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Determination of Levonorgestrel and Ethinyl Estradiol in Pharmaceutical Formulations by H-Point Standard Addition Method in Nonaqueous Solvent Using Simultaneous Addition of Both Analytes

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H-point standard addition method (HPSAM) with simultaneous addition of both analytes in nonaqueous solution was applied for determination of levonorgestrel (LEV) and ethinyl estradiol (EE) in LD and HD tablets. The results showed that, simultaneous determination could be performed with the ratio 1 to 5 of LEV-EE. The LOD for LEV and EE were obtained 5.1×10^{-7} and 9.6×10^{-7} M, respectively, and the corresponding values of %RSD (n=4) for LEV and EE were 1.96 and 2.69, respectively. Underworking conditions, the proposed method was successfully applied for simultaneous determination of LEV and EE in several synthetic mixtures and LD and HD tablets.

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Introduction

Levonorgestrel (LEV), (17-α)-(+/−)-13-ethyl-17-hydroxy-18, 19-dinorpregn-4-en-20-yn-3-one, is a synthetic female contraceptive hormone used in pregnancy prevention in humans [1]. Ethinyl estradiol (EE), 19-norpregna-1,3,5 (10)-trien-20-yne 3,17 diol,(17α), is mainly used in hormone therapies for androgen dependent disorders, acne, hirsutism, seborrhea [2] (see Fig. 1). Recently it isshown that, the continuous daily LEV-EE (90/20 μg) have a suppress ovarian activity and eliminate 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 forfemale patients with androgenic symptoms [5]. Many brand and generic combinations of these compounds have been introduced to pharmaceutical market [6]. The LD and HD (the Iranian trade names) are containing of LEV and EE and are widely used for treatment of contraceptive and reduction of post-menopausal symptoms [7]. Therefore, determinationof the components in these drugs is relatively importantand frequently is an analytical problem in quality control industry. Detailed survey of literature revealed several analytical techniquesfor simultaneous determination of investigated drugs.The HPLC/tandem mass spectrometry [8],HPLC/ion-trap mass spectrometry [9], molecular imprinted polymer-HPLC [10], immune-affinity chromatography [11] have been mostly used to determine of LEV and EE inbiological and pharmaceutical matrixes.The above-mentioned hyphenated methods bring high cost andtime consumption and require expensive and rather complicateddevices at the same time there is a need for prior separation stepsduring analysis. Hence the two proposed drugs in this study exhibit a very strong overlapping between their absorption spectra, direct spectrophotometry is not suitable and cannot be employed. Although, the Uv-vis method offers desired advantages but itsuffers from some practical difficulties. The advantages of this technique is often overshadowed by interference of LEV which its peak overlap with EE spectrum. Therefore, accurate determination of these two drugs in presences of each other is not possible by a common manner. For overcome to this problem, the principal component analysis (PCA) and chemometric methods can be proposed without using a preliminary separation and in presence of the overlapping spectra of drugs [12]. But, deep understanding of these methods is not easy for some users. The H-point standard addition method (HPSAM) has been introduced by Campins-Falco and et al. as a simple two variable chemometric technique for determination of the concentration of the analytethat is free from both proportional and constant errors in the presence of a known interfere [13]. Also, in 1995 Campins-Falco and coworkers proposed a modified HPSAM, H-point standard addition with simultaneous addition of both analytes,which permitted the resolution of both species from a unique calibration, set by simultaneous addition of both analytes [14]. The HPSAM was firstly developed for spectroph-otometric determination of two analytes which are soluble in aqueous solutions, while it is also capable of developing for determination of two lipophilic analytes in nonaqueous solutions [15]. In this work the H-point standard addition method with simultaneous addition of LEV and EE, has been applied for analyzing of binary mixtures of LEV and EE in methanol using the Uv-vis spectrophotometric method and comparison of results with the results of HPLC analysis.

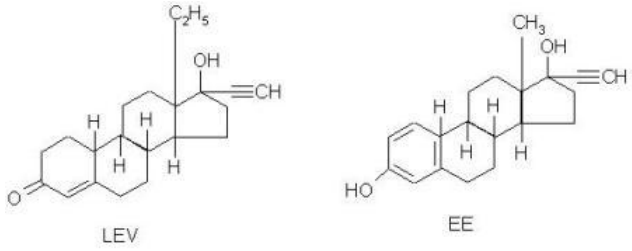

Fig. 1. The structure of Levonorgestrel (LEV) and Ethinyl Estradiol (EE).

Materials and Methods

Experimental section

Reagents

Levonorgestrel (LEV) and ethinyl estradiol (EE) were purchased from Sigma (St. Louis, USA). All other reagents and solvents were purchased from Merck. A stock solution of LEV (1.0×10^{-3} M) and EE (1.0×10^{-3} M) were prepared by dissolving an

appropriate of EE in MeOH and diluting to the mark with MeOH in a 10.0 ml volumetric flask. LD and HD tablets of three different batches were purchased from a local pharmacy store.

Apparatus

Spectrophotometric spectrums were obtained by anAgilent (hp8453) Uv–vis spectrophotometer, equipped with a 1-cm path length quartz cell and a peltier (Agilent89090A). The spectrophotometer was interfaced to a personal computer, and furnished with Chemstation software. The chromatographic analysis was carried out by a KNAURE instrument equipped with a power supplier and a UV detector. The HPLC was controlled by EZ-Chrome Elite software. The separation were performed on a Eurospher 100-5C18 column (240×4.0 mm i.d.). The mobile phase, consisted of MeOH:Acetonitril: H_2O (50:30:20 v/v%). The column was placed at an oven in 40˚C. The flow rate was 1.0 mL min^{-1}, and the detection wavelength was 220 nm. The injection volume was 20 µL and the run time was 7.0 min.

Results and discussions

The theoretical background

Due to the low solubility of LEV and EE in water, the MeOH was used as a suitable medium for development of HPSAM with simultaneous addition of two analytes. In our laboratory, the same theory was used for simultaneous adsorptive stripping voltammetric determination of cadmium and uranium by using LDOPA as a complexing agent [16] and simultaneous determination of atorvastatin and amlodipine [15]. Consider a binary mixture of LEV and EE with typical spectra is shown in Figure 2.

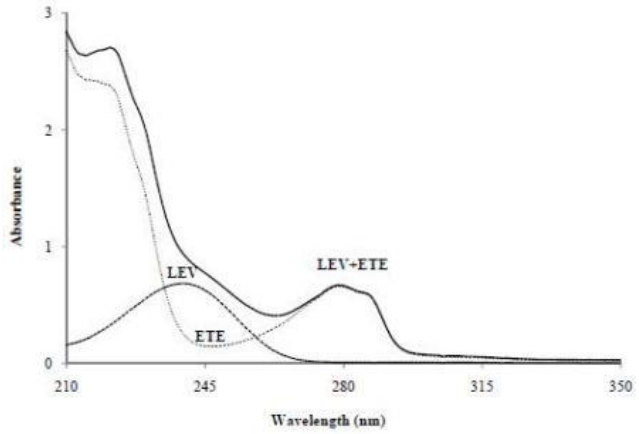

Fig. 2. The spectrum of 1.0×10^{-5} M LEV, 1.0×10^{-5} M EE and LEV+EE in the MeOH solution

As it is observed the absorbance of EE+LEV in each wavelength will be sum of EE and LEV absorbance individually, as follow:

$$A_i = K_{i,\mathrm{LEV}} C^{\circ}_{\mathrm{LEV}} + K_{i,\mathrm{EE}} C^{\circ}_{\mathrm{EE}} + A^{\circ}_i \qquad \text{(I)}$$

Where A_i is the absorbance of the mixture at wavelength λ_i, C^{o}_{LEV} and C^{o}_{EE} are concentrations of LEV and EE in MeOH solution, $Ki,_{LEV}$ and $Ki,$ EE are proportional coefficients at λ_i for corresponding drugs and $A_i{}^{o}$is the residual absorbance at λ_i, which can be nearly omitted by subtracting the absorbance of blank. So, the Equation (I) can be written as:

$$A_i = A^{\circ}_{i,\mathrm{LEV}} + A^{\circ}_{i,\mathrm{EE}} \qquad \text{(II)}$$

$A_{i,}{}^{o}{}_{LEV}$ and $A_{i,}{}^{o}{}_{EE}$ are the individual absorbances of the LEV and EE drugs in the considered sample. According to HPSAM basis the quantification of each analyte X (LEV or EE) in presence of the other as a interferentY, can be performed by the construction of two standard addition plots for the analyte,with $M_{X,1}$ and $M_{X,2}$ slopes, at two previously selected wavelengths, λ_1 and λ_2, which intersect at the H-point with ($-C_{H(X)}$, $A_{H(Y)}$) coordinates. For

example, if LEV is considered as analyte the H-point depends on its concentration C^{o}_{LEV} as:

$$-C_{H(\mathrm{LEV})} = \frac{(A^{\circ}_{\mathrm{LEV},1} - A^{\circ}_{\mathrm{LEV},2}) + (A^{\circ}_{\mathrm{EE},1} - A^{\circ}_{\mathrm{EE},2})}{M_{\mathrm{LEV},2} - M_{\mathrm{LEV},1}} = -C^{\circ}_{\mathrm{LEV}} + \frac{(A^{\circ}_{\mathrm{EE},1} - A^{\circ}_{\mathrm{EE},2})}{M_{\mathrm{LEV},2} - M_{\mathrm{LEV},1}} \qquad \text{(III)}$$

By selecting λ_1 and λ_2 in such a way that the EE absorbance values are equal:

$$A_{EE,1} = A_{EE,2}$$

Then the abscissa of the H-point will be the LEV concentration in the sample, The concentration of EE drug can be determined according to conventional HPSAM from the A_H value and a calibration plot obtained separately for EE drug. HPSAM based on simultaneous standard addition of the two species as reported by Falco *et al*.,[13] permits one to obtain concentration of both analytes in the sample from a unique calibration set. The required data to apply the method are the response of the sample and the response of the sample spiked with known amounts of both analytes at previously selected wavelengths. For simultaneous determination of LEV and EE by spectrophotometric method, let us suppose that λ_1 and λ_2 are selected according to Equation (III). Because the addition is made from a standard mixture of both analytes the relation of the added concentrations between species LEV and EE is same in all of the solutions prepared to apply the method. By representing the analytical signal, absorbance at two previously selected wavelengths λ_1 and λ_2 versus the added concentration of LEV, two lines would be obtained with intercepts $(A^{o}_{LEV,1} + A^{o}_{EE,1})$ and $(A^{o}_{LEV,2} + A^{o}_{EE,2})$ and slopes:

$$slope\ at\ \lambda_1:\ M_{\mathrm{LEV},1} + \left(\frac{C^{i}_{\mathrm{EE}}}{C^{i}_{\mathrm{LEV}}}\right) M_{\mathrm{EE},1} \quad i = 0,1,\ldots,n$$

$$slope\ at\ \lambda_2:\ M_{\mathrm{LEV},2} + \left(\frac{C^{i}_{\mathrm{EE}}}{C^{i}_{\mathrm{LEV}}}\right) M_{\mathrm{EE},2} \quad i = 0,1,\ldots,n$$

$M_{\mathrm{LEV},1}$, $M_{\mathrm{EE},1}$, $M_{\mathrm{LEV},2}$ and $M_{\mathrm{EE},2}$ are the slopes due to the addition of LEV and EE in the lines obtained at λ_1 and λ_2; and are the concentration of considered drugs added in the *i* solution; *n* is the number of additions. *i=0* corresponds to the solution when only the sample exists. Both calibration lines intersect at the H-point, with coordinates ($-C_{H(LEV)}$, $A_{H(EE)}$), where $-C_{H(LEV)}$ is the unbiased concentration of LEV. In this case the abscissa of the H-point will be:

$$-C_{H(\mathrm{LEV})} = \frac{(A^{\circ}_{\mathrm{LEV},1} - A^{\circ}_{\mathrm{LEV},2}) + (A^{\circ}_{\mathrm{EE},1} - A^{\circ}_{\mathrm{EE},2})}{(M_{\mathrm{LEV},2} - M_{LEV,1}) + \left(\frac{C^{i}_{\mathrm{EE}}}{C^{i}_{\mathrm{LEV}}}\right)(M_{\mathrm{EE},2} - M_{\mathrm{EE},1})} = \frac{(A^{\circ}_{\mathrm{LEV},1} - A^{\circ}_{\mathrm{LEV},2})}{(M_{\mathrm{LEV},2} - M_{\mathrm{LEV},1})} \qquad \text{(IV)}$$

The incorrigible error due to the presence of EE, in spite of its concentration is not constant, has been transformed into a constant systematic error as the HPSAM basis predicts. It can be proved that $A_{H(EE)}$ is equivalent to:

$$A_{H(\mathrm{EE})} = A^{\circ}_{\mathrm{EE},1} - \left(\frac{C^{i}_{\mathrm{EE}}}{C^{i}_{\mathrm{LEV}}}\right) M_{\mathrm{EE},1} C_{H(\mathrm{LEV})} = A^{\circ}_{\mathrm{EE},2} - \left(\frac{C^{i}_{\mathrm{EE}}}{C^{i}_{\mathrm{LEV}}}\right) M_{\mathrm{EE},2} C_{H(\mathrm{LEV})} \qquad \text{(V)}$$

In the same way as for LEV, by selecting two potentials λ_3 and λ_4 in such a way that the LEV presents the same absorbance similar expressions can be obtained for EE. The analogous expressions for Eqs IV and V in analysis of EE are:

$$-C_{H(EE)} = \frac{(A^{\circ}_{\mathrm{EE},3} - A^{\circ}_{\mathrm{EE},4}) + (A^{\circ}_{\mathrm{LEV},3} - A^{\circ}_{\mathrm{LEV},4})}{(M_{\mathrm{EE},4} - M_{\mathrm{EE},3}) + \left(\frac{C^{i}_{\mathrm{LEV}}}{C^{i}_{\mathrm{EE}}}\right)(M_{\mathrm{LEV},4} - M_{\mathrm{LEV},3})} = \frac{(A^{\circ}_{EE,3} - A^{\circ}_{\mathrm{EE},4})}{(M_{EE,4} - M_{\mathrm{EE},3})} \quad \text{(VI)}$$

And

$$A_{H(\mathrm{LEV})} = A^{\circ}_{\mathrm{LEV},3} - \left(\frac{C^{i}_{\mathrm{EE}}}{C^{i}_{\mathrm{LEV}}}\right) M_{\mathrm{LEV},3} C_{H(\mathrm{EE})} = A^{\circ}_{\mathrm{LEV},4} - \left(\frac{C^{i}_{\mathrm{LEV}}}{C^{i}_{\mathrm{EE}}}\right) M_{\mathrm{LEV},4} C_{H(\mathrm{EE})} \quad \text{(VII)}$$

For selection of appropriate wavelengths of λ_1, λ_2, λ_3 and λ_4 for application HPSAM with simultaneous addition of both analytes, as mentioned above, the following principles must be considered. At two selected wavelengths (λ_1 and λ_2 or λ_3 and λ_4) the analyte signals (LEV or EE) must be linear with the concentration, the interferent signals or second analyte (EE or LEV) must remain equal, and the analytical signals of the mixture composed of both compounds should be equal to the sum of the individual signals of two compounds. In addition, the slope difference of the two straight lines obtained at λ_1 and λ_2 or λ_3 and λ_4 must be as large as possible in order to get good accuracy and sensitivity. As can be seen from the spectra (Fig. 2), there are several pairs of wavelengths that fulfill the requirement for application of HPSAM. The best results in terms of linearity (correlation coefficients greater than 0.99), precision and accuracy are generally obtained for the wavelengths pairs of 216 and 258 nm for determination of LEV and 238 and 274 nm for determination of EE. In addition, these two pair's wavelengths caused the largest difference between the slopes of standard addition lines $M_{LEV,\lambda 2} - M_{LEV,\lambda 1}$ and $M_{EE,\lambda 2} - M_{EE,\lambda 1}$.

At H-point, C_H is independent of the concentration of interferent and A_H is also independent of the analyte concentration. Figures 3a, 3b, 3c, 3d, and 3e clearly shows the effects of concentration of LEV and EE on the position of the H-point. However, as shown in Figures 3a, 3b, 3c, 3d, and 3e the value of C_H for each analyte (X: LEV or EE) is independent of the concentration of interferent (Y: EE or LEV) in the sample. Four synthetic mixtures with different concentrations of LEV and EE were prepared in MeOH and were analyzed by using the suggested method. The results are given in Table 1. As can be seen from Table 1, the accuracy of the analysis is satisfactory when the concentration ratio of LEV-EE varies from 1 to 5.

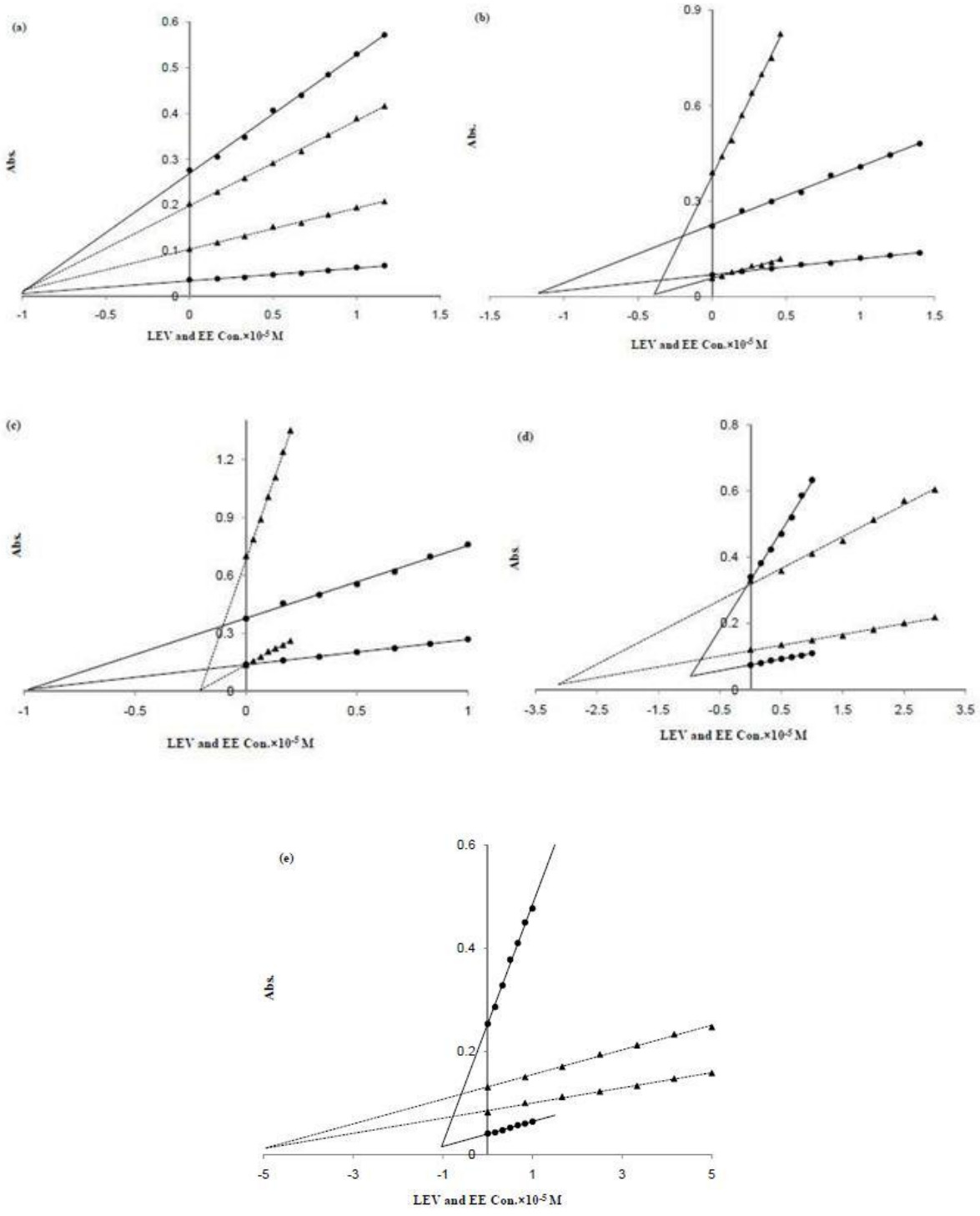

Fig. 3. Plots of HPSAM for simultaneous determination of (**a**) 1.0×10^{-5} M LEV (●) and 1.0×10^{-5} M EE (▲); (**b**) 1.2×10^{-5} M of LEV (●) and 4.0×10^{-6} M of EE (▲); (**c**) 1.0×10^{-5} M of LEV (●) and 2.0×10^{-6} M of EE (▲); (**d**) 1.0×10^{-5} M of LEV (●) and 3.0×10^{-5} M of EE (▲); and (**e**) 1.0×10^{-5} M of LEV (●) and 5.0×10^{-5} M of EE (▲).

Tablel. Results of four experiments for the analysis of LEV–EE mixtures in different concentration ratios

A-C equation	R^2	Sample ($\times10^{-5}$ M)		Found ($\times10^{-5}$ M)	
		LEV	ETE	LEV	ETE
A_{238}=0.269C+0.325	0.994	1	3	0.975±0.021	3.12±0.07
A_{274}=0.071C+0.096	0.994				
A_{216}=0.200C+0.682	0.997				
A_{258}=0.149C+0.531	0.996				
A_{238}=0.296C+0.33	0.997	1	5	1.026±0.015	4.94±0.05
A_{274}=0.035C+0.074	0.998				
A_{216}=0.096C+0.317	0.998				
A_{258}=0.032C+0.117	0.994				
A_{238}=0.182C+0.226	0.998	1.2	0.4	1.171±0.018	.392±0.011
A_{274}=0.049C+0.069	0.996				
A_{216}=0.950C+0.379	0.999				
A_{258}=0.129C+0.058	0.993				
A_{238}=0.376C+0.377	0.999	1	0.2	0.98±0.028	0.204±0.007
A_{274}=0.130C+0.136	0.996				
A_{216}=3.294C+0.681	0.999				
A_{258}=0.628C+0.135	0.997				

Reproducibility of the Method

Under optimum conditions, simultaneous determination of LEV and EE were made by using proposed HPSAM. To check the reproducibility of the method, four replicate experiments of LEV and EE determination were carried out (Table 2). A good RSD (%) was obtained for LEV and EE as is given in Table 2. The limit of detections (LOD) were measured as LOD= 3 Sc. Where Sc is the standard deviation of several (n= 4) replicated measurements of zero concentration of each analyte with HPSAM [17, 18]. The corresponding values of LOD obtained for LEV and EE were 5.1×10^{-7} and 9.6×10^{-7} M, respectively, and the corresponding values of %RSD (n=4) for LEV and EE were 1.96 and 2.69, respectively.

Table 2. Results of four replicate experiments for the analyses of LEV–EE mixtures

A-C equation	R^2	Sample(1×10^{-5} M)		Found(1×10^{-5} M)	
		LEV	ETE	LEV	ETE
A_{238}=0.260C+0.269	0.998	1	1	1.017	0.989
A_{274}=0.027C+0.032	0.997				
A_{216}=0.186C+0.197	0.996				
A_{258}=0.090C+0.102	0.998				
A_{238}=0.259C+0.258	0.994	1	1	0.971	1.047
A_{274}=0.029C+0.034	0.999				
A_{216}=0.188C+0.199	0.996				
A_{258}=0.091C+0.098	0.997				
A_{238}=0.262C+0.264	0.999	1	1	0.988	1.027
A_{274}=0.027C+0.037	0.995				
A_{216}=0.186C+0.205	0.992				
A_{258}=0.092C+0.101	0.996				
A_{238}=0.253C+0.259	0.998	1	1	0.984	0.997
A_{274}=0.024C+0.032	0.997				
A_{216}=0.187C+0.201	0.994				
A_{258}=0.091C+0.104	0.999				
Mean				0.990	1.015
Standard deviation				0.019	0.269
RSD%				1.96	2.69

Analytical application

The applicability of the developed method was further assessed through determining the LEV and EE in two different batches of LD and HD tablets. Samples were prepared according to our pervious works [19]. LEV and EE were extracted from LD and HD tablets using the MeOH as an appropriate solvent and analyzed using the proposed method. Also it was analyzed by HPLC (Fig.4a, 4b) and the results were compared with together (Tables 3-6). As it is observed from Tables 3-6 these results indicated that there are no significant differences between the outputs of the HPLC and the proposed method in non aqueous solution.

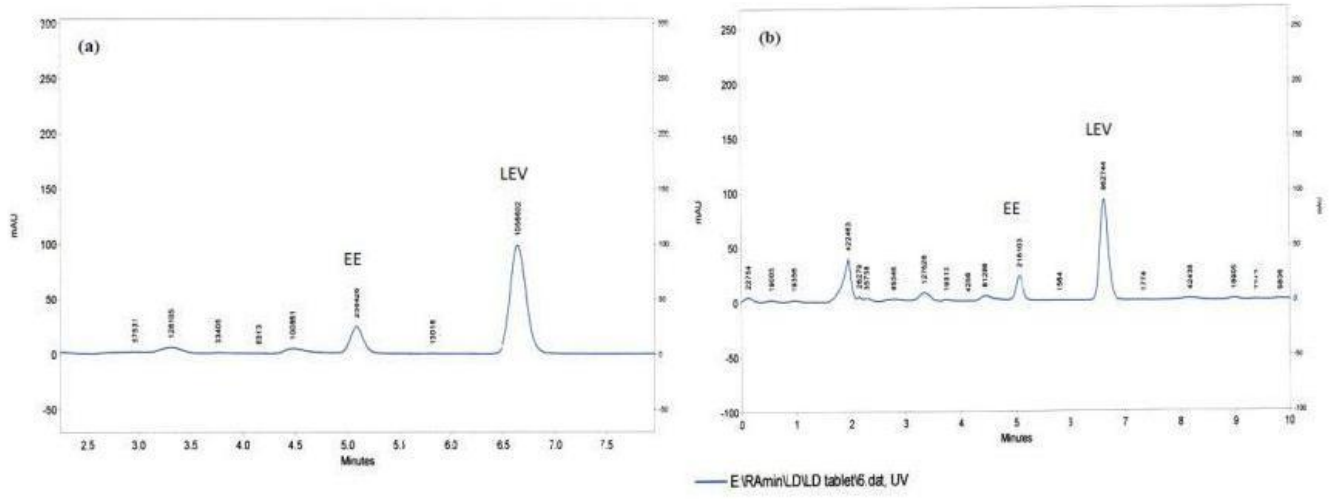

Fig. 4. A sample chromatogram of LEV and EE in (**a**) HD and (**b**) LD tablets: the mobile phase contained MeOH–acetonitrile–H_2O (50:30:20 v/v%), the wavelength of detection was 220 nm.

Table 3. Determination of LEV and EE in several LD samples at first batch using the proposed methodology and HPLC.

LD Samples	Found LEV µg/tablet	Found ETE µg/tablet	Found LEV µg/tablet	Found ETE µg/tablet
	Proposed method	Proposed method	HPLC	HPLC
1	152.30	29.42	149.61	30.37
2	153.01	29.51	150.82	29.03
3	146.35	28.67	148.03	29.71
4	155.24	30.30	148.54	28.88
5	148.70	29.05	149.12	29.49
6	153.82	29.77	147.69	27.98
Mean	151.57	29.45	148.97	29.24
Standarddeviation	3.365	0.313	2.562	0.819
RSD%	2.22	1.06	1.72	2.79

Table 4. Determination of LEV and EE in several LD samples at second batch using the proposed methodology and HPLC.

LD Samples	Found LEV µg/tablet	Found ETE µg/tablet	Found LEV µg/tablet	Found ETE µg/tablet
	Proposed method	Proposed method	HPLC	HPLC
1	150.44	28.46	150.20	29.02
2	148.36	29.12	147.66	27.69
3	149.55	30.07	146.13	30.96
4	149.86	27.89	149.60	28.76
5	150.29	29.00	149.38	29.54
6	146.12	30.19	149.81	29.37
Mean	149.10	29.12	148.80	29.22
Standarddeviation	2.864	0.895	1.574	1.072
RSD%	1.92	3.38	1.06	3.67

Table 5. Determination of LEV and EE in several HD samples at first batch using the proposed methodology and HPLC.

HD Samples	Found LEV µg/tablet	Found ETE µg/tablet	Found LEV µg/tablet	Found ETE µg/tablet
	Proposed method	Proposed method	HPLC	HPLC
1	243.28	49.44	249.13	47.24
2	251.22	49.07	246.27	49.55
3	248.35	51.03	250.41	49.38
4	248.64	48.52	250.02	48.49
5	246.89	48.25	247.59	50.72
6	248.97	49.86	247.15	47.95
Mean	247.89	49.36	248.41	48.89
Standard deviation	2.656	0.817	1.670	1.248
RSD%	1.07	1.66	0.68	2.55

Table 6. Determination of LEV and EE in several HD samples at second batch using the proposed methodology and HPLC.

HD Samples	Found LEV µg/tablet	Found ETE µg/tablet	Found LEV µg/tablet	Found ETE µg/tablet
	Proposed method	Proposed method	HPLC	HPLC
1	249.02	48.34	249.81	48.33
2	244.41	48.87	247.27	49.50
3	250.64	50.95	246.54	50.13
4	247.97	46.41	245.98	46.66
5	245.75	49.15	250.10	48.07
6	248.36	48.79	243.36	49.54
Mean	247.69	48.75	247.18	48.70
Standarddeviation	5.104	1.460	2.512	1.092
RSD%	2.06	2.99	1.02	2.24

Conclusions

The present study demonstrated that LEV and EE drugs can be determined by using H-point standard addition method with simultaneous addition of both analytes in non-aqueous solution. This method offers a practical potential for simultaneous determination of LEV and EE in LD and HD tablets with an acceptable selectivity, sensitivity, simplicity and speed.

Conflict of interest

Authors certify that no actual or potential conflict of interest in relation to this article exists.

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