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Simultaneous determination of levonorgestrel and ethinyl estradiol in combined dosage form utilizing spectrophotometric methods and high performance thin layer chromatographic method on nanosilica gel plates

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ABSTRACT

Simultaneous quantification of levonorgestrel (LEV) and ethinyl estradiol (EE) was performed utilizing five different spectrophotometric methods and a high performance thin layer chromatographic method (HPTLC). The applied spectrophotometric methods were based on either ratio spectra namely; ratio difference, ratio subtraction and derivative ratio or the presence of isosbestic point specifically; absorbance subtraction and amplitude modulation. The proposed methods had the ability to resolve the overlapped spectra of the drugs with a linear relationship in the concentration range 1-65 µg/mL and 1-95 µg/mL for LEV and EE, respectively. The developed HPTLC method has revealed a good separation of the drugs upon utilizing Nano Silica Gel on TLC plates with fluorescent indicator 254 nm glass plates as the stationary phase and chloroform: methanol (99:1, *v:v*) as the mobile phase. The proposed HPTLC method has shown high sensitivity, where the linearity range was 0.02-3.00 µg/band and 0.5-20.0 µg/band, for LEV and EE, respectively. The proposed methods were successfully applied for the analysis of laboratory prepared mixtures as well as combined dosage form. Validation for all methods was conducted in compliance with the ICH guidelines proving the methods to be selective, linear, precise and accurate. The proposed methods were statistically compared with the pharmacopoeial method, where the obtained results showed no significant difference regarding accuracy and precision.

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1. Introduction

Levonorgestrel ((8R,9S,10R,13S,14S,17R)-13-ethyl-17-ethynyl-17-hydroxy-1,2,6,7,8,9,10,11,12,14,15,16-dodeca hydrocyclopenta[a]phenanthren-3-one) (Figure 1a) is a synthetic female hormone which is usually administered for pregnancy prevention in humans [1]. Ethinyl estradiol; ((8R,9S,13S,14S,17R)-17-ethynyl-13-methyl-7,8,9,11,12,14,15,16-octahydro-6*H*-cyclopenta[a]phenanthrene-3,17-diol) (Figure 1b) is an orally bioactive estrogen usually present in many combined formulations of oral contraceptive pills. It is mainly utilized in hormone therapies for androgen dependent disorders, acne, hirsutism and seborrhoea [2].

Recently, it was shown that, the continuous daily LEV-EE dose of (150-30 µg) suppresses ovarian activity and eliminates cyclic fluctuations in estradiol [3], progesterone, luteinizing hormone and follicle-stimulating hormone [4]. In addition, the combination of these drugs was used as an oral contraceptive for female patients with androgenic symptoms [5]. Many brands and generic combinations of these compounds were released in the pharmaceutical market [6]. Microcept® coated tablets (the Egyptian trade name); containing LEV and EE is widely used as a contraceptive and for the reduction of post-menopausal symptoms [7]. Therefore, the analysis of the drugs in these pharmaceutical formulations is quite important and is a common analytical problem in quality control industry.

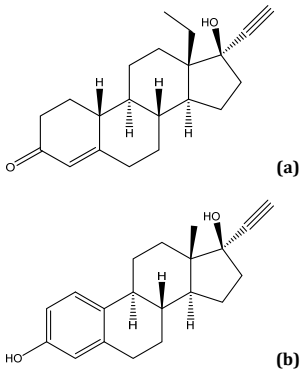

Figure 1. Molecular structures of (a) levonorgestrel (LEV) and (b) ethinyl estradiol (EE).

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Literature survey has revealed several analytical techniques for the simultaneous analysis of the studied drugs. This combination is official in United States Pharmacopoeia (USP), which describes a chromatographic method for its estimation [8]. In addition, RP-HPLC [9], HPLC-tandem mass spectrometry [10,11], molecular imprinted polymer-HPLC [12], immune-affinity chromatography [13], HPTLC [14-15], micellar electrokinetic chromatography (MEKC) [16], voltammetry [17], multivariate calibration technique of partial least squares (PLS) and principal component regression (PCR) [18], first derivative spectrophotometry [19,20] and H-Point standard addition spectrophotometry [21-23] were developed for the determination of LEV and EE in biological and pharmaceutical matrixes. It is obvious that most of the reported methods for the determination of LEV and EE have utilized hyphenated instrumentation whose use is considered a tedious process. These hyphenated analytical techniques are considered time consuming and require high cost as they usually involve several separation steps with the utilization of complex components. On the contrary are the mathematical spectrophotometric and chemometric techniques which have the ability to overcome the above drawbacks in addition they can efficiently resolve complex mixtures of analytes. However, chemometric techniques are less advantageous as they require the availability of specific software for performing the manipulation steps [24,25].

Although LEV/EE is an old combination and is released in the market long time ago, however, few spectrophotometric methods were described in the literature for the analysis of this combination. The reported first derivative spectrophotometric methods [19,20] have shown the disadvantage that the zero crossing point of EE at λ 249 nm does not correspond to a peak maximum or valley for LEV which affects the accuracy and sensitivity of the methods. While the reported H-point standard addition methods [21-23] had the disadvantage of several calculation steps which could be considered complicated. Thus, one of the aims of the presented work was to develop simple spectrophotometric methods based on smart original mathematical techniques with few manipulation steps for analyzing the binary mixture of LEV and EE, consequently, was to conduct a comparative study between the different proposed methods; this comparison was mainly regarding the ability of the developed methods to resolve binary mixture of spectral interfering problem without preliminary separation in terms of specificity and validation. Another aim was to perform an HPTLC method on Nano Silica Gelplates where the small particle size of the plates would add privileges of enhancing the sensitivity, offering better separation and providing wider linearity range than the previously reported HPTLC methods [14-15]. All the proposed methods were found to be simple, precise and accurate in addition, they were independent on any sophisticated apparatus or computer programs.

2. Experimental

2.1. Apparatus and software

The spectrophotometric measurements were carried out with a JASCO V-530 double beam UV-VIS spectrophotometer. Spectral acquisition and elaboration of the obtained data was done using a Spectra Manager Program (JASCO). Quartz cuvettes (1 cm path length) were utilized for measuring the light absorption in the ultraviolet region (200-350 nm).

The HPTLC measurements were carried out using a CAMAG TLC Scanner 3 S/N 130319 operated with win CATS software, Linomat 5 autosampler (CAMAG, Muttenz, Switzerland), CAMAG microsyringe (100 µL), and Nano Silica Gel on TLC plates for HPTLC with fluorescent indicator 254 nm glass plates (10 × 20 cm) with 6.0-9.0 µm particle size (Sigma-Aldrich by Merck KGaA, Darmstadt, Germany) were used.

2.2. Chemicals

Pure samples: Levonorgestrel and ethinyl estradiol were kindly supplied by NODCAR (El Haram, Giza, Egypt). Their purities were found to be 99.40%±1.01 and 99.72%±0.75 for LEV and EE, respectively, by the pharmacopoeial method [8].

Market sample: Microcept labeled to contain 0.150 mg LEV and 0.03 mg EE (Batch number: 1402217), manufactured by Chemical Industries development CID (Giza, Egypt) was purchased from local market.

Solvents: Methanol was obtained from Merck (Darmstadt, Germany) and chloroform was of analytical grade obtained from POCh (Gliwice, Poland).

2.3. Standard solutions

Stock solutions: Separate (1 mg/mL) solutions of LEV and EE were prepared in methanol. Working solutions: Solutions of (0.1 mg/mL) for each of LEV and EE were freshly prepared by dilution from the stock solutions with methanol.

2.4. Procedures

2.4.1. Spectral characteristics

The zero-order absorption spectra (D_0) of standard solutions of final concentration 1-65 µg/mL LEV and 1-95 µg/mL EE were recorded over the wavelength range 200-350 nm against methanol as a blank.

2.4.2. HPTLC-densitometric method

The samples were applied to the Nano HPTLC plates as bands using a 100 µL syringe. The band width was 6 mm and the bands were spaced at about 1 cm apart from each other and 1 cm from the bottom edge of the plate. The developing system was chloroform: methanol (99:1, *v*:*v*). The chromatographic tank was left to be saturated with the developing system at room temperature for 1 h then linear ascending development was performed to a distance of approximately 8 cm from the lower edge. The developed plates were air-dried and scanned at 254 nm. The detection was carried out with the aid of CAMAG TLC Scanner 3 which was operated in the absorbance mode using a deuterium lamp as the source of radiation, keeping the slit dimension at 3 mm × 0.45 mm, and the scanning speed at 20 mm/s.

2.4.3. Construction of calibration curves

For ratio subtraction method (RS); a calibration curve relating the zero order spectra of LEV at 240 nm versus its corresponding concentrations was constructed and the regression equation was computed.

For ratio difference (RD) and derivative ratio (DR) methods; the absorption spectra of LEV were divided by the absorption spectrum of 75 µg/mL EE. While the absorption spectra of EE were divided by the absorption spectrum of 25 µg/mL LEV. The obtained ratio spectra were recorded. These ratio spectra were utilized for both (RD) and (DR) methods. In the (RD) method; calibration curves relating the difference between the amplitudes of the obtained ratio spectra at 247 and 268 nm and between 282 and 247 nm versus the corresponding concentrations of LEV and EE, respectively, were constructed. Then, the regression equations were computed. However, for the DR method; the first derivative of these ratio spectra was computed. Calibration curves were constructed by plotting the amplitudes at 254 and 296 nm against the corresponding concentrations of LEV and EE, respectively. Then, the regression equations were computed.

For absorbance subtraction method (AS); calibration curves relating the zero order spectra of LEV or EE at 267 nm

versus the corresponding concentrations were constructed and the regression equations were computed. The absorbance factor of EE [A_{267}/A_{280}] was calculated and was found to be 0.47.

For Amplitude modulation method (AM); the absorption spectra of the standard solutions were divided by the EE normalized divisor spectrum and the obtained ratio spectra were recorded. Calibration curves relating the amplitudes of LEV or EE at 267 nm against their corresponding concentrations were plotted and the regression equation was computed.

For HPTLC; separate aliquots were accurately transferred from the working standard solutions of each drug to prepare solutions of 4 and 100 µg/mL for LEV and EE, respectively, which were used to construct the calibration curves. The chromatographic conditions were applied, and the chromatograms were recorded. The calibration curves were constructed in the range of 0.02-3 µg/band and 0.5-20 µg/band for LEV and EE, respectively, by plotting the relative peak area at 254 nm against the corresponding concentration; consequently the regression equations were calculated.

2.4.4. Application to laboratory prepared mixtures

For the spectrophotometric methods; seven mixtures containing different ratios of LEV and EE were prepared by transferring accurate aliquots from their working solutions into a series of 10-mL volumetric flasks; methanol was used for completing the volumes. Then, the spectra of the mixtures were recorded at 200-350 nm. The concentration of each drug was calculated by substitution in the corresponding regression equation after applying the corresponding manipulating steps for each method. In case of ratio subtraction method, the spectra of the mixture were divided by the spectrum of 75 µg/mL EE as a divisor to obtain the corresponding ratio spectra. The constant was measured at the plateau region (274-292 nm) then it was subtracted from the ratio spectra. The obtained spectra were then multiplied by the spectrum of 75 µg/mL of EE.

For the HPTLC densitometric method; five mixtures containing different ratios of the cited drugs were prepared by transferring accurate aliquots from their working solutions into a series of 10 mL volumetric flasks, then the volumes were completed with methanol.

2.4.5. Application to pharmaceutical preparation

Twenty tablets were triturated and mixed well. An accurate amount of the powder equivalent to 2.89 mg LEV and 0.578 mg EE was weighed and transferred into a beaker. The powder was mixed with 25 mL methanol and sonicated for about 30 min. The obtained solution was then filtered into 50 mL volumetric flask and the volume was completed with methanol, thus a stock solution was prepared.

For the spectrophotometric methods; an aliquot equivalent to 8.65 mL from the stock solution were transferred to 10 mL volumetric flask and the volume was completed with methanol to obtain a final concentration of 50 µg/mL for LEV and 10 µg/mL for EE. The concentration of each drug was calculated using the corresponding regression equation after applying the corresponding manipulating steps for each method.

While, for the HPTLC method; 4.35 mL aliquots from the stock solution were transferred to 100 mL volumetric flask and the volume was completed with methanol to obtain a final concentration of 2.5 µg/mL for LEV and 0.5 µg/mL for EE. The concentration of each drug was calculated using the corresponding regression equation.

To apply the standard addition technique, different known concentrations of pure standard of each drug were added to the dosage form before proceeding in the previously mentioned methods.

3. Results and discussion

Resolution of binary or ternary mixtures possessing overlapped spectra is an interesting as well as challenging issue for the analytical chemistry. Although, HPLC-UV, LC-MS and GC-MS, *etc*. are the methods of choice for analyzing such mixtures, however, in the last few years the development of mathematical spectrophotometric methods has greatly replaced these techniques with the privilege of being easy to apply, rapid, do not require optimization of conditions such as pH, temperature or flow rate, sensitive and yet highly economical [24,25]. Thus, we were motivated to develop sensitive spectrophotometric methods for the simultaneous analysis of LEV and EE in their bulk powders and dosage form with acceptable accuracy and precision specifically as the literature reveals only few spectrophotometric methods for their determination as previously cited in the introduction. We were aiming to perform simple methods with few manipulating steps to overcome the disadvantage of the complicated calculations of the previously developed H-point standard addition methods [21-23].

Besides, the emergence of Nano Silica Gel plates possessing small particle size and pore diameter; an advantage which is expected to enhance the sensitivity and to demonstrate better separation has encouraged us to carry out an HPTLC method with the aim to determine LEV and EE at lower concentrations and wider range than the previously reported densitometric methods [14-15].

3.1. Spectrophotometric methods

By scanning the absorption spectra of LEV and EE, severely overlapped spectral bands were observed in the wavelength region of 200-350 nm (Figure 2). Although EE can be determined at its λ_{max} at 280 nm, however, this could not provide simultaneous determination of both drugs. In addition, EE had an absorbance at 240 nm hindering the determination of LEV at its λ_{max} 240 nm. Thus, different mathematical spectrophotometric methods were applied for achieving best resolution and quantitative determination of each drug without any interference from the other.

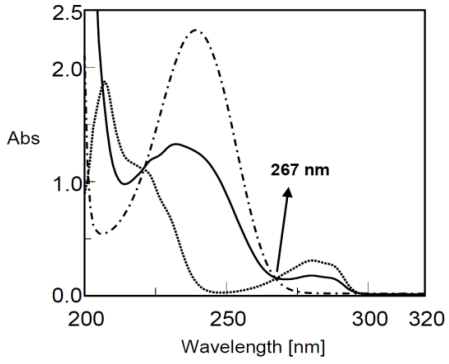

Figure 2. Absorption spectra of 40 µg/mL LEV (-·-·-·-), 40 µg/mL EE (·····) and a binary mixture of 20 µg/mL of each LEV and EE (—).

3.1.1. Ratio difference method (RD)

The overlapped spectra of LEV and EE have suggested that the ratio difference spectrophotometric method could be a suitable method for their simultaneous determination. Lotfy and Hegazy [26] have introduced the ratio difference spectrophotometric method (RD) which depended on measuring the difference between two amplitude values at two wavelengths

using the ratio spectra [27-29]. In the ratio difference method the noise is cancelled because the difference between the amplitude values was measured by subtraction of the two values at the two selected wavelengths.

For the determination of the drugs; LEV (1-65 µg/mL) spectra were divided by the spectrum of EE (75 µg/mL) (Figure 3a) and EE (1-95 µg/mL) spectra were divided by the spectrum of LEV (25 µg/mL) (Figure 3b). The concentration of LEV was calculated by using the regression equation representing the linear relationship between the differences of amplitudes of the ratio spectra at 247 and 268 nm versus the corresponding concentration of the drug. The selection of these two wavelengths was based on the fact that the ratio spectrum of LEV has shown significant difference in the amplitude values at these two wavelengths with different concentrations, while the ratio spectrum of EE has shown the same amplitudes at these wavelengths (i.e. constant). Similarly, EE could be determined in the same manner at the selected wavelengths (282 and 247 nm).

In order to attain the least noise, the smoothest ratio spectra and the highest sensitivity; the effect of the divisor concentration was tested. Thus, different concentrations of LEV (15, 25 and 35 µg/mL)and EE (20, 60 and 75 µg/mL) were tried as a divisor but the concentrations 25 µg/mL of LEV and 75 µg/mL of EE has shown the best results. In addition, the selected wavelengths have proved to be the best concerning the average recovery percent when used for estimating the concentrations of LEV and EE in bulk powder and in laboratory prepared mixtures.

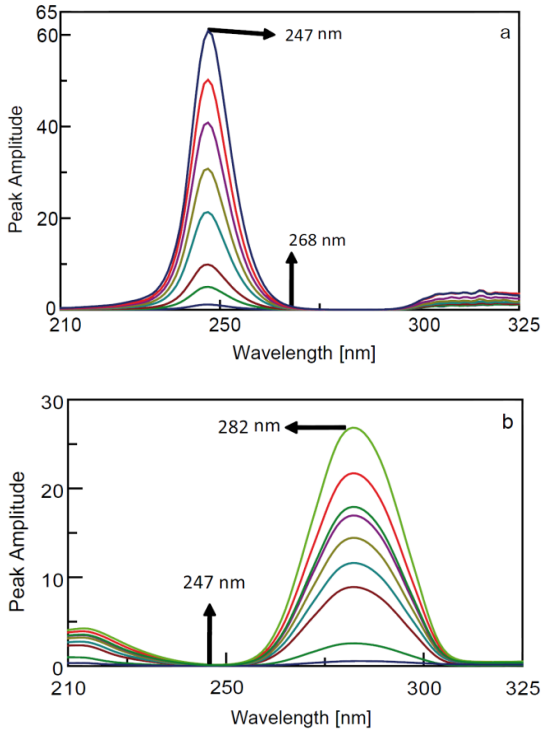

Figure 3. Ratio spectra of (a) LEV (1.0-65.0 µg/mL) using 75.0 µg/mL EE as a divisor and (b) EE (1.0-95.0 µg/mL) using 25.0 µg/mL LEV as a divisor, both spectra showing the two selected maxima and minima.

3.1.2. Ratio subtraction method (RS)

The ratio subtraction method (RS) [30] was used for the determination of LEV concentration in the binary mixture of LEV and EE. The spectrum of the binary mixture was divided by a known concentration of EE as a divisor (75 µg/mL) (Figure 4a). The constant obtained from the division was measured at the plateau (274-292 nm). By subtracting this constant value (Figure 4b), then multiplying the obtained curve after subtraction by the spectrum of 75 µg/mL of EE (the divisor), we could recover the zero order absorption spectrum of LEV present in the mixture. Thus its concentration could be measured at its λ_{max} (240 nm) (Figure 4c).

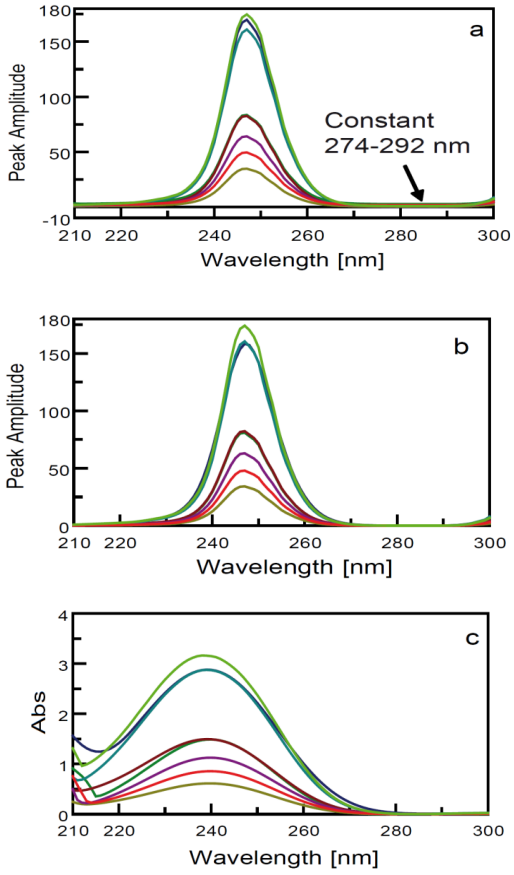

Figure 4. (a) Division spectra of laboratory prepared mixtures using 75 µg/mL EE as a divisor, (b) Division spectra after subtraction of the constants, (c) The obtained zero order spectra of LEV after subtraction of constant and multiplication by the spectrum of 75 µg/mL EE.

3.1.3. Derivative ratio method (DR)

This method has depended on division of the absorption spectrum of one component by the second one (as a divisor) and then the first derivative of the obtained ratio spectra was computed [31-33]. The overlapped spectra of LEV and EE have suggested the utilization of derivative ratio method for their determination. Thus, LEV (1-65 µg/mL) spectra were divided by the spectrum of EE (75 µg/mL) and EE (1-95 µg/mL) spectra were divided by the spectrum of LEV (25 µg/mL) followed by computing the first derivative of the obtained ratio spectra as shown in Figure 5. The concentration of LEV and EE were calculated by using the regression equations representing the linear relationship between the peak amplitudes at 254 and 296 nm versus the corresponding concentrations of the two drugs, respectively. In order to optimize the developed derivative ratio spectrophotometric method, the influence of different variables including divisor concentration, smoothing factor and working wavelengths, were studied. Consequently 25 µg/mL of LEV and 75 µg/mL of EE as divisors have given minimum noise, smoother ratio spectra and maximum sensitivity. The selected wavelengths have proved to be the best concerning the average recovery

percent when used for estimating the concentrations of LEV and EE in bulk powder and in laboratory prepared mixtures.

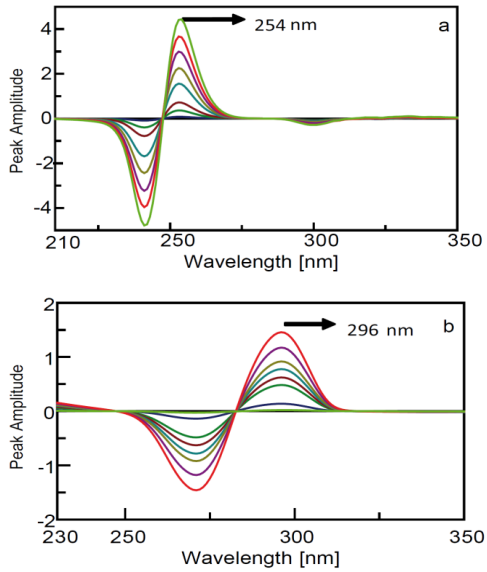

Figure 5. First derivative of ratio spectra of: (a) LEV (1-65 µg/mL) using 75 µg/mL EE as a divisor and (b) EE (1-95 µg/mL) using 25 µg/mL LEV as a divisor.

3.1.4. Absorbance subtraction method (AS)

This method was based on the same principle as the absorption factor method [34-37]. The method has mainly depended on the presence of an isoabsorptive point in the zero order absorption spectra of the drugs (λ_{iso} 267 nm) where the drugs have equal absorptivities at this point. In addition, the spectrum of EE was extended over the LEV. For the determination of LEV and EE, their isosbestic point at 267 nm was utilized (Figure 2). The absorbance of EE at 267 nm was calculated using its absorbance factor at this point (where $[A_{267}/A_{280}]$ for EE was found to be 0.47), and then the absorbance of LEV was obtained by subtraction. The absorbance values of EE and LEV at λ_{iso} 267 nm were used to calculate each of their concentration using the unified regression equation at the same wavelength.

$$A_{EE} \text{ at } 267 \text{ nm} = [A_{267}/A_{280}] \ (A_{mix} \text{ at } 280 \text{ nm}) \quad (1)$$

$$A_{LEV} \text{ at } 267 \text{ nm} = A_{mix} \text{ at } 267 \text{ nm} - A_{EE} \text{ at } 267 \text{ nm} \quad (2)$$

where A_{mix} is the absorbance of the binary mixture, A_{EE} and A_{LEV} are the absorbance of EE and LEV, respectively, and $[A_{267}/A_{280}]$ is the absorbance factor of pure EE at 267 nm to that at 280 nm.

3.1.5. Amplitude modulation method (AM)

This proposed method has depended on the presence of isosbestic point in the absorption spectrum which would be retained at the same point in the ratio spectrum (after division by one component as a divisor). In addition, the extension of the spectra of one component was another requirement [34,36]. The AM method has depended on the utilization of the normalized divisor to eliminate the effect of the choice of the divisor which greatly affects the results of manipulating ratio spectra techniques. Thus, to eliminate the effect of the divisor, the normalized spectrum of EE was used.

By dividing the spectrum of the binary mixture by the normalized EE divisor spectrum, the ratio spectrum was obtained (Figure 6). The amplitude value of the constant was determined at the plateau region at 274-292 nm, which was equal to the amplitude of the constant of EE along the whole spectrum. Since at the isosbestic point (λ_{iso}) at 267 nm, the amplitude of the ratio spectra at this point was equal to the sum of the amplitudes of EE and LEV. Thus, by subtracting the previously obtained constant, the corresponding amplitude of LEV was obtained, which was equivalent to the recorded concentration of LEV in the mixture ($C_{Recorded\ of\ LEV}$).

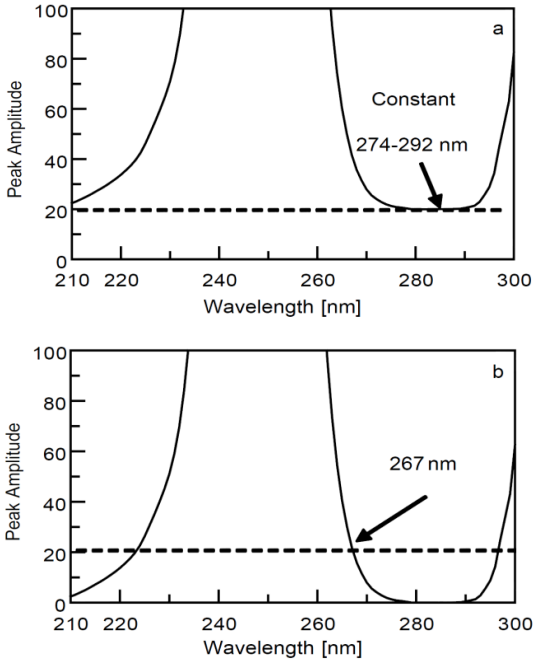

Figure 6. (a) Ratio spectra of laboratory prepared mixture of 20.0 µg/mL of each of EE and LEV using normalized EE as a divisor and (b) ratio spectra of laboratory prepared mixture of 20.0 µg/mL of each of EE and LEV using normalized EE as a divisor after subtraction of constant.

To eliminate any error due to signal to noise ratio, the actual concentration of EE or LEV were calculated by using their corresponding unified regression equation at λ_{iso} = 267 nm.

$$C_{Recorded} = 1.005\ C + 0.070 \quad (3)$$

where $C_{Recorded}$ represented the recorded amplitude of ratio spectrum which was equal to the recorded concentration of LEV and Crepresented corresponding concentration of LEV.

The developed spectrophotometric methods have introduced several advantages; where the RD method has the advantage of complete elimination of the interfering component in the form of a constant so there is no need for critical measurements; this leads to reproducible and robust results. The advantage of RS is that the component of interest is recovered in its zero order spectra and is measured at its λ_{max} thus, minimizing the noise error. The main advantage of DR method is; allowing the use of the wavelength with the highest value either maximum or minimum. Additionally, the presence of a lot of maxima and minima gives a good chance for the analysis of an active compound in the presence of other active ingredients which can possibly interfere with the analysis. The advantage of the AS and AM methods is that both components in a mixture can be determined using unified regression equation at λ_{iso}. An additional privilege for the AM method is the use of the normalized divisor, where the obtained amplitude at the ratio spectrum will directly repre-

Table 1. Assay parameters and validation sheet obtained by applying the proposed methods.

Parameter	RD		RS	DR		AM		AS		HPTLC	
	LEV	EE	LEV	LEV	EE	LEV	EE	LEV	EE	LEV	EE
Wavelength (nm)	P1-P2 247-268	P1-P2 282-247	D_0 at 240	P at 254	P at 296	P at 267		D_0 at 267		At 254	
Calibration range [a]	1-65 µg/mL	1-95 µg/mL	1-65 µg/mL	1-65 µg/mL	1-95 µg/mL	1-65 µg/mL		1-65 µg/mL		0.02-3.00 µg/band	0.5-20.0 µg/band
Slope	0.9971	0.2778	0.0529	0.0732	0.0151	1.0060		0.0035		0.1083	0.2219
Intercept	0.9218	0.0297	0.0578	0.0206	0.0076	0.0700		0.0030		0.0018	0.0770
Correlation coefficient	0.9996	0.9998	0.9995	0.9995	0.9999	0.9997		0.9997		0.9998	0.9995
Mean [a]	99.4	100.1	99.9	100.1	99.9	99.9	99.4	99.4	99.7	99.7	101.1
RSD%	1.01	1.07	1.14	1.35	0.97	0.93	0.76	1.38	0.98	1.31	1.36
SE of slope	8.28×10^{-3}	1.97×10^{-3}	4.53×10^{-4}	9.99×10^{-4}	9.26×10^{-5}	8.17×10^{-3}		2.48×10^{-5}		1.19×10^{-5}	8.62×10^{-4}
SE of intercept	3.06×10^{-1}	1.08×10^{-1}	1.624×10^{-2}	3.72×0^{-2}	5.09×10^{-3}	2.76×10^{-1}		9.16×10^{-4}		1.58×10^{-2}	9.64×10^{-3}
LOD	0.13	0.25	0.18	0.22	0.30	0.28		0.17		0.015	0.42
LOQ	0.39	0.76	0.55	0.67	0.91	0.85		0.52		0.047	1.27
Repeatability [a,b]	0.69	0.59	0.66	0.55	0.65	0.79	0.65	0.75	0.64	0.32	0.86
Inter-day precision [a,b]	0.64	0.72	0.74	0.95	0.71	0.91	0.84	0.72	0.80	0.48	1

[a] Average of three experiments.
[b] Relative standard deviation of three concentrations (10, 35 and 60 µg/mL) of each drug for the spectrophotometric method and of three concentrations of LEV (0.3, 0.5 and 1.0 µg/band) and EE (5, 10 and 15 µg/band) for HPTLC method.

sent the concentration of each component and thus the absorbance factor step is eliminated.

3.2. HPTLC method

This method has offered a simple way to separate and quantify LEV and EE directly on HPTLC plates by measuring the optical density of the separated bands. Several solvent systems were tried for the best separation of the drugs. However, chloroform: methanol (99:1, *v*:*v*) has provided good resolution and sharp symmetrical peaks. The band width was chosen to be 6 mm in order to minimize band diffusion. Moreover, the scanning wavelength was chosen to be 254 nm which has resulted in sharp and symmetrical peaks with minimum noise. The R_f values were 0.55 and 0.40 for LEV and EE, respectively. A typical chromatogram is demonstrated in Figure 7 in which the separation has allowed for the determination of the drugs without any interference from each other. The HPTLC method has the advantages of requiring simple developing systems with no pH adjustments, additionally, several samples can be run simultaneously with a small quantity of the mobile phase, thus offering less analysis time and cost per analysis. In comparison to other developed HPTLC methods for the simultaneous analysis of LEV and EE [14-15] our proposed method has offered higher sensitivity and/or wider concentration range which is referred to the utilization of the Nano HPTLC plates. LEV and EE were determined in the concentration range of 0.02-3 ug/band and 0.50-20 ug/band, respectively.

3.3. Methods validation

Methods validation was performed according to ICH guidelines [38] for the proposed methods as follows:

3.3.1. Linearity and range

The linearity of the proposed methods was estimated by constructing the different calibration curves on three different days. The linearity of the calibration curves were validated by the high value of correlation coefficients. The analytical data of the calibration curve including concentration ranges, calibration equations, standard error of the slope and intercept were summarized in Table 1.

3.3.2. Accuracy

The procedures under construction of the calibration curves, for both drugs using the different proposed methods, were repeated three times for the determination of seven concentrations of pure LEV and EE within the linearity range. The accuracy expressed in terms of percentage recoveries (mean) and standard deviation was shown in Table 1. Good accuracy of the developed methods was indicated by the obtained results.

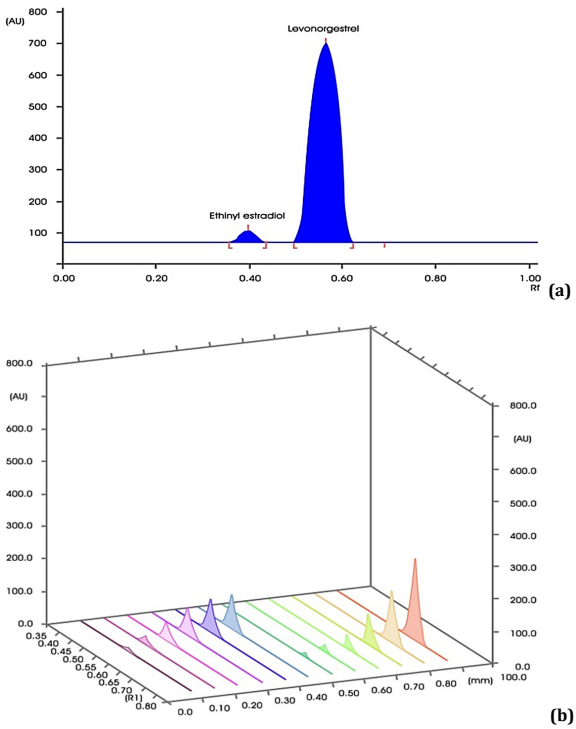

Figure 7. (a) HPTLC chromatogram and (b) Three dimensional (3D) HPTLC chromatogram of: the separated peaks of LEV (R_f = 0.55) over the concentration range of 0.02-3.00 µg/band and EE (R_f = 0.40) over the concentration range of 0.5-20.0 µg/band using chloroform: methanol (99:1, *v*:*v*) as the mobile phase.

3.3.3. Precision

The intra-day and inter-day precision of the proposed methods were determined by the analysis of three different concentrations of pure LEV and EE, each three times on a single day and on three consecutive days, respectively. The results were illustrated in Table 1.

Table 2. Analysis of laboratory prepared mixtures by the proposed methods.

Conc. in µg/mL LEV : EE	Spectrophotometric methods									HPTLC method		
	RD		RS	DR		AM		AS		Conc. in µg/band LEV : EE	LEV	EE
	LEV	EE	LEV	LEV	EE	LEV	EE	LEV	EE			
25 : 25	100.4	100.6	100.7	101.1	99.6	100.4	101.0	98.9	99.9	1:2	100.1	98.2
50 : 25	99.3	100.9	101.8	100.7	99.9	101.8	101.4	99.7	99.9	2:1	101.9	101.2
25 : 5 [b]	101.2	97.8	100.9	100.8	99.3	100.8	99.8	100.9	100.3	3:0.6 [b]	98.7	101.8
50: 10	99.6	101.8	99.9	100.8	101.07	101.4	101.5	100.5	100.6	1:5	99.9	98.5
10 : 10	101.0	99.5	101.6	101.8	100.9	100.5	99.9	101.8	100.3	2:2	101.9	99.7
20 : 20	101.2	101.3	101.1	100.8	100.9	100.5	101.0	98.5	100.2			
60: 12 [b]	100.4	99.9	98.7	99.4	99.7	100.2	100.8	101.9	99.4			
Mean [a]	100.4	100.3	100.7	100.8	100.2	100.8	100.8	100.3	100.1		100.5	99.9
RSD%	0.75	1.33	1.06	0.71	0.74	0.59	0.65	1.34	0.51		1.39	1.59
SEM	0.28	0.37	0.39	0.27	0.28	0.22	0.24	0.51	0.19		0.616	0.586
Variance	0.56	1.78	1.13	0.51	0.55	0.36	0.43	1.82	0.26		2.48	1.97

[a] Average of three experiments.
[b] Ratio present in dosage form.

Table 3. Application of standard addition technique to the analysis of dosage form by applying the proposed methods.

Methods	LEV			EE		
	Found [a, b]	Found [c] Recovery % ± SD	Pure added [d, e] Recovery % ± SD	Found in µg/mL [a, b]	Found [c] Recovery % ± SD	Pure added [d, e] Recovery % ± SD
RD	50.19 µg/mL	100.4±1.09	99.9±1.24	10.03	100.3±0.79	99.7±0.75
RS	50.06 µg/mL	100.1±1.06	99.9±1.07			
DR	50.12 µg/mL	100.2±0.87	100.1±1.00	10.07	100.7±0.91	99.8±0.78
AM	50.07 µg/mL	100.1±0.76	99.8±0.92	10.06	100.6±0.58	100.0±0.86
AS	50.04 µg/mL	100.1±0.77	99.9±1.20	10.01	100.1±0.89	99.9±0.90
HPTLC	2.49 µg/band	99.8±1.08	100.7±1.05	0.5002	100.0±1.37	100.2±1.26

[a] For the spectrophotometric methods; LEV is claimed to be 50 µg/mL and EE is claimed to be 10 µg/mL.
[b] For the HPTLC method LEV is claimed to be 2.5 µg/band and EE is claimed to be 0.5 µg/band.
[c] Average of five experiments.
[d] Average of three experiments (added equivalent to 2.5, 5, 7.5, 10, 15 µg/mL LEV and 10, 20, 30, 40, 50 µg/mL EE) for the spectrophotometric methods.
[e] Average of three experiments (added equivalent to 0.1, 0.2, 0.3, 0.4, 0.5 µg/band LEV and 1, 2, 3, 4, 5 µg/band EE) for HPTLC method.

Table 4. Statistical comparison between the results obtained by the proposed methods and reported methods [8] for the determination of LEV and EE in pure powder form.

Items	LEV						
	RD	RS	AM	AS	DR	HPTLC	Reported method
Mean [a]	99.40	99.90	99.90	99.40	100.10	99.70	99.40
RSD%	1.01	1.14	0.93	1.38	1.35	1.31	1.01
SEM	0.38	0.40	0.35	0.52	0.51	0.59	0.41
Variance	1.01	1.30	0.87	1.88	1.84	1.72	1.02
n	7	7	7	7	7	5	6
Student´s *t*-test [b, c]	0.8280	0.8517	0.9970	0.0038	1.0180	0.3940	
F value	1.807 [d]	1.281 [e]	1.174 [d]	1.854 [e]	1.809 [e]	1.689 [f]	
	EE						
Items	RD	-	AM	AS	DR	HPTLC	Reported method
Mean [a]	100.10	-	99.40	99.70	99.90	101.10	99.70
RSD%	1.07	-	0.76	0.98	0.97	1.36	0.75
SEM	0.40	-	0.29	0.37	0.37	0.62	0.31
Variance	1.13	-	0.57	0.95	0.93	1.89	0.56
n	7	-	7	7	7	5	6
Student´s *t*-test [b, c]	0.0510	-	0.7094	0.0080	0.4264	2.1670	
F value	1.128 [e]	-	1.661 [e]	1.024 [e]	1.696 [e]	3.382 [f]	

[a] Average of three experiments.
[b] The corresponding tabulated value of *t* equals to 2.201 at $p = 0.05$ for the spectrophotometric methods.
[c] The corresponding tabulated value of *t* equals to 2.2621 at $p = 0.05$ for the HPTLC method.
[d] The corresponding tabulated value of F equals to 4.39 at $p = 0.05$.
[e] The corresponding tabulated value of F equals to 4.95 at $p = 0.05$.
[f] The corresponding tabulated value of F equals to 5.19 at $p = 0.05$.

3.3.4. Selectivity

Selectivity was established by analyzing different mixtures of the drugs in different ratios within the linearity range. Satisfactory results were shown in Table 2. Good recovery percentages with accepted standard deviations were obtained in all cases.

3.3.5. Limits of detection and quantification (LOD and LOQ)

The LOD is the lowest concentration of the drug that can be detected, but not necessarily quantitated, under the stated experimental conditions. The LOQ is the lowest concentration of the analyte that can be determined with acceptable precision and accuracy. Both LOD and LOQ were calculated and the results were abridged in Table 1.

3.4. Application of the proposed methods

The proposed procedures were applied for the determination of LEV and EE in Microcept® tablets where the obtained recovery and standard deviation proved no interference from the excipients. The validity of the proposed procedures was further evaluated by applying the standard addition technique. The results obtained were shown in Table 3.

3.5. Statistical analysis

Table 4 showed statistical comparisons of the results obtained by the proposed methods and the pharmacopoeial method [8]. No significant difference between the proposed and the reference method with respect to accuracy and

Table 5. One way ANOVA testing for the different proposed and reported method [8] used for the determination of LEV and EE *.

Drug	Source of variation	DF	Sum of squares	Mean square	F value
LEV	Between experiment	6	7.429	1.2380	0.935 (2.342)
	Within experiment	39	51.663	1.3247	
EE	Between experiment	5	10.91758	2.183516	2.258 (2.502)
	Within experiment	33	31.91307	0.967063	

* The values between parentheses are the theoretical F values, the population means are not significantly different.

precision was ascertained as the calculated t and F values were less than the theoretical ones. One-way ANOVA was performed for the purpose of comparison of developed methods; where Table 5 showed no significant difference between the developed methods.

4. Conclusions

This work introduced the application of sensitive, selective, accurate and precise spectrophotometric resolution techniques and HPTLC method for the analysis of binary mixtures of levonorgestrel and ethinyl estradiol. The spectrophotometric methods were dependent either on the utilization of the ratio spectra of the drugs or the presence of isosbestic point. The advantages of each of the developed spectrophotometric methods were discussed. The HPTLC (on Nano silica gel plates) method besides its high sensitivity, had the advantages of being simple and economic where up to 20 samples could be applied to a single plate and analyzed per one development. The proposed methods were directly applied for the multicomponent determination in laboratory prepared mixtures and in pharmaceutical formulation without the need for any priory chemical treatment such as derivatization or extraction and with minimum mathematical manipulating steps. As a final conclusion, the proposed methods could be effectively used for the routine analysis of the studied drugs in quality control laboratories with satisfactory accuracy and precision.

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