Addis Ababa University

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CHEMOMETRICS - ASSISTED DETERMINATION OF EMTRICITABINE AND TENOFOVIR DISOPROXIL FUMARATE COMBINATION IN COMMERCIAL TABLETS

A thesis submitted to the School of Graduate Studies of Addis Ababa University in partial fulfillment of the requirements for the degree of the master of science in pharmaceutical analysis and quality assurance.

By

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Acronyms

ART	Antiretroviral therapy
CLS	Classical Least Square
CV	coefficient of variation
^{1}D	First derivative
DDU	2-,3-didoxyuridine
DNA	Deoxyribonucleic acid
fmol	femtomoles
FTC	Emtricitabine
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
LC–MS	Liquid chromatography mass spectroscopy
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NRTIs	Nucleoside reverse transcriptase inhibitors
NtRTIs	Nucleotide reverse transcriptase inhibitors
PCR	Principal component regression
PI	Protease inhibitors
RNA	Ribonucleic Acid
RP	Reverse-phase
SPE	Solid phase extraction
TDF	Tenofovir disoproxil fumarate
WHO	World health organization

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Abstract

In this thesis, derivative spectrophotometric and multivariate method of assaying truvada® combination tablets, which contain tenofovir disoproxil fumarate 300 mg, and emtricitabine 200 mg, is described. Emtricitabine, 4-amino-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-pyrimidin-2-one, and tenofovir DF, 1-(6-aminopurin-9-yl)propan-2-yloxymethylphosphonic acid, are anti-HIV drugs of nucleoside / nucleotide reveres transcriptase inhibitor class. Emtricitabine was determined at 294.5 nm using the "zero crossing" technique because the signals of tenofovir DF is zero at this wavelength. Tenofovir DF was determined at 270.5 nm by the same technique because emtricitabine have no contribution at this wavelength. Tenofovir DF was quantified at 249.5nm and 270.5 nm wavelengths by using derivative ratio technique because the signal of emtricitabine was zero at these wavelengths. Emtricitabine was quantified at 288.5 and 303.5 nm because the signal of tenofovir DF was zero at these wavelengths. By classical least square (CLS) and principal component regression (PCR) methods good reproducible and repeatable results were obtained. In choosing the optimal magnitudes for the simultaneous determination of both drugs, the following criteria were considered: the linearity, the intercept, the sensitivity, the degree of interference, the relative percent error, the relative recovery, and the reproducibility. The R^2 values which are in excess of 0.999, which indicated the good linearity of the calibration graphs. Sample recovery rates were found satisfactory (almost all are $\geq$ 99%) with relative standard deviations (RSD) of less than 5%. The mentioned chemometrics-assisted techniques were successfully applied to simultaneous determination of emtricitabine and tenofovir DF in the laboratory-prepared mixtures and commercial tablets with considerable precision and good recoveries.

1. Introduction

1.1. The prevalence of HIV AIDS

AIDS (Acquired Immune Deficiency Syndrome) is caused by the Human Immunodeficiency Virus (HIV). More than twenty years after the first clinical evidence of acquired immunodeficiency syndrome was reported; AIDS has become one of the most devastating diseases of humankind has ever faced. Since the epidemic began, more than 60 million people have been infected with the virus. HIV/AIDS has become the fourth-largest cause of death worldwide (WHO, 2007).

At the end of 2005, an estimated 39 million people globally were living with HIV. In that year alone, there were an estimated 2.8 million AIDS deaths and over 4 million new HIV infections. In many parts of the developing world, the majority of new infections occur in young adults, with young women especially vulnerable. About one-third of those currently living with HIV/AIDS are aged 15-24 (WHO, 2007; AVERT, 2007).

1.2. Antiviral therapy

Antiviral therapy is a treatment consisting of drugs that have to be taken every day for the rest of the patient's life. Antiretroviral treatment for HIV infection consists of drugs, which work against HIV infection itself by slowing down the replication of HIV in the body. The drugs are often referred to as; antiretroviral, anti-HIV drugs and HIV antiviral drugs (United States DHHS, 2004).

Antiviral agents are used for different purposes.

1. Treatment of HIV-infected adults, adolescents and children
2. Prevention of mother-child transmission by the use of antiretroviral agents perinatally
3. Prevention of HIV infection after exposure in the healthcare worker or after non-occupational exposure

1.2.1. Classes of antiretroviral drugs

Antiretroviral drugs are broadly classified by the phase of the retrovirus life-cycle that the drug inhibits in to:-

- **Nucleoside & nucleotide reverse transcriptase inhibitors (NRTI)** are chemicals, which inhibit reverse transcription by being incorporated into the newly synthesized viral DNA and preventing its further elongation.
- **Non-nucleoside reverse transcriptase inhibitors (nNRTI),** which inhibit reserve transcriptase directly by binding to the enzyme and interfering with its function.
- **Protease inhibitors (PIs)** proceed through by inhibiting the activity of protease, an enzyme used by HIV to cleave nascent proteins for final assembly of new virons.
- **Integrase inhibitors** inhibit the enzyme integrase, which is responsible for integration of viral DNA into the DNA of the infected cell. There are several integrase inhibitors currently under clinical trial, and raltegravir became the first to receive FDA approval in October 2007.
- **Entry inhibitors** (**or fusion inhibitors**) interfere with binding, fusion and entry of HIV-1 to the host cell by blocking one of several targets. Maraviroc and enfuvirtide are the two currently available agents in this class.

- **Maturation inhibitors** inhibit the last step in gag processing in which the viral capsid polyprotein is cleaved, thereby blocking the conversion of the polyprotein into the mature capsid protein. Because these viral particles have a defective core, the virions released consist mainly of non-infectious particles. There are no drugs in this class currently available, though two are under investigation, bevirimat and Vivecon™ (Hess, 2005).

1.3. Nucleoside /Nucleotide reverse transcriptase inhibitors

Natural Nucleosides have structural units comprising nucleobase (or simple base) and ribose or deoxyribose sugar that are linked to one other as shown in Figure 1. Nucleotides are chemical compound that consists of three portions: a nitrogenous base, a sugar, and one or more phosphate groups, see figures 1. They are the monomers of nucleic acids. Nucleotides are the structural units of RNA, DNA, and several cofactors (Kers *et al.,* 1996; Westheimer, 1987). Nucleosides differ from nucleotides by having a hydroxyl group attached to carbon number 5 (the one that isn't in the ring) of the ribose, rather than one or more phosphate groups. (Jankowska *et al*., 1994).

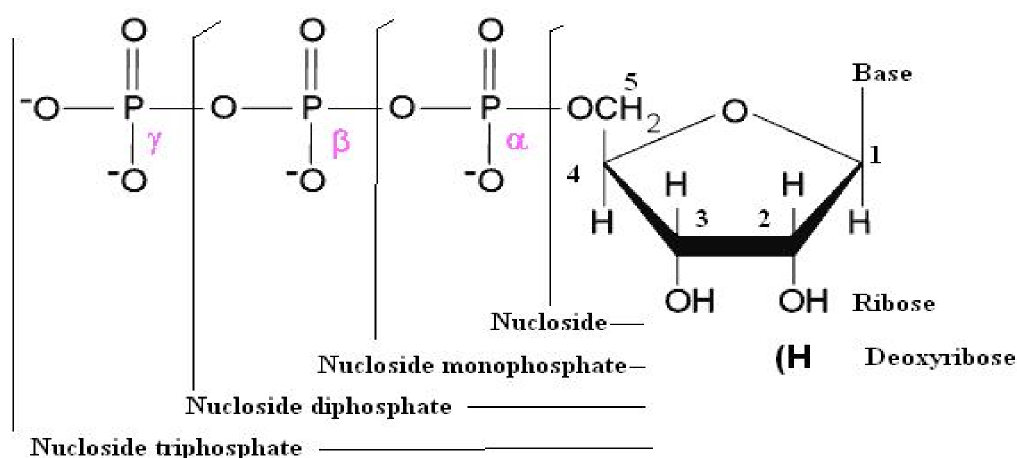

Figure 1: Structural parts for different nucleosides / nucleotides.

1.3.1. Emtricitabine

Emtricitabine is a nucleoside reverse transcriptase inhibitor (NRTI) for the treatment of HIV infection in adults. The IUPAC name of emtricitabine is 4-amino-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-pyrimidin-2-one (USDHHS, 2008). Emtricitabine have molecular formula $C_8H_{10}FN_3O_3S$, molecular weight equal to 247.25g/mol and percent composition of C 38.86%, H 4.08%, F 7.68%, N 16.99%, O 19.41%, S 12.97%. It is white solid from ether and methanol, mp 136-140°C. Optical rotation of $[\alpha]_D$ 25 -133.60° (c = 0.23 in MeOH). UV max in water 287.8 nm (pH 2); 280.0 nm (pH 7) and 279.8 nm (pH 11) (ε 14210, 11090, 11810). Its melting point is equal to 136-140°C (USDHHS, 2007).

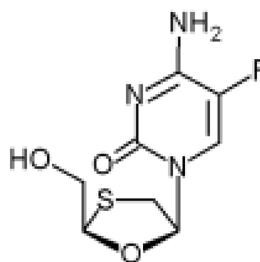

Figure 2: Chemical structure of emtricitabine

Dr. Dennis C. Liotta, Dr. Raymond Schinazi and Dr. Woo-Baeg Choi of Emory University discovered Emtricitabine. The FDA approved it as HIV treatment on July 2, 2003 (Investment Dealers' Digest, 2006). Emtricitabine is also marketed in a fixed-dose combination with tenofovir disoproxil fumarate (TDF). The FDA approved the fixed-dose on July 12, 2006 (Núñez and Soriano, 2005).

Emtricitabine is an analogue of cytidine. The drug works by inhibiting reverse transcriptase, the enzyme that copies HIV RNA into new viral DNA, which is central to the replication of HIV (De Clercq, 2005; Frampton and Perry, 2005).

Emtricitabine 5'-triphosphate inhibits the activity of HIV-1 RT by competing with the natural substrate deoxycytidine 5'-triphosphate and by incorporating into nascent viral DNA (De Clercq, 2002). Following three phosphorylation steps, if incorporated into the DNA chain they lead to termination of the DNA chain elongation, as it does not provide the 3-hydroxyl function on the deoxyribose moiety that is needed for the formation of the ester linkage with the next nucleotide (Roberts *et al.,* 1988; Dennis *et al*., 2004).

1.3.2. Tenofovir disoproxil fumarate

Tenofovir, which belongs to a class of antiretroviral drugs known as nucleotide analogue reverse transcriptase inhibitors (NtRTIs) (Emmet *et al.*, 2007), was discovered through a collaborative research effort between Antonín Holý at the Academy of Sciences of the Czech Republic in Prague, and Erik DeClercq, Rega Catholic University of Leuven, Belgium (Official Viread website, 2004 ; Naesens *et al*., 1997). Tenofovir DF have molecular formula $C_{19}H_{30}N_5O_{10}P.C_4H_4O_4$, molecular weight 635.52 g/mol, and percent composition C43.47%, H5.39%, N11.02%, O35.25%, P4.87% . It is white to off-white crystalline solid, which is soluble in water at room temperature (USDHHS, 2007). Tenofovir DF the anti-HIV properties were first described in 1993 (Balzarini *et al*., 1993). FDA approved the compound for clinical use of HIV infections on October 26, 2001 (Núñez M, and Soriano V., 2005). Tenofovir is not sufficiently bioavailable by the oral route. Therefore, an oral prodrug has been developed and formulated as its salt, tenofovir disoproxil fumarate (Arimilli *et al*., 1997; Naesens *et al*., 1998). Tenofovir DF is also available in a fixed-dose combination with emtricitabine for once-a-day dosing (Emau *et al*, 2006; FHI, 2007).

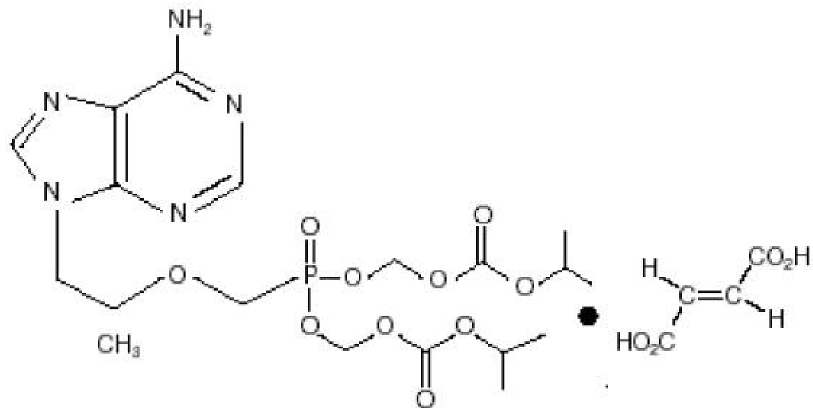

Figure 4: The chemical structure of tenofovir disoproxil fumarate

Tenofovir DF inhibits the activity of HIV-1 RT by competing with the natural substrate deoxyadenosine 5'-triphosphate and by incorporating into DNA, which results in chain termination. Directly taking NtRTI preparations allow the conversion steps to be skipped, then cause less toxicity (De Clercq, 2003). In the absence of long-term safety and efficacy data, tenofovir should be reserved for patients with HIV and HBV co-infection who exhibit lamivudine resistance (Emmet, 2007; Damian and Michael, 2003).

TDF acts as a false building block similar to nucleoside analogs targeting the enzyme reverse transcriptase (Hoffmann and Mulcahy, 2007).

1.5. Emtricitabine and tenofovir disoproxil fumarate fixed combination

For antiretroviral treatment to be effective for a long time, it has been found that taking more than one antiretroviral drug at a time is necessary (Hoffmann and Fiona Mulcahy, 2007). The need to take two or more antiretroviral drugs at a time is one of the reasons to begin antiretroviral combination therapy (Miller *et al*., 2001; Hooper, 1999).

Truvada is a fixed-dose tablet containing two synthetic nucleoside analogues: emtricitabine and tenofovir disoproxil fumarate. Each of the tablets contains emtricitabine 200 mg and tenofovir DF 300 mg (Truvada prescribing information, 2007). FDA approved it on August 2, 2004, for the treatment of HIV-1 infection in adults (FDA, 2004; Gilead Sciences, 2007). Truvada provides the nucleotide part for once-daily dosing, as a component of highly active antiretroviral therapy (HAART) (USDHHS, 2004; AIdS alert, 2006). Components of truvada tablet are not metabolized by cytochrome P450, which confers little potential for interactions with drugs metabolized by these enzymes. Resistance mutation K65R is selected by tenofovir and confers a two to four fold reduced susceptibility to this drug (Muñoz, 2006). The co-formulation is clinically potent combination that is free of short-term irritating toxicity. The limited published data indicated that emtricitabine and lamivudine have equivalent potency (Gazzard, 2006). Truvada has good tolerability, but monitoring of renal function is needed (Dando and Wagstaff, 2004; Gallant *et al*., 2005).

1.6. Analysis of emtricitabine and tenofovir DF

A high-performance liquid chromatographic assay was developed for the determination of tenofovir in plasma. A solid–liquid extraction procedure was coupled with a reversed-phase HPLC system. The system requires a mobile phase containing phosphate buffer, tetrabutylammonium hydrogen sulfate and acetonitrile for different elution's' through a C18 column with UV detection. The method proved to be accurate, precise and linear between 10 and 4000 ng/ ml. (Sentenac *et al.*, 2003).

The quantification of tenofovir in human plasma is important due to a recent increase in its use. HPLC, however, cannot easily detect and quantify tenofovir because of interfering peaks.

Therefore, rapid and conventional LC-MS method was developed and validated by estimating the precision and accuracy for inter- and intraday analysis in the concentration range of 0.019 - 1.567 mg/ml. The mobile phase was a mixture of 0.3% trifluoroacetic acid (A), 100% acetonitrile (B) and 100 mM ammonium acetate (C). An isocratic mobile phase consisting of A–C (95: 5) was used during the first 3 min of the run, followed by a linear gradient elution consisting of A–B–C (45: 50: 5) for the next 5 minute. The mass spectrometer was operated in positive ion electrospray mode. Average accuracy ranged from 95.9 to 100.7%. Relative standard deviations of both inter- and intraday assays were less than 11.6%. Recovery of tenofovir was more than 80.2%. This novel method provides a conventional, accurate and precise way to determine tenofovir in human plasma samples (Takahashi, *et al*., 2007).

An LC–MS–MS method was developed and validated for the determination of tenofovir-diphosphate concentrations, which directly correspond with the intra-human peripheral blood mononuclear cells concentration. The assay was linear in the range of 50–10,000 fmol per sample. The lower limit of quantitation of the method was 10 fmol per million cells with 5 million human peripheral blood mononuclear cells used. Column temperature of LC was 35 °C and isocratic flow rate at 0.200 ml/min. The mobile phase was made by adding 10 ml aqueous acetic acid and 30 ml acetonitrile to 960 ml Deionized /distilled water. The signal was achieved in positive ion mode with electron spray ionization and selected reaction-monitoring detection (King *et al*., 2006).

Two methods by reversed phase liquid chromatography were developed for the analysis of 19 antiretroviral molecules used in medicinal products. Both of these HPLC techniques used a C18 column and UV detection (Rebiere, 2007). The mobile phase for nucleoside inhibitors was methanol-water (5: 95) mixture, and acetonitrile-ammonium acetate buffer (40:60) mixture for

the other two families. The first method was for the family of nucleoside inhibitors analysis and allows eight molecules (zalcitabine, lamivudine, amdoxovir, emtricitabine, didanosine, stavudine, zidovudine and abacavir) to be separated. The second method is for non-nucleoside inhibitors and protease inhibitors family analysis and allows 11 molecules (fosamprenavir, nevirapine, indinavir, amprenavir, saquinavir, atazanavir, ritonavir, lopinavir, efavirenz, nelfinavir and tipranavir) (Alphabetical list of drugs for HIV, 2006) to be separated. The combination of these two methods makes possible the quality control of mono-, bi- or tri-therapy pharmaceutical products.

Two simple, rapid, sensitive and accurate UV- Spectrophotometric and first order derivative UV spectrophotomeric has been developed for estimation of tenofovir in bulk and tablets (Shirkhedkar *et al*., 2007). In methanol – water (3:7), the lamed max of tenofovir was found to be 260 nm and the same spectrum was derivatized into first order derivative, using UV probe software of instrument (shimadzu 2450). The amplitude of the trough was recorded at 273 nm. In both the methods, linearity was observed in the concentration range of 5 – 40 µg/ml. The assay results were found to be in good agreement with label claim. The methods were validated statistically and recovery studies.

Simultaneous quantitative determination of emtricitabine and tenofovir in human blood plasma using reverse-phase high-performance liquid chromatography assay was described as follows by Naser (2005). Using 200 µL of plasma and BOND ELUT-C18 Varian columns, the solid phase extraction (SPE) method results in a clean baseline and high extraction efficiencies (100% for emtricitabine and 98.6% for tenofovir). An AtlantisTM dC-18 analytical column was used along with an 18 min linear gradient elution with phosphate buffer (pH 5.7) and methanol to provide sharp peaks for emtricitabine at 280 nm, tenofovir at 259 nm, and the internal

standard 2-,3-didoxyuridine at 262 nm. The method was validated over the range of 10–10,000 ng/ml for both analytes, and found to be accurate over a range of 98 to 105% concentrations for emtricitabine and 97 to 103% for tenofovir. It was also precise within and between - day precision ranging from 1.7 to 3.7% and 3.7 to 5.2% respectively.

Chemmometrics-assisted determination of the emtricitabine and tenofovir DF was not reported in any literature until now. The proposed, chemmometrics-assisted method for the determination of the combined formulations is easy to handle and quite reasonable cost wise.

2. Objectives

2.1. General objective

- To develop a chemometrics-assisted spectrophotometric method for the quantitative determination of emtricitabine and tenofovir disoproxil fumarate in some binary laboratory prepared mixtures and commercial tablets.

2.2. Specific Objective

- To develop a multivariate calibration model for the studied pure drugs and laboratory mixtures of emtricitabine and tenofovir disoproxil fumarate.
- To test and validate a multivariate calibration model developed.
- To use the so developed model for the prediction of both emtricitabine and tenofovir disoproxil fumarate concentration in commercial dosage forms present in the Ethiopian market.

3. Experimental

3.1. Instrumentation and chemicals

3.1.1. Apparatus

A UNICO-UV-2102 PC spectrophotometer (UNICO products and instruments Inc. Shanghai, China) equipped with 1 cm quartz cells was used. The spectrophotometer is supported with UV PC software UNICO SB-2.53.

3.1.2. Chemicals

Pure standard emtricitabine (Gilead Science, Canada), tenofovir DF (Gilead Science, Canada) and the brand truvada tablets labeled to contain 200 mg emtricitabine and 300 mg tenofovir disoproxil fumarate (Gilead Science, Canada) were supplied by The Ethiopian drug quality control authority. The manufacturing and expire date of the tablets were 04/06/2006 and 03/06/2008 respectively). Distilled water was used throughout all the work done.

3.2. Methods

3.2.1. Preparation of tenofovir DF, and emtricitabine standard solutions

An accurately weight amount (50 mg) of either tenofovir DF or emtricitabine was transferred quantitatively to 100 ml volumetric flask, and then dissolved by shaking in about 80 ml of distilled water. After completion of dissolution, the volume was made up to the mark using distilled water to give a stock solution of 500 µg/ml.

3.2.2. Preparation of solutions for calibration steps

Solutions with 10 different concentrations (5-50 µg/ml) were prepared by dilution of specified amounts of the stock solution with water. This concentration range was previously established to obey Beer's law for each of the studied compounds in the distilled water and it was used to obtain the calibration matricies for applying the first derivative, first derivative ratio, CLS and PCR analysis. Laboratory prepared mixtures were prepared by mixing known amounts of emtricitabine with tenofovir DF in ratios varying from 45:5 to 5:45 respectively as indicated in Table 1. The prepared solutions were scanned using UV spectrophotometer and the scanned data were collected. The absorption data in the range of 200-350 nm were analysized in order to obtain the calibration K matrix for each drug.

The mixtures are used to verify the precision of the method for analysis of such mixtures and matching the commercial tablets with those having comparable concentration ratios.

Table 1: Ratios of the laboratory prepared mixtures of emtricitabine and tenofovir DF.

Concentration of emtricitabine (µg/ml)	45	40	35	30	25	20	15	10	5
Concentration of tenofovir DF (µg/ml)	5	10	15	20	25	30	35	40	45
Ratio (tenofovir DF /emtricitabine)	9:1	4:1	7:3	3:2	1:1	3:2	3:7	1:4	1:9

3.2.3. Sample preparation from the commercial tablet

Ten truvada tablets were weighted and the average weight of one tablet was calculated. The tablets were then finely powdered using mortar and pestle. An amount of the powdered tablets equivalent to the average weight of one tablet was transferred to a clean 100 ml graduated volumetric flask. About 80 ml of distilled water was added to the contents of the flask and then shaked by hand for about 20 minutes. The volume was adjusted to the mark with distilled water and the mixture was then filtered. The final concentration of the solution became 5000 µg/ml. 1 ml of the solution was diluted using water and 100ml volumetric flask to prepare sample solution having concentration equal to 50 µg/ml. Then the sample solution was measured using UV spectrophotometer.

3.2.4. Data processing

For derivative and classical least square calculations the data collected was analysized and manipulated using Microsoft excel 2003 with standard curve fit package and Harvard graphics version 2.00 programs. MVSP version 3.13 (1985-2003) and VISTA 6.4.3436-EWU (2001) software were used in combination with the above mentioned application softwares for principal component regression (PCR) analysis.

4. Results and discussion

4.1. Zero-crossing first derivative spectrophotometric analysis

First derivative spectrophotmetry is one of the chemometric methods, which is suggested for assay of mixtures with two or more components. In this work, first derivative spectrophotometric technique was inspect for the assay of emtricitabine and tenofovir DF drug mixtures. Figure 4 show that absorption spectrum of emtricitabine completely overlapped with that of tenofovir DF.

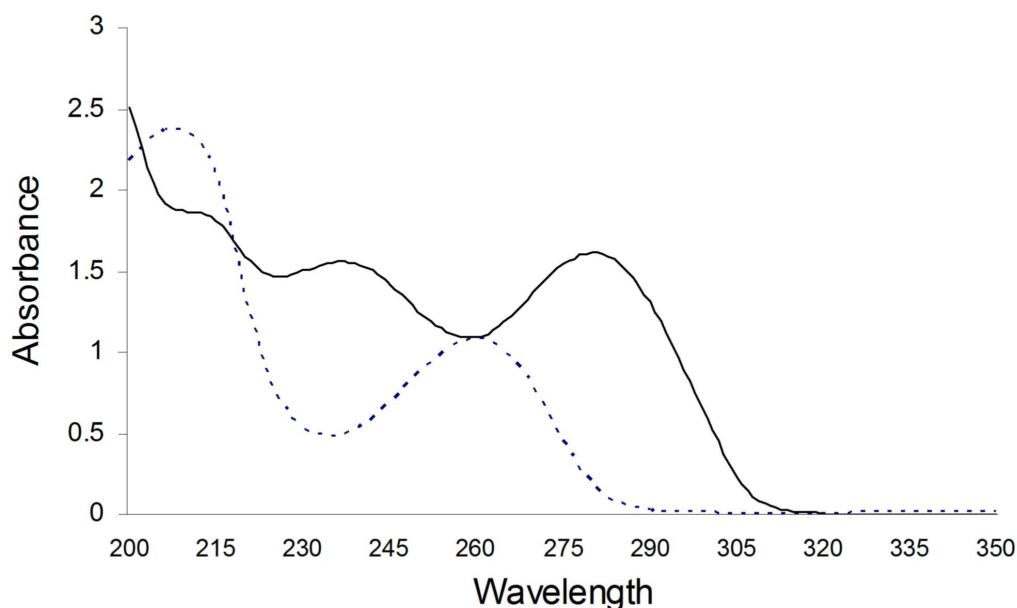

Figure 4: Degree of overlapping as indicated by absorption spectra of emtricitabine (——) (45 µg/m1) and tenofovir DF (-------) (45 µg/ml).

In the corresponding first derivative (^1D) curve as shown in Figure 5, the emtricitabine absorption maximum was located at the zero crossing point of tenofovir DF (λ = 294.5 nm). At

longer wavelengths, tenofovir DF exhibited an absorption maximum of ^{1}D value (λ= 278.5 nm) where emtricitabine absorbance is nil.

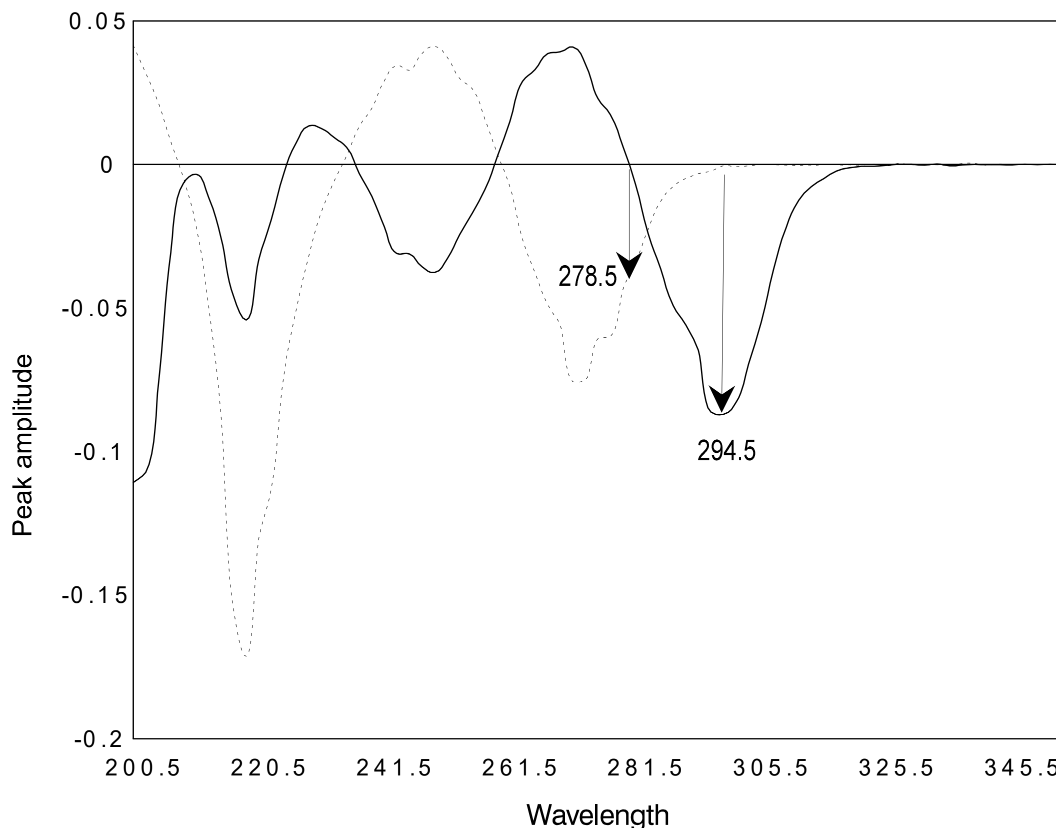

Figure 5: First derivative spectra of emtricitabine (—) and tenofovir DF (-----) with zero-crossing at 294.5 nm and 278.5 nm respectively.

The regression equations for both drugs were derived using the least-squares regression analysis and at the specified wavelength for each drug. The obtained results are summarized in Table 2, which include the intercepts, slopes, correlation coefficients, and determination coefficients, limits of detections (LOD) and limits of quantifications (LOQ).

The slopes are used as a measure of sensitivity of the proposed methods, while intercepts indicate the magnitude of background interferences. Results listed in Table 2 indicate the high sensitivity and low background effects of the determination.

The limits of detection and quantification were calculated on the bases of the following relations

$$\mathbf{LOD=3\sigma/S}$$

$$\mathbf{LOQ=10\sigma/S}$$

Where σ is the standard deviation of intercept, and S is the sensitivity parameter, (sensitivity expressed here by the slopes of the calibration curves).

Table 2: Analytical parameters for determination of emtricitabine and tenofovir DF with the zero crossing first derivative spectrophotometric technique in the concentration range from 5 to 50 µg/ml for both drugs

Standard solution	λ (nm)	Linear regression equation parameters					
		Intercept	Slope	correlation coefficient	Determination coefficient	LOD (µg/ml)	LOQ (µg/ml)
Emtricitabine	294.5	-0.00046	-0.00173	0.9998	0.9997	0.58323	1.9441
Tenofovir DF	278.5	-0.00038	-0.00094	0.9994	0.9989	1.08819	3.6273

Table 3: Actual and predicted amounts of emtricitabine given by applying first derivative spectrophotometric technique at 294.5 nm, for pure, laboratory prepared mixtures, with tenofovir DF and commercial tablets.

Analyzed components	Used concentrations (μg/ml)	Found		
		μg/ml	%	CV
	10	9.9	98.8	3.1
Pure emtricitabine	15	15.1	100.7	0.8
	20	20.0	100.2	2.0
	25	24.6	98.6	2.2
	30	27.9	93.0	1.9
	35	34.2	97.8	1.3
	40	39.4	98.4	1.7
	45	45.6	101.4	0.7
	50	50.1	100.3	0.9
	Emtricitabine/Tenofovir DF			
Laboratory prepared mixtures	45:5	45.0	100.0	0.8
	40:10	39.8	99.4	0.3
	35:15	35.3	100.8	0.5
	30:20	30.1	100.5	1.4
	25:25	25.2	100.7	1.2
	20:30	20.0	100.0	2.6
	15:35	14.9	99.2	2.4
	10:40	10.0	100.1	2.9
Truvada tablets	20:30	19.7	98.7	0.2

Table 4: Actual and predicted amounts of tenofovir DF given by applying first derivative spectrophotometric technique at 278.5 nm for pure and laboratory prepared mixtures with emtricitabine and commercial tablets.

Analyzed components	Used concentrations (µg/ml)	Found		
		µg/ml	%	CV
Pure tenofovir DF	10	10.1	100.6	3.2
	15	14.9	99.6	2.5
	20	19.9	99.3	1.2
	25	24.7	98.9	0.6
	30	29.7	99.0	1.6
	35	34.7	99.1	0.7
	40	40.1	100.3	2.1
	45	45.3	100.6	0.6
	50	49.8	99.6	1.0
Laboratory prepared mixtures	Tenofovir DF/Emtricitabine			
	10:40	9.9	99.2	2.0
	15:35	14.4	96.0	0.6
	20:30	19.5	97.6	0.9
	25:25	25.0	100.1	0.8
	30:20	29.4	97.9	1.3
	35:15	35.2	100.5	0.8
	40:10	39.7	99.2	0.3
	45:5	45.2	100.5	2.2
Truvada tablets	30: 20	29.8	99.4	1.5

4.2. Derivative ratio spectrophotometric analysis

Determination of emtricitabine:-

The absorption spectra of standard solutions of emtricitabine were divided (amplitude by amplitude) at appropriate wavelengths, by absorption spectrum of a standard solution of tenofovir DF (25 µg/m1) to obtain the corresponding ratio spectra as shown on Figure 6. Then the first derivative of the obtained ratio spectra were calculated with the interval of $\Delta\lambda$ = 3nm (Figure 7). Figure 7 shows that, emtricitabine can be determined in the given mixture by measuring the amplitude at 288.5 and 303.5 nm where there is no contribution of tenofovir DF.

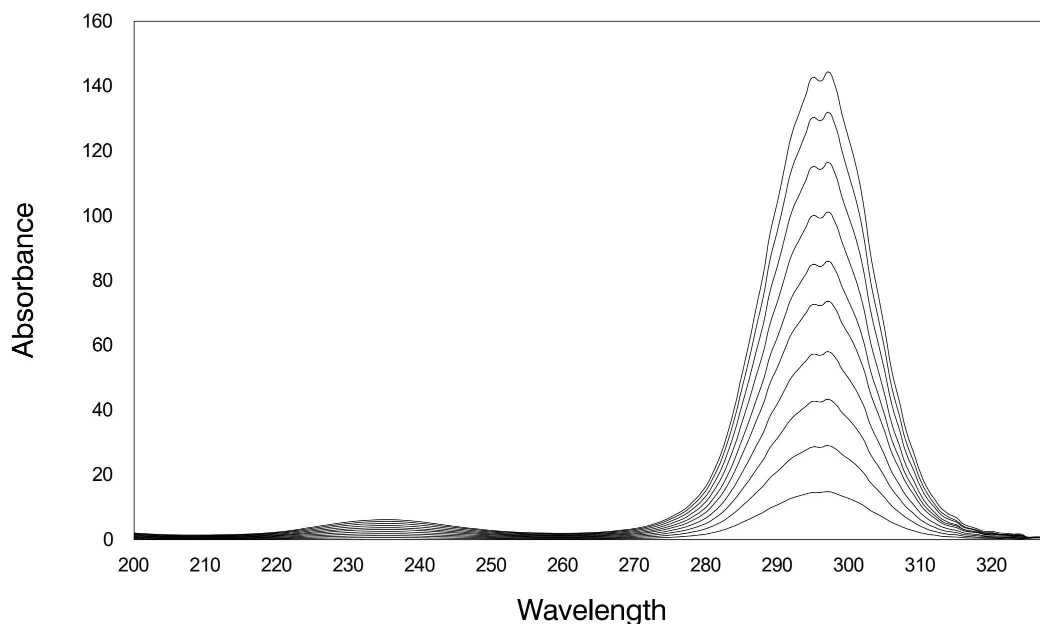

Figure 6: The ratio spectra of emtricitabine (5-50 µg/ml) (The divisor is 25 µg/ml tenofovir DF).

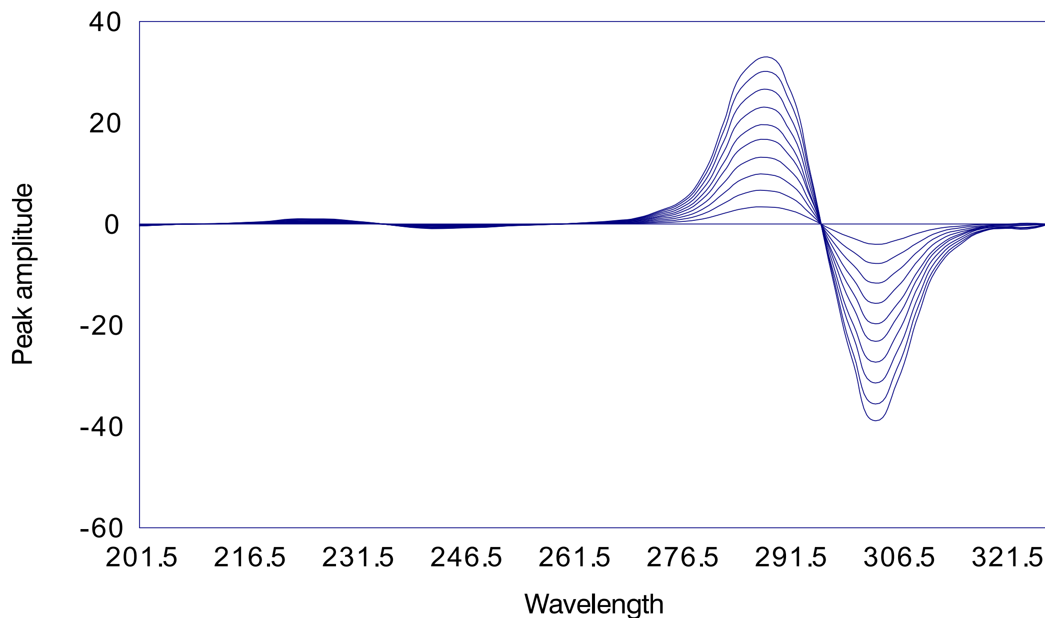

Figure 7: First derivative ratio spectra of emtricitabine (5-50 μg/m1) (Divisor is 25 μg/m1 tenofovir DF).

Determination of tenofovir DF:-

A similar procedure used for emtricitabine was followed. The absorption spectra of tenofovir DF was divided by that of a solution with concentration of emtricitabine (25 μg/m1) as shown in Figure 8, and the first derivative of the developed ratio spectra was calculated with the interval of $\Delta\lambda$ = 3nm difference as indicated in Figure 9.

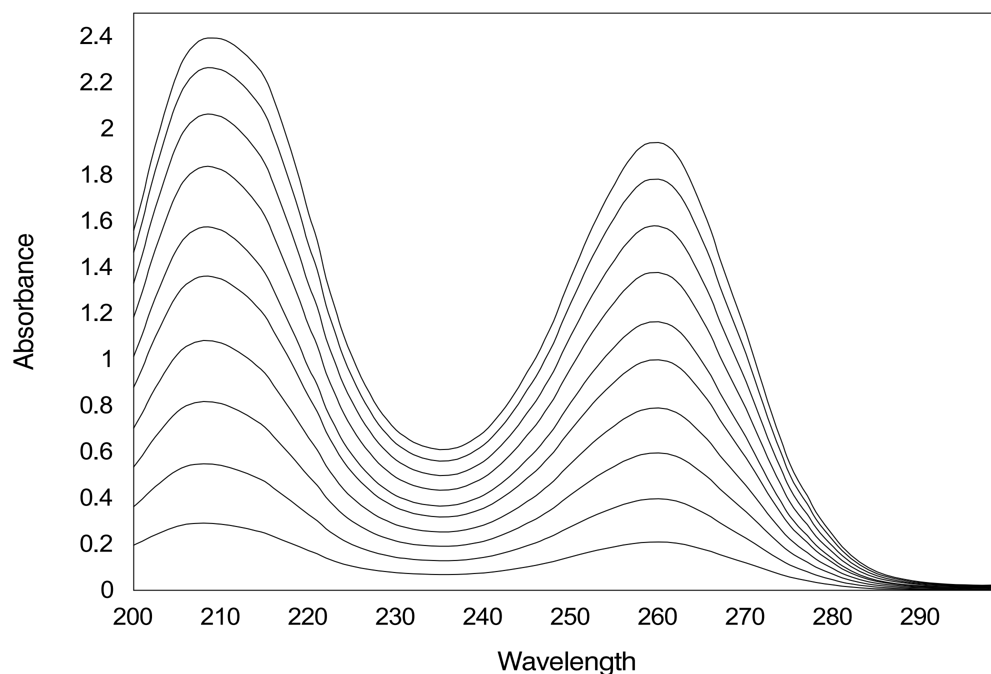

Figure 8: Ratio spectra of tenofovir DF (5-50 µg/m1) (Divisor is 25 µg/m1 emtricitabine).

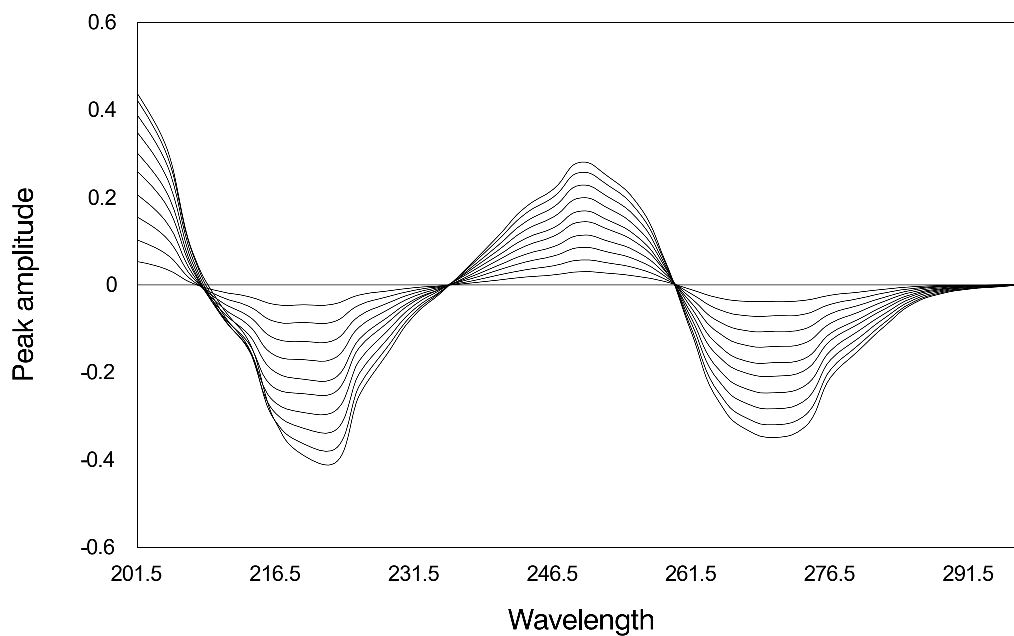

Figure 9: First derivative ratio spectra of tenofovir DF (5-50 µg/m1) (Divisor is 25 µg/m1 emtricitabine).

Results listed in Table 5 indicate the high sensitivity and low background effects for the determination of the components of the given fixed dosage drug.

Figure 9 shows that, tenofovir DF can be determined by measuring the amplitude at three wavelengths (225.5, 249.5 and 278.5 nm) where emtricitabine has no contribution, but it was found that tenofovir DF gave best results at amplitudes 249.5 and 278.5nm.

The influence of $\Delta\lambda$ for obtaining the first derivative of the ratio spectra was tested to obtain the optimum wavelength interval. Intervals of wavelength 1, 2, 3, 4, and 5 were examined and $\boldsymbol{\Delta\lambda}$ = 3 nm was found the most suitable. The effect of the divisor concentration on the calibration graphs was also studied. The results obtained indicated that the divisor concentrations have no significant effect on the assay results. Among the concentration ranges 25 µg/ml of both drugs was found to be the most suitable divisor.

Table 5: Analytical parameters for determination of emtricitabine and tenofovir DF with derivative ratio spectrophotometric techniques in the concentration range from 5 to 50 µg/ml for both drugs.

Standard solution	λ (nm)	Linear regression equation parameters					
		Intercept	Slope	Correlation coefficient	Determination coefficient	LOD (µg/ml)	LOQ (µg/ml)
Emtricitabine	288.5	-0.08315	0.665125	0.9999	0.9999	0.27057	0.90189
	303.5	-0.00055	-0.78208	0.9999	0.9999	0.19935	0.66451
Tenofovir DF	249.5	0.001703	0.005226	0.9999	0.9998	0.45028	1.50095
	270.5	-0.00022	-0.00665	0.9998	0.9995	0.71844	2.39479

Results obtained by the proposed first derivative techniques for some suggested laboratory prepared mixtures were summarized in Tables 6 and 7. The coefficient of variation (CV) was found to be range from 0.1 to 2.66 for emtricitabine and from 0.1 to 2.8 for tenofovir DF. As can be seen, the recovery and repeatability of the technique was satisfactory and within the limit.

Table 6: Actual and predicted amounts of emtricitabine given by applying derivative ratio spectrophotometric technique at 288.5 and 303.5 nm for pure, laboratory prepared mixtures with tenofovir DF, and commercial tablets.

		Found at 288.5 nm			Found at 303.5 nm		
Analyzed component	Used concentrations (µg/ml)	µg/ml	%	CV	µg/ml	%	CV
Pure emtricitabine	5	5.1	102.3	1.9	5.1	101.2	2.3
	10	10.0	100.0	1.4	10.0	100.1	1.8
	15	15.0	99.9	1.3	14.9	99.5	2.5
	20	20.0	99.8	1.5	20.0	100.2	2.1
	25	25.1	100.4	1.5	25.1	100.3	1.8
	30	29.8	99.2	1.7	29.9	99.5	1.8
	35	34.8	99.4	1.2	34.9	99.7	1.3
	40	40.1	100.2	1.7	40.1	100.3	2.4
	45	45.1	100.1	1.5	44.9	99.8	2.3
	50	50.1	100.2	1.8	50.1	100.2	2.4
Laboratory prepared mixtures	Emtricitabine/Tenofovir DF						
	45: 5	44.7	99.3	0.5	44.8	99.6	0.9
	40:10	40.0	99.9	0.6	39.8	99.5	1.2
	35:15	35.2	100.6	0.9	35.1	100.4	0.7
	30: 20	30.0	99.9	1.2	30.0	100.0	1.1
	25: 25	25.5	101.3	0.5	24.9	101.3	2.1
	20: 30	20.0	100.0	1.8	20.2	100.8	1.9
	15: 35	15.3	100.1	1.3	15.2	101.9	1.2
	10: 40	10.0	98.1	2.6	10.1	97.5	1.6
Truvada tablets	20:30	20.4	101.9	1.6	19.7	98.3	0.3

Table 7: Actual and predicted amounts of tenofovir DF given by applying derivative ratio spectrophotometric technique at 249.5 and 270.5 nm for pure, laboratory prepared mixtures with emtricitabine, and commercial tablets.

Analyzed component	Used concentrations (µ/ml)	Found at 249.5 nm			Found at 270.5 nm		
		µg/ml	%	CV	µg/ml	%	CV
Pure tenofovir DF	10	9.9	98.5	1.9	9.9	98.9	1.3
	15	14.9	99.6	1.3	14.9	99.6	1.2
	20	19.9	99.3	1.9	19.9	99.5	1.4
	25	25.1	100.5	1.4	25.2	100.7	1.4
	30	29.7	99.0	1.5	29.6	98.6	1.4
	35	35.1	100.1	1.1	35.0	100.1	1.2
	40	39.9	99.7	1.4	39.9	99.8	1.7
	45	45.4	101.0	1.4	45.5	101.1	1.2
	50	49.5	99.0	1.6	49.6	99.1	1.5
	Tenofovir DF/Emtricitabine						
Laboratory prepared mixtures	5:45	5.0	100.6	2.8	5.4	108.6	0.3
	10:40	9.9	99.2	1.4	9.9	99.4	1.4
	15:35	15.1	100.7	1.2	15.1	100.8	1.7
	20:30	19.9	99.3	1.0	20.0	99.9	2.2
	25:25	25.1	100.3	1.9	24.8	99.2	0.8
	30:20	29.8	99.3	1.9	29.7	99.1	1.4
	35:15	35.0	100.0	1.7	35.1	100.3	1.4
	40:10	39.6	99.1	0.7	39.9	99.7	0.4
	45:5	45.3	100.7	0.7	45.6	101.4	0.9
Truvada tablets	30: 20	30.0	99.8	1.5	30.0	99.9	0.9

4.3. Classical Least squares analysis (CLS)

In CLS, the assumption was existence of a linear relationship between absorbance and the components concentration at each wavelength. As can be seen from the absorption spectra of the studied drugs presented in Figure 4, a considerable degree of spectral overlapping occurs in the region from 200 to 285 nm for emtricitabine and tenofovir DF. The degree of spectral overlapping is practically given by $(D_i)^{0.5}$, where D_i is the magnitude of dependency. In case of the currently studied compounds the spectra shown in Figure 6 lead to $D_i = 0.83813$ implying a 90.13 % of spectral overlap.

Conversely, the achieved data showed the following characters:

1. Absorption spectra and spectral constants of the targeted drugs harmonize well with the reported spectral data.
2. There were no noticeable outliers, marked errors or data clustering were detected in the collected data points for C- or A-values (see Figures 10 - 13).

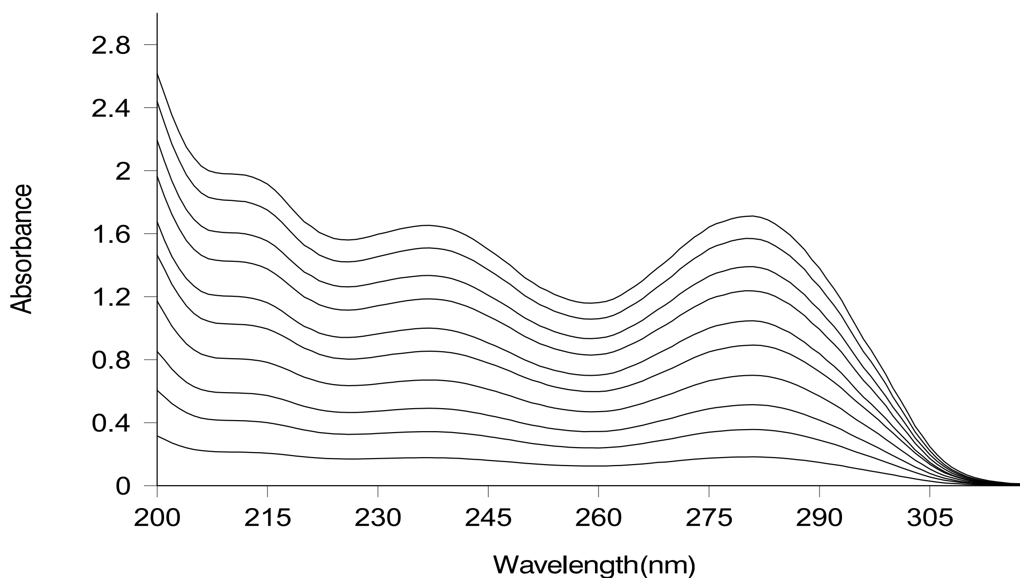

Figure 10: Overlay absorption curves of emtricitabine in the calibration range (5 - 50 µg/ml).

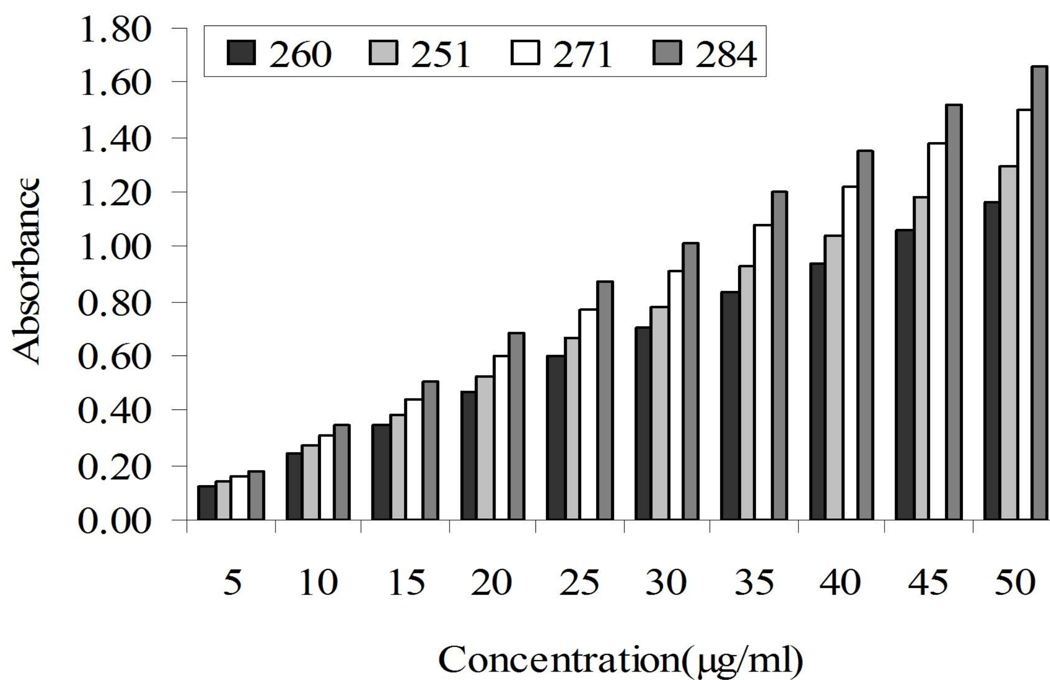

Figure 11: Histogram of C-values of emtricitabine at four different wavelengths

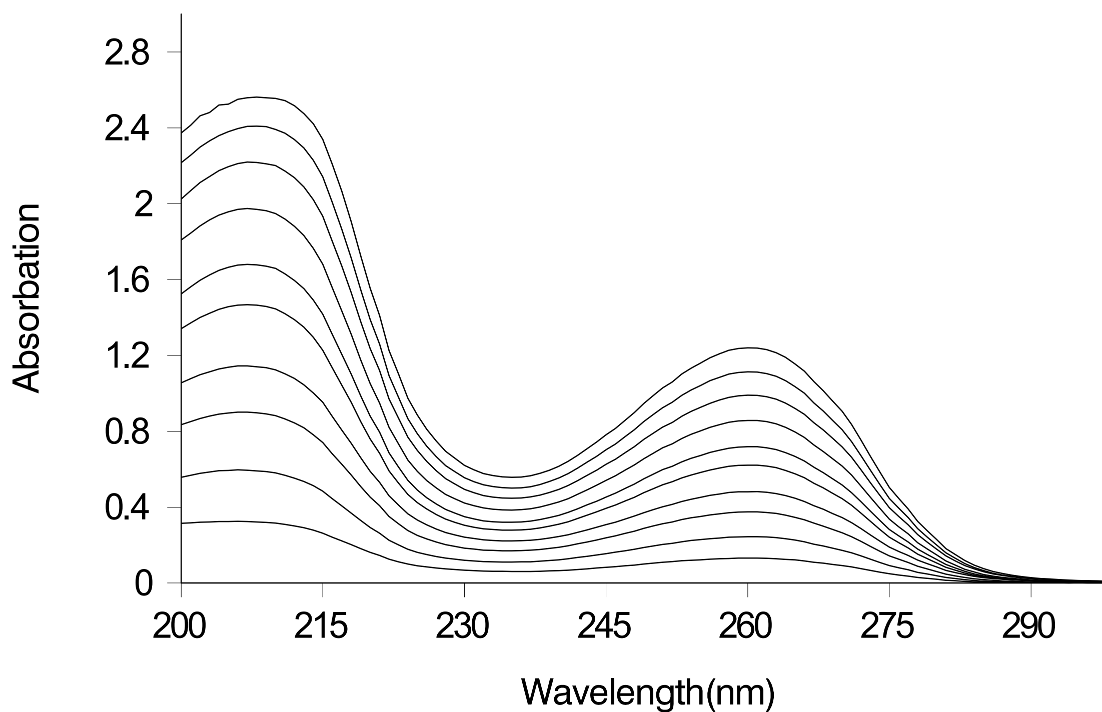

Figure 12: Overlay absorption curves of tenofovir DF in the calibration range (5 - 50 μg/ml).

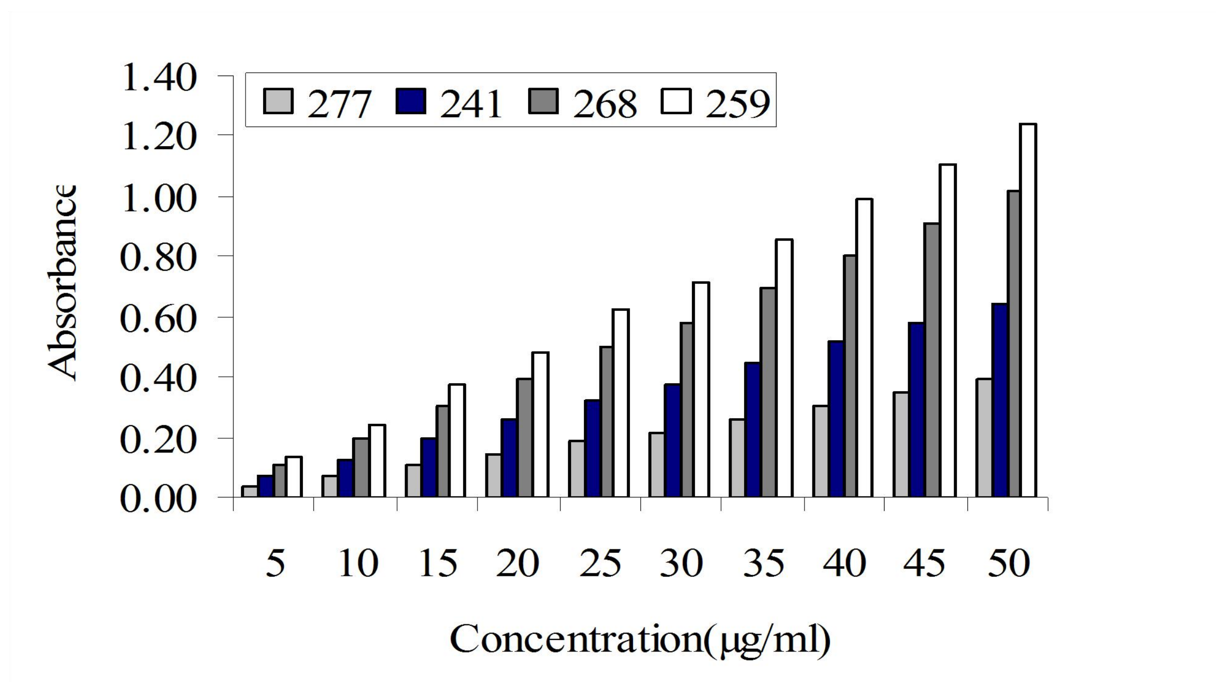

Figure 13: Histogram of C-values of tenofovir DF at four different wavelengths.

3. Calibration break for each compound covers the expected prediction space according to the ratios used for the prepared laboratory prepared mixtures and that amount tagged for the commercial dosage form.
4. There was a strong correlation between the absorbance and concentration as indicated by their calibration equations and correlation coefficients as given by the CLS of the spectral data obtained experimentally in the range from 200 to 320 nm.

Several laboratory prepared mixtures were subjected to the CLS analysis in order to confirm the suitability of the calibration model for determination of the studied drugs in the pharmaceutical sample solutions. As we see from tables 8 and 9, the results obtained for the pure and laboratory prepared mixtures were good for both drugs. In addition, the results of pharmaceutical preparation of both drugs also give a considerable value.

Table 8: Actual and predicted amounts of tenofovir DF given by applying CLS for pure, laboratory prepared mixtures with emtricitabine, and commercial tablets.

Analyzed components	Used concentrations (µg/ml)	Found (µg/ml)	Found (%)	CV
Pure tenofovir DF	5	5.1	101.6	4.4
	10	10.1	101.5	1.9
	15	15.0	100.2	1.2
	20	20.0	99.8	1.7
	25	25.3	101.1	1.5
	30	29.7	98.8	1.8
	35	34.8	99.5	1.3
	40	40.0	99.9	2.1
	45	44.0	97.8	1.7
	50	48.0	96.0	1.3
	Tenofovir DF/Emtricitabine			
Laboratory prepared mixtures	10: 40	9.9	98.9	2
	15: 35	15.0	99.9	1.5
	20: 30	19.8	99.0	0.2
	25: 25	24.6	98.2	0.4
	30: 20	29.6	98.8	1.7
	35: 15	34.5	98.5	0.3
	40: 10	39.6	99.0	1.9
	45:5	44.5	98.8	1.6
Truvada tablets	30 : 20	29.9	99.62	0.9

Table 9: Actual and predicted amounts of emtricitabine given by applying CLS for pure, laboratory prepared mixtures with tenofovir DF, and pharmaceutical preparations

Analyzed components	Used concentrations (μg/ml)	Found (μg/ml)	Found (%)	CV
Pure emtricitabine	10	10.1	100.6	1.5
	15	15.0	99.8	1.1
	20	20.0	100.1	1.3
	25	25.4	101.4	1.1
	30	29.9	99.5	0.7
	35	35.0	100.1	1.3
	40	40.3	100.6	0.6
	45	45.2	100.5	0.9
	50	49.4	98.9	1.2
	Emtricitabine/Tenofovir DF			
Laboratory prepared mixtures	45 : 5	44.3	98.4	0.5
	40 : 10	39.2	98.1	0.4
	35 : 15	34.5	98.7	1
	30 : 20	29.5	98.4	0.8
	25 : 25	25.0	100.0	0.9
	20 : 30	19.8	99.0	1.1
	15: 35	14.9	99.0	0.5
	10 : 40	9.9	99.3	1.7
	45 : 5	44.3	98.4	0.5
Truvada tablets	20:30	19.8	99.07	0.3

4.4. Principal component regression method (PCR)

By the same manner stated in the CLS method, the same laboratory prepared mixtures were subjected to the analysis by the proposed PCR technique to confirm the efficiency of the given model for subsequent determination of the study compounds in pharmaceutical preparations. The correlation between the first three principal components in case of PCR models, (PC1, PC2 and PC3) was that all data points come about in the same plane as seen in figures 14- 19.

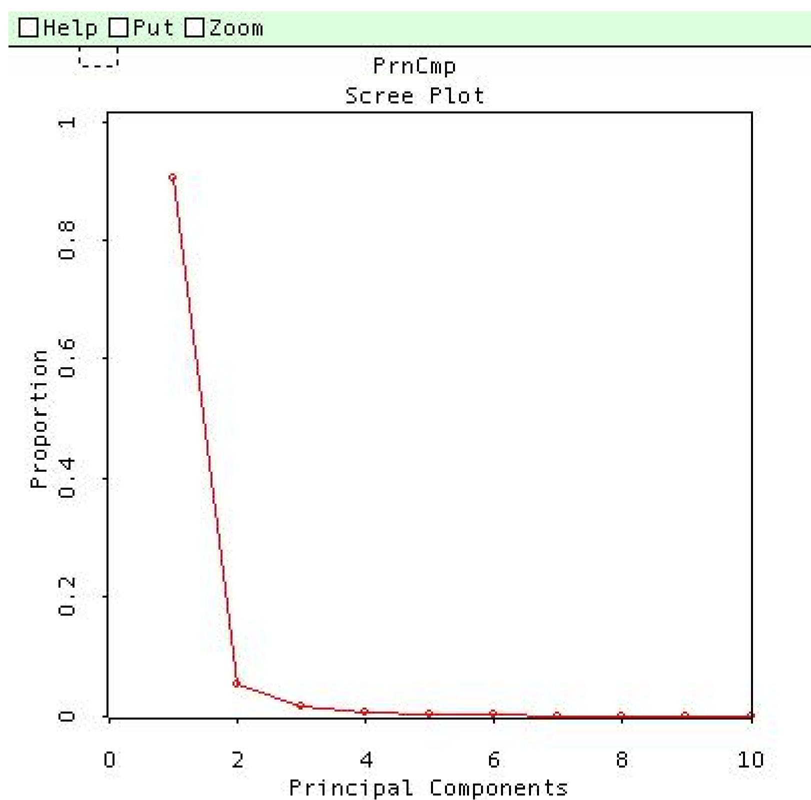

Figure 14: The scree plot emtricitabine pure drug

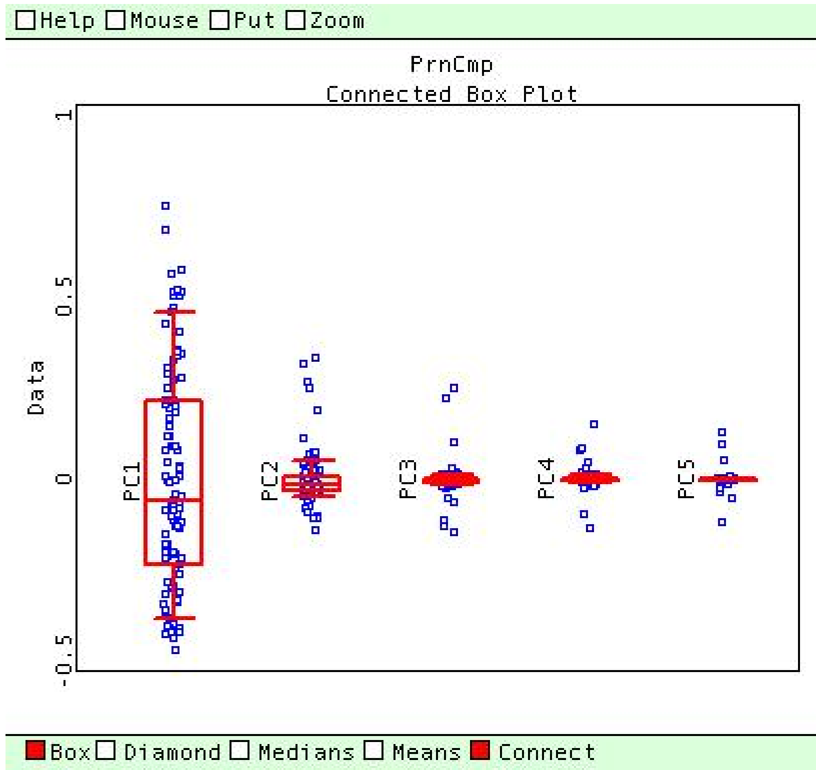

Figure 15: The connected box plot for emtricitabine pure drug

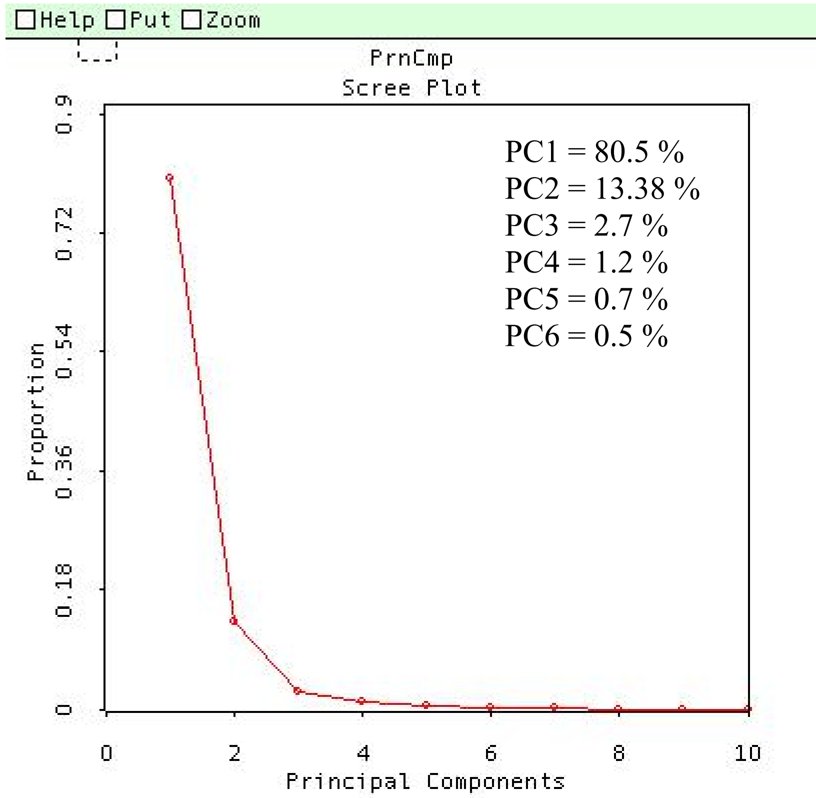

Figure 16: The scree plot for tenofovir DF pure drug

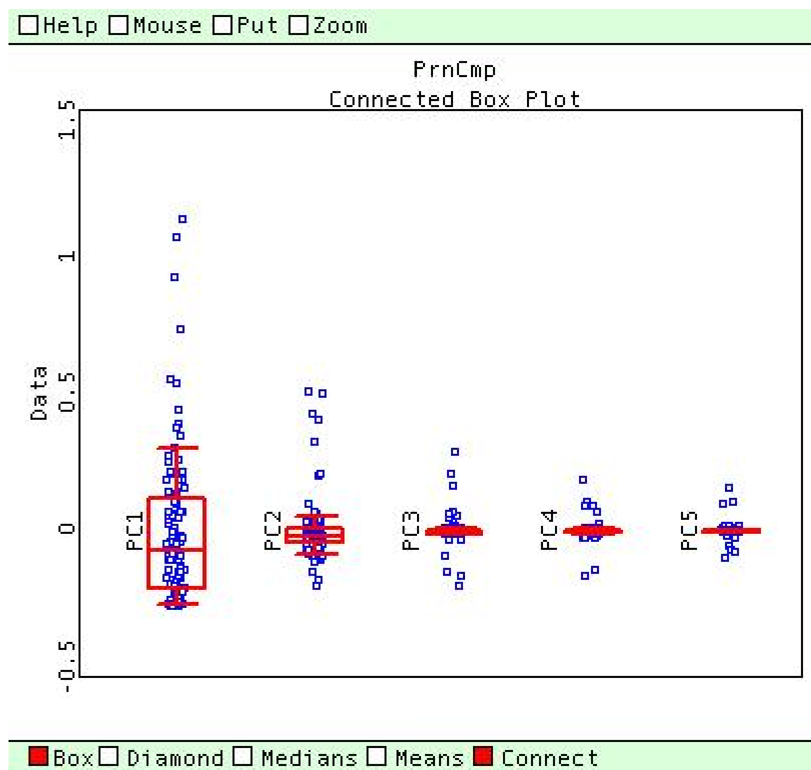

Figure 17: The connected box plot for tenofovir DF pure drug

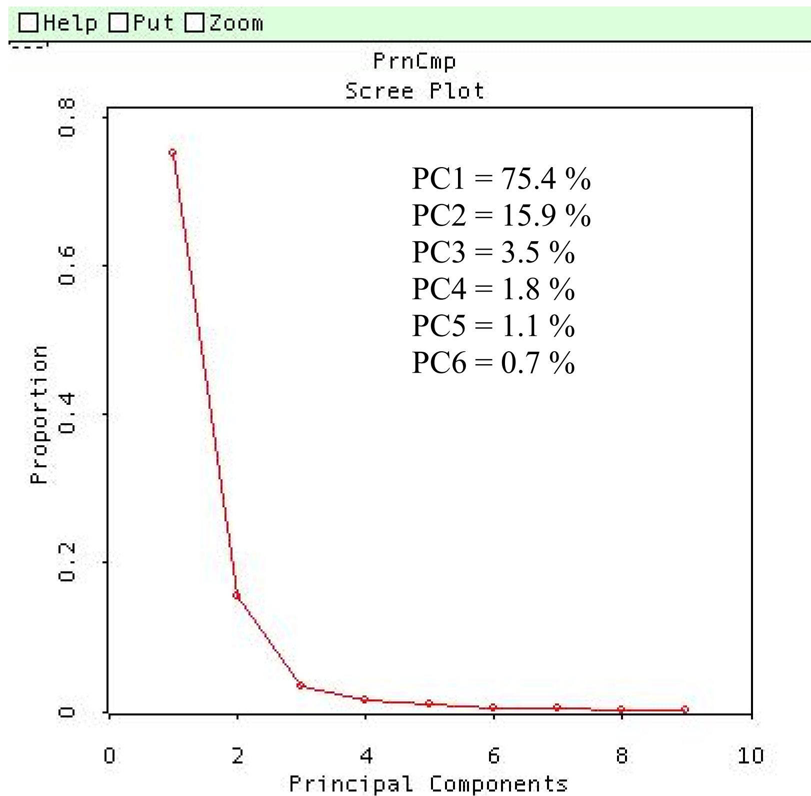

Figure 18: The scree plot for mixture of emtricitabine and tenofovir DF

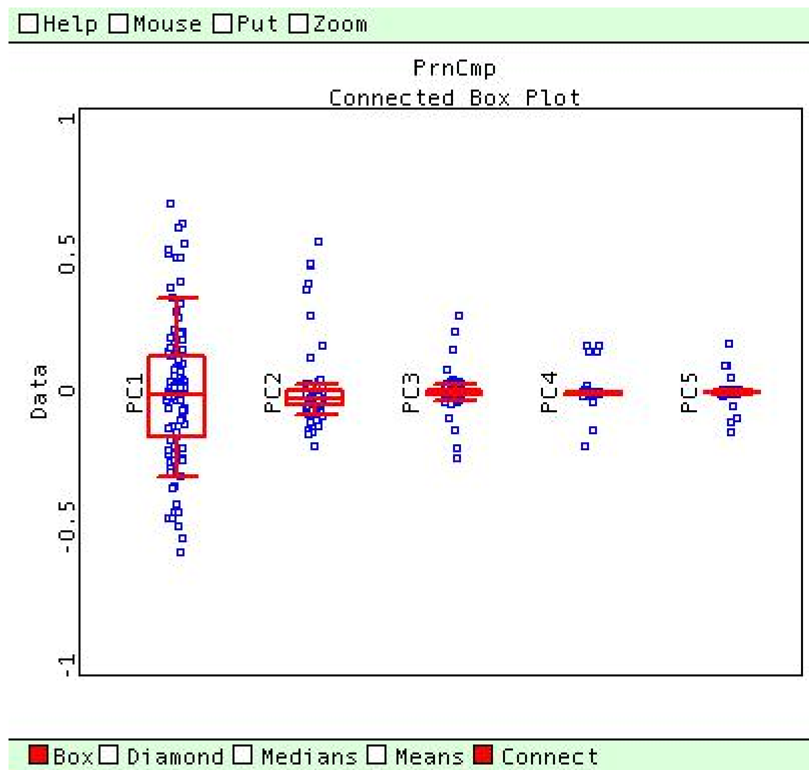

Figure 19: The connected box plot for the mixture of emtricitabine and tenofovir DF

Table 10: Actual and predicted amounts of emtricitabine in pure, laboratory prepared binary mixture with tenofovir DF, and commercial tablets by using PCR Technique

Analyzed component	Used concentrations	Found		
	(µg/ml)	(µg/ml)	(%)	CV
Pure Emtricitabine	5	5.08	101.60	1.66
	10	10.07	100.77	1.92
	15	14.92	99.47	0.94
	20	19.94	99.70	1.35
	25	25.08	100.32	1.11
	30	30.13	100.43	0.95
	35	34.89	99.69	1.55
	40	39.33	98.33	1.64
	45	44.56	99.02	1.76
	50	50.23	100.46	1.96
Laboratory prepared mixtures	Emtricitabine/ Tenofovir DF			
	45 : 5	44.58	99.07	1.22
	40 : 10	40.35	100.88	1.65
	35 : 15	35.47	101.34	1.98
	30 : 20	29.48	98.27	1.78
	25 : 25	24.72	98.88	1.55
	20 : 30	20.07	100.35	0.94
	15: 35	14.87	99.13	0.77
	10 : 40	10.062	100.62	1.44
	5 : 45	5.03	100.60	1.73
Truvada tablet	20 : 30	20.082	100.41	1.93

*Relative errors as calculated from the corresponding calibration model (n = 3)
*Relative errors as calculated from the corresponding calibration model (n = 3 & 7 for laboratory prepared mixtures and tablets respectively)

Table11: Actual and predicted amounts of given tenofovir DF by applying PCR for pure, laboratory prepared mixtures with emtricitabine, and commercial tablets.

Analyzed components	Used concentrations (µg/ml)	Found		
		(µg/ml)	(%)	CV
Pure tenofovir DF	5	4.92	98.40	1.45
	10	9.93	99.30	1.98
	15	14.88	99.20	0.96
	20	19.85	99.25	1.67
	25	25.29	101.16	1.34
	30	29.66	98.87	0.88
	35	35.07	100.20	1.11
	40	40.11	100.28	1.37
	45	45.73	101.62	0.79
	50	50.13	100.26	1.22
Laboratory prepared mixtures	Tenofovir DF /Emtricitabine			
	5: 45	4.94	98.80	1.67
	10: 40	9.88	98.78	1.88
	15 : 35	14.83	98.87	1.91
	20 : 30	19.79	98.95	1.95
	25: 25	25.11	100.44	1.87
	30 :20	30.24	100.80	1.33
	35:15	35.17	100.49	1.54
	40: 10	39.45	98.63	1.39
	45:5	45.33	100.73	1.11
Truvada tablet	30 : 20	30.22	100.73	1.99

*Relative errors as calculated from the corresponding calibration model (n = 3)

*Relative errors as calculated from the corresponding calibration model (n = 3 & 7 for Laboratory prepared mixtures and tablets respectively)

As indicated in tables 10 and 11 in addition to pure forms, quite a lot of Laboratory prepared mixtures were subjected to PCR analysis in order to substantiate the suitability of the calibration model for determination of the studied drugs in the pharmaceutical sample solutions. As indicated by the calibration equations and correlation coefficients given by the PCR spectral data that obtained experimentally in the range from 200 to 320 nm, there was a strong correlation between the absorbance and concentration.

The tables, Tables 10 and 11, also illustrate the actual and predicted amounts ± errors (%) of the studied drugs. The domino effect verifies the high degree of agreement and indicates that the method is suitable for analysis of the given drug.

According to the plots we obtain, the concentrations predicted by the model are very close to the actual concentrations, the recoveries in all cases were acceptable. It can be observed from this set of results that the drug mixture determination is perfectly practicable and the multivariate calibration model allows a considerable reduction of errors in relation to the determination by multivariate calibration.

Results for laboratory prepared mixtures and the tablets with comparable concentrations were found closely harmonized. This pointed out that, the added or originally present excipients and additives did not affect the determination.

4.5. Comparison of the results obtained from the four chemometrics methods

Table 12: Summary of the results obtained for the quantitative determination of the truvada tablet components by the ^{1}D, first derivative ratios, CLS and PCR methods

Technique	λ_{max}	% Recovery of emtricitabine	CV	λ_{max}	% Recovery of tenofovir DF	CV
Zero crossing first derivative	278.5	98.7	0.2	294.5	99.4	1.5
Derivative ratio	249.5	101.9	1.6	288.5	99.8	1.5
	270.5	98.3	0.3	303.5	99.9	0.9
CLS	-	99.07	0.3	-	99.62	0.9
PCR	-	100.41	1.93	-	100.73	1.99

The recovery and the coefficient of variation of the studied components of the drug were summarized in table 12. It can be observed from the results that the compounds under investigation could be analyzed by all the suggested methods with a good precision and accuracy. CLS and PCR are found to be more suitable for the determination of the given combination tablets. Except derivative ratio technique, which gave very good result for tenofovir DF, results of the PCR technique were found to be more accurate than the results of other techniques. In addition, the results confirmed that the compounds mixtures were determined with sufficient degree of precision by all the method, but the factorized multivariate calibration models (PCR) allows a more significant reduction of errors in relation to the other used methods.

5. Conclusion

Trial to develop a method of assay for simultaneous determination of the components of truvada table with out prior separation of the components is found to be successful. Because of the spectral overlap in the range of 200-285 nm of emtricitabine and tenofovir DF, direct quantitative determination of these drugs in their combined state had been difficult. The components of this binary mixture drug were being simultaneously quantified by the proposed chemometrics-assisted technique. All the analysis methods (first derivative spectrophotometric, derivative ratio PCR and CLS techniques) were able to provide results that are within the limit. Among the given methods, derivative ratio and PCR techniques are the best choices for simultaneous determination of the truvada tablets. Especially PCR technique can be manipulated for quantitative determination of the components of the mixture without prior separation of the components. This technique was able to eliminate the unwanted background and spectral interferences significantly during determination. This method of determination has also advantages over the methods discussed in the introduction part interms of determination with out prior separation of the components, less sample preparation time, usage of less expensive instrumentation, usage of less expensive solvents and less analysis time.

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