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Simultaneous determination of emtricitabine and tenofovir disoproxil fumarate in pharmaceutical preparations using spectrophotometric, chemometric and chromatographic methods

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Abstract: Simple, accurate and sensitive spectrophotometric, chemometric and chromatographic methods were used for the simultaneous determination of emtricitabine (ETC) and tenofovir disoproxil fumarate (TDF) in tablets. In 1st derivative spectrophotometry, the first derivative spectra of the solution of ETC and TDF in water were recorded as $\Delta\lambda$ = 4 nm and the first derivative absorbances were measured at the zero-crossing points at 297.3 and 281.2 nm for ETC and TDF, respectively. In ratio of the 1st derivative spectrophotometry measurements were recorded at 239.0 and 270.2 nm for ETC and TDF, respectively. Then analytical signals were measured at the wavelengths corresponding to either maximum or minimum for both drugs. For these spectrophotometric methods Beer's law is obeyed in the concentration range of 2–15 μg mL^{-1} for both drugs. As chemometric method, the PLS technique was used. In chromatographic method, the separation was achieved on a C18 column with DAD (262 nm) and isocratic elution of methanol, acetonitrile and 0.1 % orthophosphoric acid in the volume ratio of 40:40:20, respectively, containing the mobile phase. The mean recovery and the relative standard deviation of the methods were found as 97.51–100.17 % and 0.55–1.26 % respectively. All these methods were statistically compared, and they were successfully applied to a pharmaceutical preparation.

Keywords: tenofovir disoproxil fumarate; emtricitabine; first derivative; ratio; PLS; HPLC.

INTRODUCTION

ETC (Fig. 1) is a nucleoside reverse transcriptase inhibitor also, chemically named 4-amino-5-fluoro-1-[(2*R*,5*S*)-2-(hydroxymethyl)-1,3-oxathiolan-5yl]-1,2--dihydropyrimidin-2-one. Both it and TDF are active drugs against hepatitis B

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virus and used for the treatment of human immunodeficiency virus (HIV) infection in adults and children.[1,2]

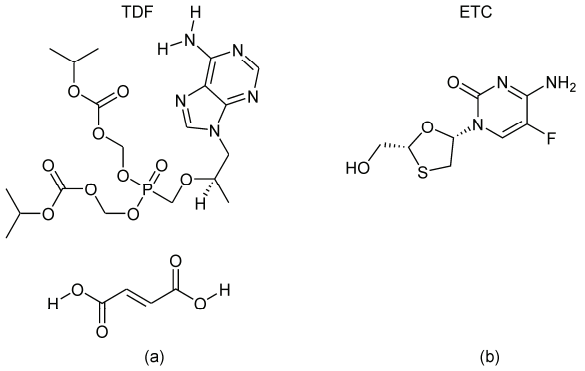


Fig. 1. Chemical structures of: a) tenofovir disoproxil fumarate b) emtricitabine.

TDF (Fig. 1) is an acyclic nucleoside phosphonate di ester analogue of adenosine monophosphate, a nucleoside reverse transcriptase inhibitor. The molecular formula is $C_{23}H_{34}N_5O_{14}P$ and chemically named [[(2*R*)-1-(6-aminopurin--9-yl)propan-2-yl]oxymethyl-(propan-2-yloxycarbonyl-oxymethoxy)phosphoryl]-oxymethyl propan-2-yl carbonate; (*E*)-but-2-enedioic acid, respectively.[3,4]

Several analytical methods were studied using the reverse-phase HPLC (RP--HPLC),[4–8] ultra-performance liquid chromatography,[9] UV–Vis spectroscopic methods,[10–15] HPTLC[16] and LC–MS[17–20] which have been reported in literature for the assessment of TNF and ETC in their single formulations and in combination together or with other drugs. Limited number of studies[21] are available in literature about chemometric approach for these two substances and so far there is no study for the determination and the comparison of chemometric approach of spectrophotometric method and chromatographic method.

The derivative spectrophotometry is an analytical technique of a highly useful method for both qualitative and quantitative analysis of the spectra composed of unresolved bands. The zero-crossing approach involves measuring the absolute value of the total derivative spectrum at an abscissa value matching the derivative spectra, of the first derivative in this study (^{1}D) of each individual component's zero-crossing wavelengths. The wavelength that is chosen because it has the best linear correlation to the analyte concentration in terms of the absolute value of the derivative absorbances at that wavelength.[22] In ratio of the 1st derivative spectra method; the ratio spectra of the solutions of ETC or TDF at different concentrations were obtained by dividing each with the stored standard spectrum of the TDF or ETC and then the first derivatives of these spectra are traced (^{1}DD). The unknown analyte concentrations can be calculated by reading the analytical signals at various points within the selected wavelength range.[23]

The chemometric approach is the easiest technique for the determination of active ingredients in mixtures and of pharmaceutical preparation, because using different solutions that contain various concentrations of active ingredients, without separation methods, each ingredient concentration can be calculated in a few seconds. There are many techniques available for this purpose, the principal component regression (PCR) and the partial least-squares (PLS) are the mainly preferred. These techniques have some advantages over other multicomponent analytes.[24–27] By creating the matrixes that include such variables, as for instance the absorbance value and concentration, the assessment of the exact concentration of the active ingredients of preparation can be reached.

The aim of this study was to develop the appropriate spectrophotometric (derivative and ratio spectra derivative) and chemometric methods for the determination of ETC and TDF simultaneously in their binary mixture and comparing these techniques with a new RP-HPLC method. In addition, it was also aimed to apply all methods developed in order to enable the determination of TDF and ETC in marketed pharmaceutical formulations, such as a tablet, containing these two drugs.

EXPERIMENTAL

Materials

Reference standards of ETC, 99.8 % purity and TDF, 99.7 % purity were obtained as a gift from İLKO Drug Ind. (İstanbul, Türkiye). H_3PO_4 were purchased from Sigma Aldrich. HPLC-grade acetonitrile and methanol were purchased from Merck. Ortho-phosphoric acid, analytical-grade hydrochloric acid and analytical-grade sodium hydroxide from Merck. Quercetin (internal standard) was purchased from Merck. “Hivent” is a commercial pharmaceutical preparation which includes both TNF and ETC, used for the treatment of HIV, were obtained as a gift from İLKO Drug Ind. (İstanbul, Türkiye and Batch no: 2203910002).

Instrumentation and chromatographic conditions

The spectrophotometric analyses were performed using Jasco V-730 (C246261798) UV–Vis spectrophotometer connected to a computer. The standard quartz cuvette (10 mm) was used for the measurement of the absorbance values. In the PLS method, the multi-variate analysis Add-ing for Excel v. 1.3 software (Brereton, 2002) was used.[28] The zero-order absorbance spectra were recorded over the wavelength range of 200–320 nm for the 1st derivative with the zero-crossing and for the ratio spectra of the the 1st derivative spectra methods. The suitable settings were response time, 0.015 s; scan speed, 1000 nm min^{-1}; spectral slit width, 1 nm; data interval, 0.2 nm; $\Delta\lambda = 4$ nm.

The chromatographic system consisted of a Shimadzu liquid chromatograph equipped with a pump (LC-10AT VP), a controller (SCL-10A VP) connected to a computer using a software (Class-VP 5.03), an autosampler (SIL-10AD VP), 30 µL injection loop and diode array detector (DAD, SPD-10A VP). The system was controlled through a system controller (SCL-10A) using a personal computer using a CLASS-VP 5.0 workstation with a data processing system (Shimadzu, Kyoto, Japan) installed on it. The separation was performed on a XTerra, C18 (100 mm×4.6 mm i.d., 3.5 µm) analytical column (Waters, Milford, MA, USA). The column temperature was set to 25 °C. The mobile phase consisted of 0.1 vol. % ortho-

-phosphoric acid, (pH adjusted to 2.5 with NaOH), acetonitrile and methanol in the volme ratio of 40:40:20 in the isocratic mode and DAD detector was set to the wavelength of 262 nm. 10 µL of the sample solutions were injected into the HPLC system at the flow rate 0.5 mL min^{-1}. Quercetin was chosen as the internal standard as its peak was very well resolved from the two drugs peaks and baseline. All solutions were prepared in type 1 water (Simplicity 185 Water System, Millipore Corp., Bedford, MA, USA). The mobile phase was filtered through a membrane filter with a pore diameter of 0.45 µm and kept in an ultrasonic bath for 15 min to remove the soluble gases. At the end of the analysis column was flushed with approximately 20 times of column volume of HPLC grade water and then methanol. Finally, the column was stored in pure methanol. This procedure was applied for every analysis.

Chemicals and solutions

The stock solutions of ETC and TDF (1 mg mL^{-1}) were prepared in 0.05 M HCl and stored at –20 °C for one week. Quercetin (IS, 1 mg mL^{-1}) was prepared in methanol. The standard solutions were prepared daily by diluting the stock solutions with water to the desired concentrations. All solutions were prepared in type 1 water (Simplicity 185 Water System, Millipore Corp., Bedford, MA, USA).

Sample preparation

Twenty Hivent tablets (each tablet contains 200 mg ETC and 300 mg TDF) weighed accurately and crushed in a mortar. An amount equivalent to one tablet was transferred into a 100-mL volumetric flask. Then, it was dissolved in 0.05 M 50 mL HCl, and the volume was completed to the mark with distilled water. After 30 min of shaking the solution was filtered through the filter paper and it was used for the spectrophotometric and chemometric analysis after the dilution with the distilled water in order to obtain the final concentrations within the specified range of ETC and TDF (Final solution). In RP-HPLC method, this final solution was sonicated for 45 min. and filtered through a membrane filter with a pore diameter of 0.45 µm.

RESULT AND DISCUSSION

Method development

For the validation, studies were performed within the scope of International Conference on Harmonization (ICH) requirements.[29,30] In this context, the retention time, the capacity factor, the tailing factor and the theoretical number of plates for each active ingredients were calculated according to the data obtained from the HPLC method. For the validation studies, the specificity, the limit of detection (*LOD*), the limit of quantification (*LOQ*), the linearity, the accuracy and the intra-day and the inter-day precision parameters were investigated for spectrophotometric, chemometric and HPLC methods.

For the accuracy, a stock solutions of active ingredients of 1000 µg mL^{-1} were prepared in 0.05 M HCl. The working standard solutions were prepared in the range of 1–15 µg mL^{-1}. The linearity was calculated using the regression equation ($y = mx + n$) data including concentration the ranges, the correlation coefficients, and the standard error of intercept.

Using the standard addition method at various concentration the levels were studied and compared by the difference between the found and the actual value

show the accuracy of the method. The precision studies were carried out by analysing three different concentration levels at six replicates. Fig. 2 shows the zero--order absorption spectra of the solution of ETC and TDF in water. In the first derivative by the zero-crossing method the first derivative spectra were traced with the interval of 4 nm (1D). Fig. 3 shows the 1st derivative spectra obtained at the increasing concentrations for ETC and TDF. The derivative absorbances ($\Delta A/\Delta\lambda$ values) read at 281.2 and 297.3 nm (zero-crossing points for ETC and TDF, respectively) in their 1D spectra were used for the analysis of ETC and TDF in pure form and in pharmaceutical tablets.

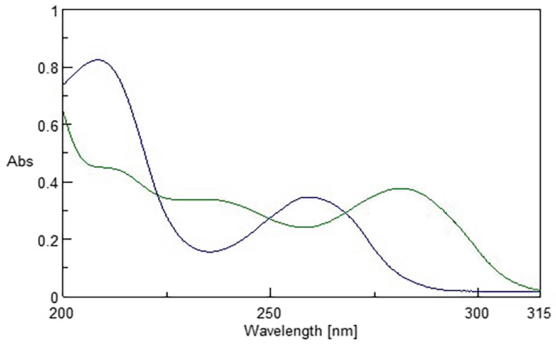


Fig. 2. Zero-order absorption spectra of 15 μg mL^{-1} solutions of ETC and TDF in water.

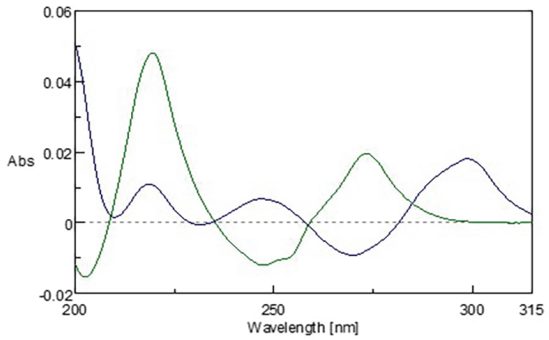
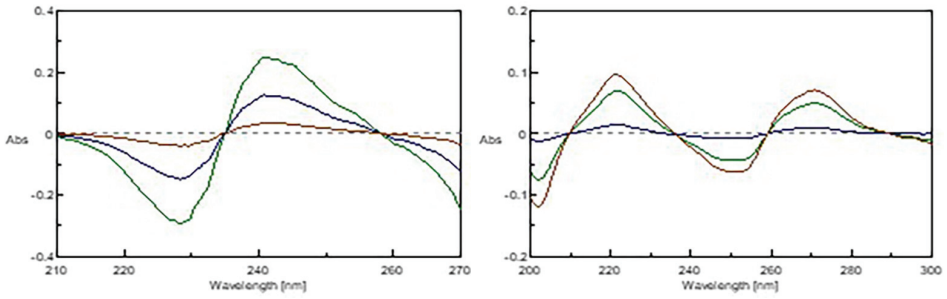


Fig. 3. 1st Derivative absorption spectra of 10 μg mL^{-1} ETC and 10 μg mL^{-1} TDF solutions in water.

For the ratio of the 1st derivative spectra studies, the solutions containing both ETC and TDF were prepared in their increasing concentrations. The solutions of the co-existing component were also prepared in a constant concentration (selected as 10 μg mL^{-1} for both, because the optimum condition were obtained with this concentration) as divisor and their spectra are stored. The ratio spectra of different standards at the increasing concentrations were obtained by dividing each with the stored spectrum of the stored spectra of another drug (divisor).

Then, their first derivative spectra were recorded as $\Delta\lambda$ = 4 nm. The analytical signals were read at 239.0 and 270.2 nm, for ETC and TDF respectively, which gave a linear correlation for these substances. The obtained spectra are shown in Fig. 4a and b according to the increasing concentrations of ETC (each solution composed of 10 μg mL^{-1} TDF and increasing concentration of 2, 5, 7.5, 10, 15 μg mL^{-1} ETC) and TDF (each solution composed of 10 μg mL^{-1} ETC and the increasing concentration of 2, 5, 7.5, 10, 15 μg mL^{-1} TDF). The divisor concentration was kept constant at 10 μg mL^{-1} for both TDF and ETC. Table I summarized the wavelengths selected and the calibration results for ETC and TDF. The calibration curves were prepared by plotting the analytical signals read against to the concentrations.



(a) (b)

Fig. 4. Ratio 1st derivative spectra samples obtained increasing concentrations (2, 7.5, 15 μg mL^{-1}) of both ETC and TDF in water.

TABLE II. Results of regression analysis for TDF and ETC in 1st Derivative and ratio 1st derivative spectra and HPLC method; *SE* – standard error

Parameter	1st Derivative – zero crossing		Ratio 1st D. spec.		HPLC	
	ETC	TDF	ETC	TDF	TDF	ETC
λ / nm	297.3	281.2	239.0	270.2	262	262
Linearity range, μg mL^{-1}	2–15	2–15	2–15	2–15	1–15	1–15
Slope (m) ± *SE* ($y = mx+n$)	0.001681± 0.0001	0.001858± 0.0002	0.0151± 0.003	–0.0191± 0.003	0,396± 0.0004	0,447± 0.0005
Intercept (n) ± *SE*	0.000606± 0.0001	0.000815± 0.00015	–0.0007± 0.00016	–0.0006± 0.0001	–0.,064± 0.0004	0.075± 0.0001
R^2	0.9988	0.9973	0.9999	0.9998	0.9998	0.9998

To optimize the simultaneous determination of ETC and TDF using the ratio of the 1st derivative spectra method, it is necessary to test the divisor concentration. For this purpose, some different divisor standard concentrations were studied. 10 μg mL^{-1} was found optimal as the divisor concentration. The first derivative spectra were recorded as $\Delta\lambda$ = 4 nm interval and the analytical signals

were recorded at the selected wavelengths (239.0 and 270.2 nm for ETC and TDF, respectively) were plotted against the concentration. In the regression analysis the correlation coefficients obtained in the linear range values of all the methods studied were very close to 1 (Table I).

For PLS method the absorbance data matrix for the training set in different compositions (in the working range of 2.0–15.0 μg mL^{-1} for ETC and TDF) were obtained by the measurements of the absorbance values between 240 and 320 nm in the 4 nm wavelengths intervals in the zero-order absorption spectra.

The PLS technique was chosen as chemometric technique. In this technique, the calibration or regression was obtained using the absorbance matrix and the concentration matrix for prediction of the unknown concentrations of ETC and TDF in their binary mixtures and the pharmaceutical formulations. The standard error of prediction (*SEP*) in the prediction step can be used to describe the prediction ability of this technique:

$$SEP = \sqrt{\sqrt{\frac{\sum_{i=1}^{n} (C_i^{\text{added}} - C_i^{\text{found}})^2}{n}}} \quad (1)$$

where C_i^{added} is the added concentration of a drug and n is the total number of samples. In this method, the absorbance values (A) and the concentration values (C) are used to create the data matrix using the absorbances read in the UV–Vis spectra. The multivariate calibration technique was applied for the training set prepared using 20 mixtures in the concentration range of 2–15 μg mL^{-1} for ETC and TDF (Table II).

In RP-HPLC method, the retention times for ETC, TDF and IS (quercetin) were found as 5.623, 6.865 and 10.560 min, respectively, in the chromatogram. In this method, the calibration curve was obtained by the peak area under the analyte peak was divided to the peak area of IS peak area and the obtained values were plotted against the concentration values. For the evaluation of system suitability parameters retention time, the capacity factor, the tailing factor and the theoretical number of plates for the each active ingredient for the developed new RP-HPLC method are calculated and given in Table III. No interfering peaks from the tablet excipients were observed (Fig. 5).

The accuracy and precision were studied using three different solutions for ETC and TDF for four methods developed. The Inter-day and the intra-day precision and accuracy values are shown in Table IV. PLS algorithms were performed with four components in the range of 2–15 μg mL^{-1}. After obtaining, the data prediction was employed using the training set, and the standard errors of the prediction values were calculated by the PLS software program. The calculated values are 0.325 and 0.318 from experimental results. The limit of detection (*LOD*) and the limit of quantification (*LOQ*) are calculated by the ratio of the

standard deviation (*SD*) of the response to the slope (*m*) of the calibration curves (*LOQ* = 10(*SD*/*m*) and *LOD* = 3.3(*SD*/*m*)), where *m* is the slope of the calibration curve. *LOD* and *LOQ* values in the 1st derivative with zero-crossing, ratio spectra of the 1st derivative spectra methods and the PLS technique are 0.05 and 0.15 μg mL^{-1} for ETC and TDF, respectively. In HPLC, *LOD* and *LOQ* values are 0.1 and 0.4 μg mL^{-1} for ETC and TDF, respectively.

TABLE II. Training set used in PLS technique for ETC and TDF (*C* / μg mL^{-1})

Mixture No.	ETC	TDF
1	5	2
2	8	2
3	10	2
4	13	2
5	2	2
6	15	2
7	2	5
8	5	5
9	8	5
10	10	5
11	13	5
12	15	5
13	2	8
14	5	8
15	8	8
16	13	8
17	10	10
18	15	10
19	2	15
20	5	15

TABLE III. System suitability parameters

Active ingredients	Retention time[a] min	Capacity factor	Tailing factor	Theoretical number of plates
TDF	6.865±0.002	5.84	1.194±0.002	9841.19
ETZ	5.623±0.001	4.61	1.372±0.001	7480.12
IS	10.56± 0.002	4.23	1.241±0.001	6492.41

[a]Results are given by mean ± standard deviation, $n = 6$

Analysis of pharmaceutical preparation

When the results obtained in the pharmaceutical formulation using the 1st derivative spectra, ratio of the 1st derivative spectra, HPLC and PLS technique were summarized in Table V, it was observed that the values obtained were very close to each other when compared for the pharmaceutical formulation selected. There is no significant difference observed among the value of results using the ANOVA at the $p < 0.05$ level for the commercial formulations. It can be said that

all developed methods were found selective and specific for the simultaneous determination of ETC and TDF in commercial preparations, such as tablets. No interfering peaks from the tablet excipients were observed in chromatograms in RP-HPLC method developed (Fig. 5). When compared the spectra of ETC and TDF in standard and tablet formulation solutions showed that excipients placed in the commercial preparations did not interfere the wavelength of maximum absorbance in the zero-order spectra and the quantitation of active ingredients of in these methods.

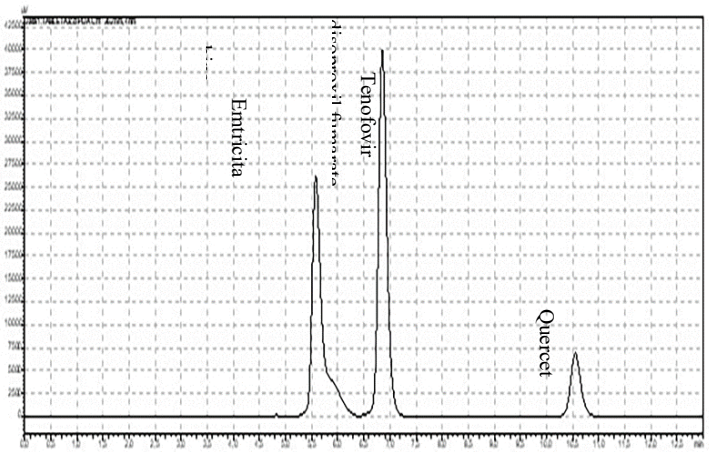


Fig. 5. Representative chromatogram of tablet form containing ETC (10 μg mL^{-1}) and TDF (15 μg mL^{-1}); IS: 1 μg mL^{-1}.

TABLE IV. Inter-day and intra-day precision and accuracy results

Added μg mL^{-1}	Inter–day			Intra–day		
	Found[a] μg m L^{-1}	Precision[b] *RSD* / %	Accuracy[c] Bias, %	Found[a] μg m L^{-1}	Precision[b] *RSD* / %	Accuracy[c] Bias, %
1st derivative–zero crossing (TDF)						
2.0	1.96±0.003	0.347	–1.90	1.97±0.05	0.587	–1.73
7.5	7.47±0.015	0.487	–0.67	7.46±0.02	0.67	–0.54
10.0	10.04±1.01	2.45	0.41	10.05±0.96	2.34	0.54
1st derivative–zero crossing (ETC)						
2.0	1.99±0.01	0.87	–0.71	1.99±0.16	1.92	–0.12
7.5	7.48±0.14	0.47	–0.18	7.51±0.29	0.96	1,33
10.0	10.10±0.62	1.49	1.02	10.12±0.52	1.27	1.15
Ratio 1st derivative (TDF)						
2.0	1.98±0.003	0.39	–0.67	1.99±0.07	0.80	–0.32
7.5	7.51±0.016	0.54	0.18	7.52±0.022	0.73	0.31
10.0	10.11±0.11	2.54	1.12	10.10±0.11	0.31	1.55

TABLE IV. Continued

Added μg mL^{-1}	Inter–day			Intra–day		
	Found[a] μg m L^{-1}	Precision[b] *RSD* / %	Accuracy[c] Bias, %	Found[a] μg m L^{-1}	Precision[b] *RSD* / %	Accuracy[c] Bias, %
Ratio 1st derivative (ETC)						
2.0	1.98±0.12	1.53	–0.65	1.97±0.014	1.72	–1.44
7.5	7.51±0.19	0.65	0.16	7.51±0.022	0.71	0.07
10.0	9.90±0.79	0.16	–0.98	9.87±0.97	2.39	–1.32
RP–HPLC (TDF)						
1.0	1.04±0.022	5.21	0.001	1.04±0.063	5.11	4.39
7.5	7.57±0.056	1.84	0.15	7.49±0.019	0.64	0.89
15.0	14.96±0.072	1.17	0.25	15.07±0.030	1.17	0.47
RP–HPLC (ETC)						
1.0	1.05±0.010	2.46	4.23	1.04±0.011	2.58	0.0005
7.5	7.53±0.043	1.40	1.11	7.42±0.029	0.96	0.44
15.0	14.93±0.085	1.40	0.86	14.87±0.026	0.60	0.48
PLS (TDF)						
2.0	2.03±0.02	2.37	1.22	2.02±0.02	2.38	1.20
7.5	7.57±0.04	1.22	1.01	7.61±0.02	0.65	1.44
10.0	9.96±0.05	0.98	–0.40	9.99±0.03	0.84	–0.067
PLS (ETC)						
2.0	1.99±0.006	0.76	–1.32	1.99±0.007	0.83	–0.37
7.5	7.52±0.017	0.56	0.20	7.52±0.017	0.56	0.20
10.0	9.95±0.062	1.52	–0.536	9.91±0.74	1.83	–0.87

[a]Mean value±standard error; [b]relative standard deviation; [c]100(amount found – amount added)/amount found

TABLE V. Results obtained for the commercial preparation "Hivent" (contains 200 mg ETC and 300 mg TDF per tablet)

Parameter	1st Derivative--zero crossing		Ratio spectra 1st derivative		RP-HPLC		PLS	
	ETC	TDF	ETC	TDF	ETC	TDF	ETC	TDF
Found, mg/tablet	197.12	298.18	197.46	300.52	199.46	299.52	195.62	299.18
RSD[a]	0.97	0.55	1.26	0.56	0.87	1.25	0.68	0.84
Bias[b]	–1.46	–0.61	–1.29	0.17	–0.27	–0.16	–2.24	–0.27
Recovery, %	98.56	99.39	98.73	100.17	99.73	99.84	97.81	99.73

[a]Relative standard deviation (n = 6), [b]100(amount found – amount added)/amount found

CONCLUSIONS

Four new analysis methods, the 1st derivative spectra zero-crossing, the ratio spectra of the 1st derivative spectra methods, the chemometric method (PLS technique), and the new HPLC method for the determination of TDF and ETC were proposed for the simultaneous determination in their mixture. These methods were also applied for the determination of TDF and ETC in pharmaceutical tablets formulation, containing their binary mixture. All developed methods were

evaluated according to the ICH guidelines for validation. The comparison of methods was assessed by the parameters such as linearity, recovery, accuracy, precision and bias values. When the results of commercial preparation according to the 1st derivative and ratio of the 1st derivative spectra and the PLS technique are compared to the HPLC method results, it was found that there was no significant difference statistically between the results using these methods in $p < 0.05$ level. The PLS technique is superior to many analysis methods. It does not require an excessive time for the preparation of samples, neither liquid-liquid extraction process, complex gradient elution program, pretreatment process nor any derivatization process. It requires only a computer program for calculations. In the literature, there are various spectrophotometric methods for the analysis of ETC and TDF, but there is no study for the simultaneous analysis of ETC and TDF in their binary mixture using chemometric techniques and spectrophotometric data. For this reason, our developed chemometric method (PLS) gives a new approach. Also, a few spectrophotometric methods were also available for the analysis of ETC and TDF in their binary mixture, such as the derivative methods in the literature, our derivative and the ratio of the 1st derivative spectra method, which are all different and new methods. After all, in this study four new accurate, precise, and selective methods for the analysis of ETC and TDF in their binary mixture were developed and the results obtained by the spectrophotometric and the chemometric technique (PLS) were compared to our developed and validated RP-HPLC method.

For the analysis of a selected pharmaceutical formulation that is currently on the market, all proposed procedures were effectively used. There was no statistically significant difference in the results. These methods were found suitable as simple, accurate, selective, and precise for the routine analysis of the pharmaceutical preparations, such as the tablets, for the simultaneous analysis of ETC and TDF.

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ИЗВОД

СИМУЛТАНО ОДРЕЂИВАЊЕ ЕМТРИЦИТАБИНА И ТЕНОФОВИР ДИЗОПРОКСИЛ-ФУМАРАТА У ФАРМАЦЕУТСКИМ ПРЕПАРАТИМА ПРИМЕНОМ СПЕКТРОФОТОМЕТРИЈСКИХ, ХЕМОМЕТРИЈСКИХ И ХРОМАТОГРАФСКИХ МЕТОДА

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У овом раду су за истовремено одређивање емтрицитабина (ETC) и тенофовир дизопроксил-фумарата (TDF) у таблетама примењене једноставне, тачне и осетљиве спектрофотометријске, хемометријске и хроматографске методе. Применом деривативне спектрофотометрије, деривативни спектри првог реда раствора ETC и

TDF у води су измерени као $\Delta\lambda$ = 4 nm, а апсорбанце су мерене на нултој тачки пресека, на 297,3 и 281,2 nm за ETC и TDF, редом. Деривативном спектрофотометријом заснованој на односу спектара урађена су мерења на 239,0 nm за ETC и на 270,2 nm за TDF. Затим су мерени аналитички сигнали на таласним дужинама које одговарају или максимуму или минимуму за оба лека. Беров закон је, за оба лека, примењен у опсегу концентрација 2–15 μg mL^{-1}. Као хемометријска метода коришћена је PLS техника. Применом хроматографије, одвајање је постигнуто на C18 колони са DAD детектором (262 nm) и изократским елуирањем мобилном фазом метанол:ацетонитрил:0,1 % ортофосфорна киселина у запреминском односу 40:40:20. Добијени резултат је у опсегу 97,51–100,17 %, а релативна стандардна девијација 0,55–1,26 %. Методе су успешно примењене у анализи фармацеутских узорака, а добијени подаци су анализирани и поређени применом статистичких тестова.

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