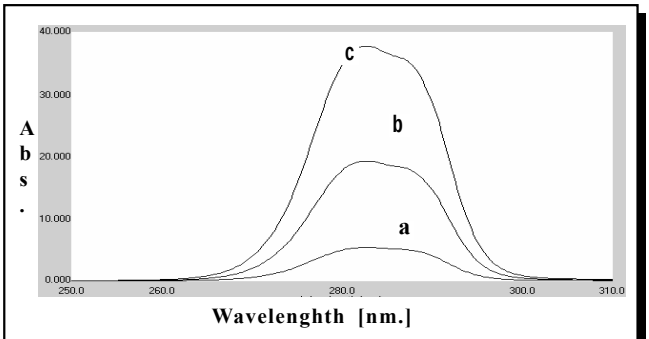

Figure 4a: Ratio spectra of (a) 8 μg ml^{-1}, (b) 30 μg ml^{-1}, (c) 60 μg $ml^{-1}$of ethinylestradiol in methanol when 32 μg $ml^{-1}$of levonorgestrel in methanol was used as divisor ($\Delta\lambda$ = 2 nm, scaling factor :1)

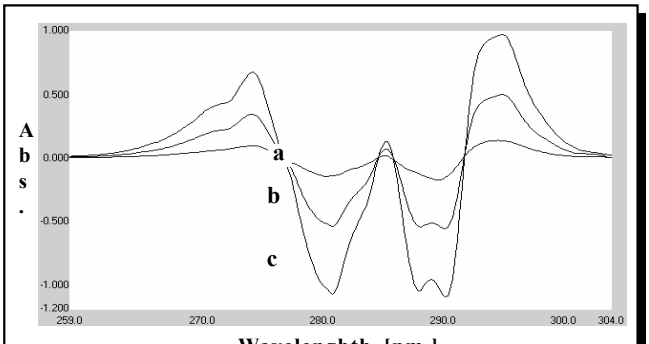

Figure 4b: Second derivative of the ratio spectra of (a) 8 μg ml^{-1}, (b) 30 μg ml^{-1}, (c) 60μg ml^{-1} of ethinylestradiol in methanol when 32 μg/mL of levonorgestrel in methanol was used as divisor ($\Delta\lambda$ = 2 nm, scaling factor :1)

Summary of the assay results for commercial preparations were shown in TABLE 4.

Precision

The precision was determined by means of a one-way ANOVA including 10 replicates carried out on three successive days using ratio spectra second derivative spectrophotometry (^{2}DD) and LC method for synthetic mixtures of EE+LNG. Snedecor F values below the tabulated levels were obtained in all cases (F=4.21, n_1=2, n_2=27; TABLE 3), so there were no significant differences between the result obtained in the determination of each drug in the presence of other on different days (TABLE 3)

Applications

Comparison of the spectra of EE and LNG in standard and drug formulation solutions showed that the wavelength of maximum absorbance in the zero-

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TABLE 3: Analysis of variance (ANOVA) for the proposed methods

Parameters	HPLC		^{2}DD	
	EE	LNG	EE	LNG
Between-days variance	0.86	0.48	0.46	0.34
Within-daysvariance	0.28	0.17	0.32	0.21
F ratio	3.07	2.82	1.43	1.61
Mean value (μg ml^{-1})	40.20	30.16	40.06	29.94
Between-days RSD %)	0.39	0.38	0.22	0.14
Within-days RSD (%)	0.28	0.16	0.08	0.04

Between-day and within-day degree of freedom 2 and 27 respectively. The critical F ratio value for 2 and 27 degree of freedom and a confidence level of 95 % is 4.21

order spectra did not change and also after addition of known amount of these active ingredients to the commercial formulations powder was found the amount of these drugs did not change. It has been decided that excipients placed in the commercial. Preparations selected (lactose, starch, avicel, povidon, sodium dodecylsulfate, aerosil and magnesium stearate) did not interfere the quantitation of EE and LNG in the methods. All the results obtained by using the methods described above were compared with each other and official method[10], no significant difference was observed between the amount of drugs found as theoretical values fort at P = 0.05 level for commercial formulations.

CONCLUSION

In this study; a new HPLC and a new spectrophotometric method, ratio spectra second derivative spectrophotometry, were developed for the simultaneous analysis of EE+LNG combination. By not needing any time consuming sample preparation procedures and using methanol as solvent, spectrophotometric method developed is easier and cheaper when compared with the HPLC methods. In our HPLC method for the analysis of EE+LNG mixture, the lower limit in the determination was found at least two times lower than those of HPLC methods given in the literature[4,5,6] for these drugs. In ratio spectra derivative spectrophotometric method, having more than one suitable wavelength for measurements is an advantage for thedetermination of these drugs in the presence of other interfering compounds when compared with the zero-crossing derivative spectrophotometric methods[1,2] especially in the assay of pharmaceutical formulations containing many excipients.

Also, in our ratio spectra derivative spectrophotometric method, linearity ranges for these drugs were wider than those of cited spectrophotometric methods[1,2,3].

The spectrophotometric and LC method proposed in this article were successfully applied for the analysis of EE+LNG containing sugar-coated tablets marketed in Turkey. Good -agreement was seen

TABLE 4: Assay results of commercial preparations

methods	EE		LNG	
	mean ± SD	t values	mean ± SD	t values
MİCROGYNON®	(Label claim= 30 μg ethnylestradiol and 150 μg levonorgestrel / sugar- coated tablet)			
HPLC	30.05 ± 0.32		150.62 ± 1.26	
^{2}DD	30.30 ± 0.48	HPLC – USP= 0.30 ^{2}DD – USP = 0.88 ^{2}DD – LC = 1.28	150.60 ± 0.62	HPLC – USP = 0.16 ^{2}DD – USP = 0.20 ^{2}DD – LC = 0.36
****Officialmethod (USP)	30.12 ± 0.42		150.59 ± 0.96	
LO/OVRAL®	(Label claim= 30 μg ethnylestradiol and 150 μg levonorgestrel / sugar-coated tablet)			
HPLC	30.15 ± 0.42		150.21 ± 1.71	
^{2}DD	30.37 ± 0.21	HPLC – USP = 0.21 ^{2}DD – USP = 0.66 ^{2}DD – HPLC = 1.21	150.66 ± 0.66	HPLC – USP = 0.40 ^{2}DD – USP = 0.44 ^{2}DD – HPLC = 1.88
****Official method (USP)	30.19 ± 0.62		150.41 ± 0.71	

***Obtained results are average of ten tablets for four techniques; **SD=standard deviation, ***Theoretical value for t at P : 0.05 level = 2.26 ****Official method(USP)[10]**

in the assay results. These results were compared with those of literature method[2] (first derivative spectrophotometry) and statistically no significant difference was observed between the results according to the student t tests (TABLE 4). These two new methods for the analysis of mixture EE+LNG were found suitable for simple and precise routine analysis of the pharmaceutical preparation selected.

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