

Full Length Research Paper

New solid phase extraction reversed phase high performance liquid chromatography ultraviolet (RP-HPLC-UV) method for simultaneous determination of tenofovir and emtricitabine in Chinese population

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The objective of this study is to develop and validate a method of reversed phase high performance liquid chromatography (RP-HPLC) assay with ultraviolet (UV) detector after solid phase extraction (SPE) for simultaneous measure tenofovir and emtricitabine in Chinese population. Detection of analytes was performed with a Phecda C₁₈ (250 × 4.6 mm, 5 μm), reversed-phase analytical column and a security guard column C₁₈ (4 × 3.0 mm, 5 μm). An obviously extrusive separation was successively followed with the SPE method using BOND ELUT-C₁₈ Varian columns and a 30 min gradient elution consisting of potassium phosphate monobasic buffer with 0.08% of triethanolamine (pH 3.52) and methanol with UV detector at 270 nm. The results of this study showed that the method used in this study was simple, accurate and sensitive with a wide linear range from 10 to 5000 ng/ml for both tenofovir and emtricitabine when 300 μl aliquots was analyzed. The intra- and inter-day precision and accuracy for both analytes were lower than ±20% at the limit of quantification (LOQ) and ±15% at the other quality control (QC) levels. The absolute recoveries for tenofovir and emtricitabine were 80.6 and 86.5%, respectively. This method is suitable for routinely monitor plasma concentrations of tenofovir and emtricitabine in Chinese population.

Key words: High performance liquid chromatography ultraviolet (HPLC-UV), solid phase extraction (SPE), pre-exposure chemoprophylaxis (PrEP), tenofovir, emtricitabine.

INTRODUCTION

With a global estimate of 2.7 million, new human immunodeficiency virus (HIV) infections occurring worldwide each year and even more undiagnosed cases waiting to be discovered, discovering novel methods to help stem the spread of this virus is critical. While behavior change programs have contributed to dramatic reductions in the number of annual infections in the U.S.

and many other nations, far too many individuals remain at high risk. With an effective vaccine years away, there is a growing body of evidence that antiretroviral drugs may be able to play an important role in reducing the risk of HIV infection (<http://www.cdc.gov>, 2008). Pre-exposure chemoprophylaxis (PrEP) is being explored as a novel strategy to interrupt the spread of HIV in at-risk patient populations such as men who have sex with men (MSM), injecting drug users (IDU), and female sex workers (Clauson et al., 2009). The Centers for Disease Control and Prevention (CDC) is sponsoring three clinical trials of PrEP, and is participating in a University of Washington-

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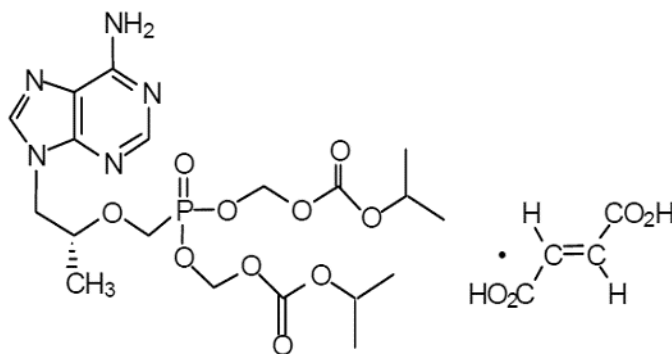


Figure 1. Chemical structure of tenofovir disoproxil fumarate (TDF).

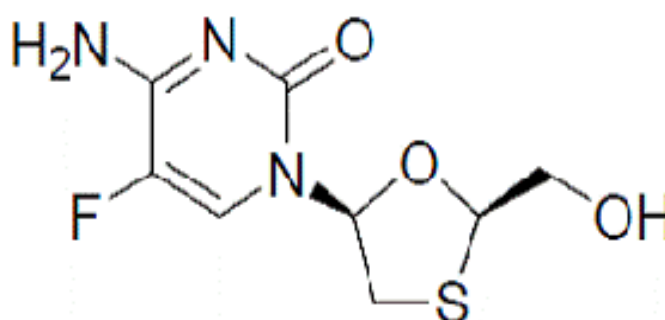


Figure 2. Chemical structure of emtricitabine.

sponsored trial in Kenya and Uganda. Similar PrEP trials are also being conducted by Family Health International (FHI), the National Institutes of Health (NIH), FHI and the Microbicide Trials Network (MTN) (<http://www.cdc.gov>, 2008). Recently, a project named feasibility study of PrEP to decrease the infection of HIV for High Risk Group in Western China has been initiated by Chongqing Medical University to study the safety, compliance and resistance of tenofovir and emtricitabine for PrEP.

Some researchers believe that an antiretroviral drug taken as a daily oral preventative is one of the most important new prevention approaches of HIV infection and several sources of data suggest that the use of antiretroviral drugs in this manner may be effective. Animal studies have demonstrated that pre-exposure administration of tenofovir disoproxil fumarate (TDF) plus emtricitabine provided significant protection to monkeys exposed repeatedly to an HIV-like virus. These data, combined with the drug's favorable resistance, safety profiles and long intracellular half times make TDF and TDF plus emtricitabine ideal candidates for HIV prevention trials (<http://www.cdc.gov>, 2008).

Tenofovir and emtricitabine are nucleoside/nucleotide reverse transcriptase inhibitors (NRTI), commonly used to treat persons infected with HIV-1. VIREAD® is a trademark for TDF of Gilead Sciences, Inc., which is a fumaric acid salt

of the bis-isopropoxycarbonyloxymethyl ester derivative of tenofovir. It has a molecular formula of $C_{19}H_{30}N_5O_{10}P \cdot C_4H_4O_4$ and a molecular weight of 635.52. Its structural formula is shown in Figure 1 (<http://www.accessdata.fda.gov>, 2008). It is an oral prodrug of tenofovir, an acyclic nucleoside phosphonate (nucleoside) analog of adenosine 5'-monophosphate, which has potent activity against retroviruses and hepadnaviruses. Upon oral administration, it is converted *in vivo* to tenofovir by esterase hydrolysis, which is taken up by cells. And then tenofovir is activated to tenofovir diphosphate through phosphorylation reaction, the active form of tenofovir. Tenofovir diphosphate competes with the natural substrate deoxyadenosine 5'-triphosphate for incorporation into DNA during HIV transcription, which blocks the action of HIV reverse transcription, prevents further DNA chain elongation, and cause termination of viral DNA replication (Kwarney et al., 2004).

Emtricitabine differs from other cytidine analogs in that it has a fluorine in the 5-position. It has a molecular formula of $C_8H_{10}FN_3O_3S$ and a molecular weight of 247.24. Its structural formula is shown in Figure 2. It is phosphorylated by cellular enzymes to form emtricitabine 5'-triphosphate, which inhibits the activity of the HIV-1 reverse transcriptase (RT) by being competing with the natural substrate deoxycytidine 5'-triphosphate and by

Table 1. The gradient elution table of mobile A and B over 30 min of run time.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0.00	90	10
2.00	90	10
18.00	80	20
19.00	10	90
21.00	10	90
22.00	90	10
30.00	90	10

being incorporated into nascent viral DNA which results in chain termination (<http://www.accessdata.fda.gov>, 2008).

In the project of feasibility study of PrEP to decrease the infection of HIV for High Risk Group in Western China, measurement of the two drug levels in plasma of Chinese people was required to define pharmacokinetic properties, monitor the drug administration compliance, design rational PrEP modalities, and understand the correlation between drug levels and protection. Several methods for determination of tenofovir or tenofovir combined with emtricitabine were described. Avolio et al. (2008) set up an assay based on solid phase extraction (SPE) procedure with liquid chromatography-mass spectrometry (LC-MS) to measure plasmatic concentrations of tenofovir and emtricitabine in HIV infected patients. Gomes developed a LC-tandem MS (LC-MS/MS) method for simultaneous determination of tenofovir and emtricitabine (Gomes et al., 2008). Also, Delahunty et al. (2006) developed and validated an LC/MS/MS assay for the determination of tenofovir. These assay methods were costly because of expensive performance apparatus and it was not really convenient for the developing countries, in which the infection percentage of HIV was in a large population. Barkil used LC method coupled to UV and single MS detection to determine tenofovir in human plasma, and concluded that both detections were allowed to obtain a reliable quantification of tenofovir (Barkil et al. 2007).

The aim of this study was to set up a simple and reliable method for simultaneous determination of tenofovir and emtricitabine in plasma of Chinese people with high performance liquid chromatography-ultraviolet (HPLC-UV) detector.

MATERIALS AND METHODS

Chemicals and reagents

Chemical standards of tenofovir and lamivudine (internal standard, IS.) were kindly provided by Yingpu Science and Technology Development Co., Ltd. (Hangzhou, China). Emtricitabine was supplied by Sichuan Baili Pharmaceuticals.

Methanol of HPLC grade was obtained from Hanbon Science and Technology Co., Ltd (Jiangsu, China). High purity water used in

the mobile phase and solution preparation was freshly prepared from Milli-Q Academic (Millipore, Bedford, USA). Potassium phosphate monobasic used for the preparation of mobile phase was provided by Bo Yi, Chongqing Chemical Regant Co., Ltd (Chongqing, China). Triethanolamine used for mobile phase A was provided by Zhengzhou Zhongtian Test Instruments (Zhengzhou, China). Phosphoric acid for pH regulation of mobile phase was obtained from Chengdu Kelong Chemical Regant Factory (Chengdu, China). Ammonium acetate of analytical grade used in SPE was purchased from Chuandong Chemical Industry Group Co., Ltd. (Chongqing, China), while sodium hydroxide was from Zhengzhou Chemical Factory.

Drug free (blank) heparinised human plasma was obtained from healthy volunteers and was stored at -20°C prior to use. SPE columns (3.0 ml, 200 mg, BOND ELUT-C₁₈) purchased from Varian (Lake Forest, CA, USA) was employed.

VIREAD® for volunteers was provided by Gilead Sciences, Inc. and emtricitabine was purchased from Hebei Pharmaceutical company, Hebei Medical University.

Chromatographic conditions and instrumentation

The chromatographic separation was performed at 35°C, on a Pheceda C₁₈ analytical column (4.6 × 250 mm, 5 μm, Hanbon Science and Technology, China), with a Phenomenex® C₁₈ security guard column (4.0 × 3.0 mm). The total run time for each sample was 30 min, delivered with a flow rate of 1.0 ml/min. Run time was achieved with a gradient elution (Table 1) consisting of potassium phosphate monobasic buffer with 0.08% of triethanolamine (pH 3.52) as mobile phase A, and methanol as mobile phase B. The buffer solution was filtered through a 0.45 μm membrane filter. For UV detection, wavelength was monitored at 270 nm in order to detect tenofovir, emtricitabine and lamivudine, simultaneously.

A HPLC system of LC-2010A HT LIQUID CHROMATOGRAPH from SHIMADZU CORPORATION run on a Dell computer (operated with Windows 2000 Professional), was used for this method.

Preparation of stock standard solutions, working standard solutions, plasma calibration curve samples and quality controls

Standards were accurately weighed and then dissolved with HPLC-grade water to produce the corresponding stock standard solutions (40 μg/ml for lamivudine, and 1 mg/ml for each for tenofovir and emtricitabine), refrigerated at 4°C until use.

A 9-point working standard solutions (both tenofovir and emtricitabine) for calibration curve were prepared by diluting the stock solutions with HPLC-grade water from 1 to 500 μg/ml; and plasma calibration curve standards were obtained from 10 to 5000

ng/ml by 4.9 ml blank plasma with 50 μ l stock standard solution of both tenofovir and emtricitabine at each point.

Quality control (QC) samples were prepared at three concentration levels of 20, 200 and 2000 ng/ml for both standards in the same manner with the preparation of plasma calibration curve samples.

Sample preparation

A 300 μ l aliquot of human plasma sample was mixed with 20 μ l of IS working solution (30 μ l/ml of lamivudine) and 180 μ l of 10% trichloroacetic acid solution. Then the solution was vortexed for 30 s and centrifuged at 13,000 rpm for 10 min, at room temperature. The supernatant fluid was neutralized by 240 μ l of 1% sodium hydroxide solution and then loaded in the SPE cartridge, which was conditioned by 3 ml methanol and 3 ml of 150 mM ammonium acetate (pH 5.0). After washed with 900 μ l of 100 mM ammonium acetate (pH 7.0) in order to obtain less interference at the retention time of each analyte, the column was eluted by 500 μ l of methanol to receive the drugs. Finally, the elute was evaporated to dryness by using a concentrator (Eppendorf®, Germany) in vacuum at room temperature. The extracted sample was reconstituted with 120 μ l of water and methanol solution (90:10,v/v) and 100 μ l was injected into the chromatographic system.

Method validation

Selectivity

Selectivity of the method was investigated by analyzing standards solutions, blank plasma samples of four different blank plasma samples and the spiked samples, in order to determine if any interference of endogenous compounds existed at the retention time of tenofovir, emtricitabine, and lamivudine.

Linearity

Both calibration curves of two drugs were established by plotting the peak area ratio (tenofovir/lamivudine or emtricitabine/lamivudine) as a function of plasma concentration of the aim analytes. Each calibration curve was obtained using 9 calibration points ($n = 5$) of plasma calibration curve samples prepared as 2.4, which were 10, 20, 50, 100, 200, 500, 1000, 2000 and 5000 ng/ml, respectively.

The limit of determination and quantification

The limit of quantification (LOQ) was the smallest analytical concentration which can be detected with a signal noise ratio more than 3. The lower LOQ (LLOQ) was the smallest analytical concentration which could be measured with accuracy and precision still less than 20%.

Accuracy and precision

Accuracy, intra- and inter-day precision were evaluated by replicate analysis ($n = 5$) of the three QC levels at the concentrations of 20, 200 and 2000 ng/ml.

Five replicates at each level of quality controls were assayed at the first day for the accuracy and intra-day precision experiment. Another five replicates at each level of quality controls were assayed within five different days for the inter-day precision experiment.

Accuracy is defined as the percent relative error (%RE) and was calculated by using the formula:

$$\%RE = (E-T)/(100/T)$$

Where E is the experimentally determined concentration and T is theoretical concentration.

Intra- and inter-day precision were expressed as relative standard deviation:

$$(\%R.S.D.) = (S.D./M)(100),$$

Where M is the mean of the experimentally determined concentration and S.D. is the standard deviation of M at each concentration.

Recovery

Recovery of the tenofovir and emtricitabine after extraction procedures was assessed by comparing the peak area of the extracted plasma samples ($n=5$) with those of non-extracted standard solutions at concentrations corresponding to the three QC values (20, 200 and 2000 ng/ml).

Stability

Stability of tenofovir and emtricitabine in spiked plasma control samples was determined in triplicate at three concentrations (20, 200 and 2000 ng/ml) including freeze-thaw, auto sampler and long-term stabilities. The acceptance criterion was $\pm 15\%$ difference between the peak area of observed and theoretical concentrations.

Freeze-thaw stability of analytes was determined by assaying the three levels of samples in triplicate over three freeze-thawing cycles. The long-term stability of spiked plasma at -20°C was also evaluated by assaying weekly in triplicate, three concentration levels of samples stored at -20°C for a 2-month period. Additionally, stability of extracted samples after the final reconstitution during storage in the auto sampler for 24 h at room temperature was also verified.

RESULTS

The mean retention times around 7.7 ± 0.2 min for the IS (lamivudine), around 6.5 ± 0.2 min for tenofovir and 12.5 ± 0.1 min for emtricitabine. No endogenous substances interfered with any of the analytes in blank plasma.

Selectivity

Chromatogram of standard for tenofovir, emtricitabine and lamivudine is shown in Figures 11, 12, and 13. Representative chromatogram of the blank human plasma is shown in Figure 3. Figure 4 shows chromatogram of the extracted spiked plasma with the concentration of 200 ng/ml for tenofovir, emtricitabine and IS.

Linearity

The peak area ratios of analyte to IS for calibration

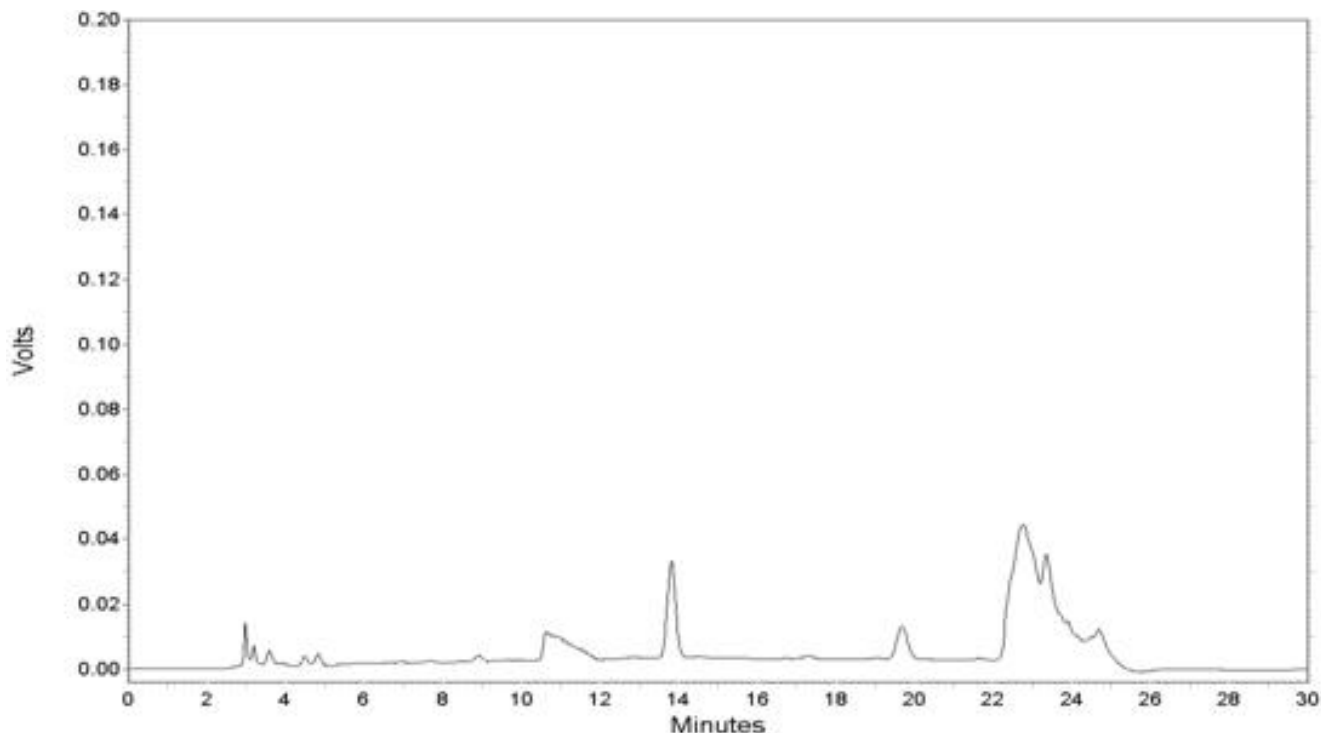


Figure 3. Chromatogram of drug-free human plasma of volunteers.

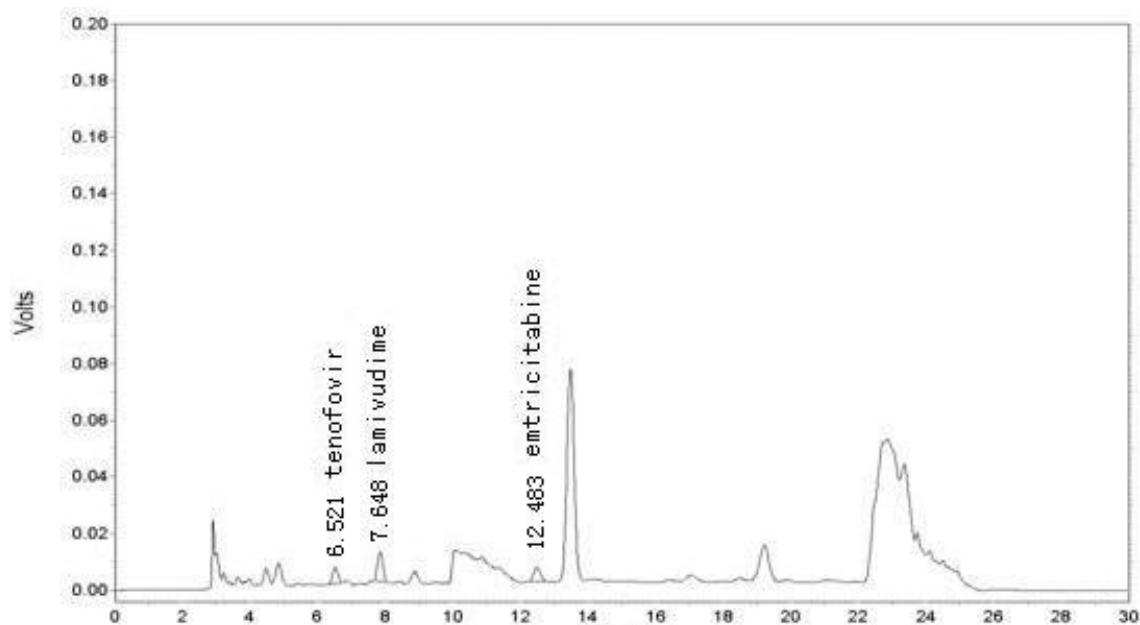


Figure 4. Chromatogram of plasma spiked with tenofovir and emtricitabine at 200 ng/ml.

standards were proportional to the concentration of each drug in plasma over the range tested. The calibration curves recorded have satisfied the requirement of regression coefficient (R^2) higher than 0.999, with 0.9998

for tenofovir and 0.9997 for emtricitabine. Across the 9 points taken as calibration standards, the R.S.D. and %RE obtained were within $\pm 20\%$ at the LLOQ and $\pm 15\%$ for the rest of the tested concentration.

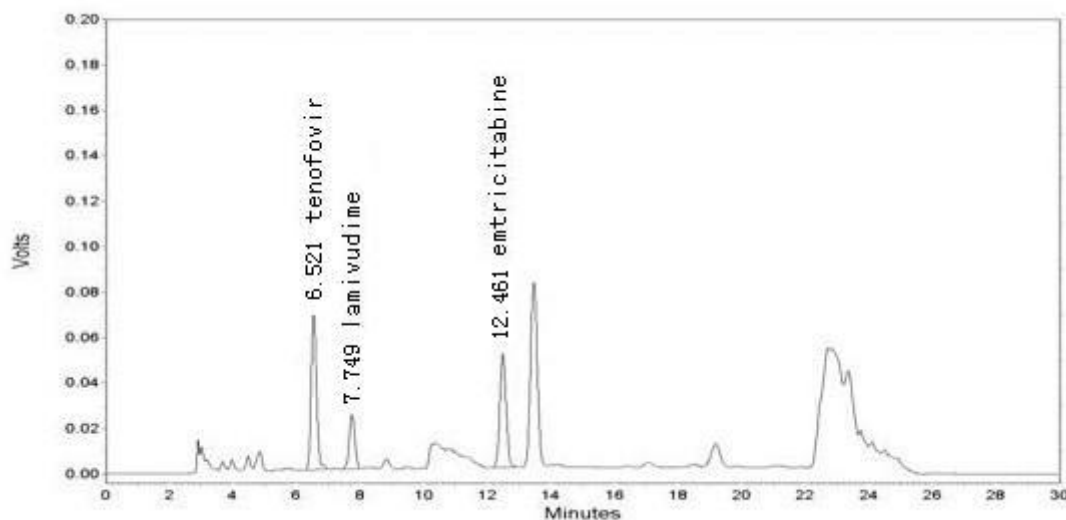


Figure 5. Chromatogram of plasma spiked with tenofovir and emtricitabine at the highest level of QC concentration (2000 ng/ml).

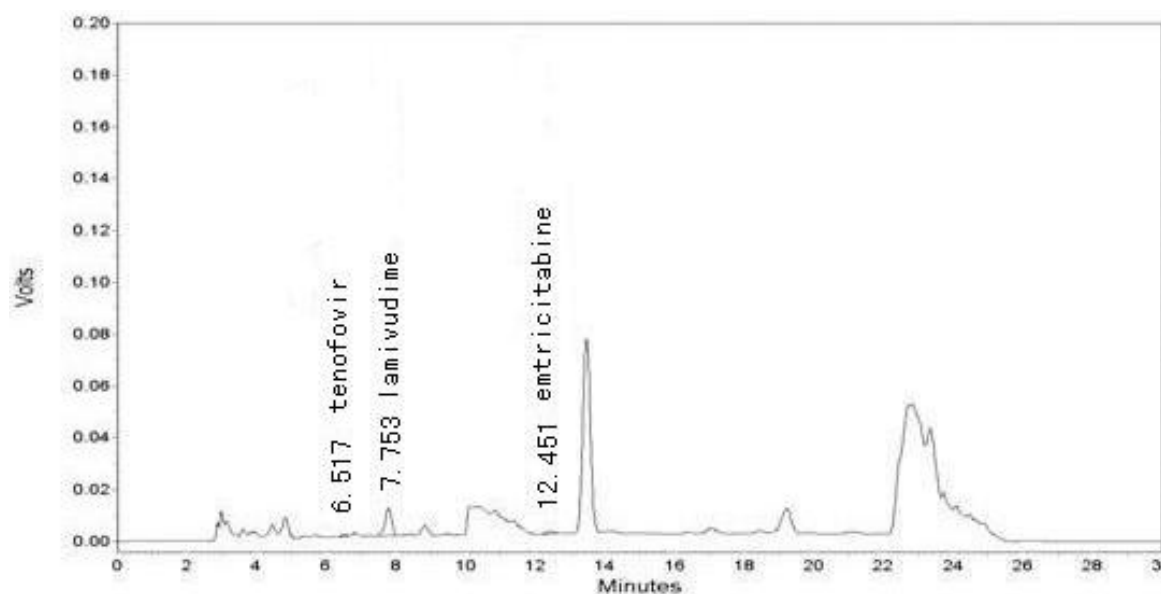


Figure 6. Chromatogram of plasma spiked with tenofovir and emtricitabine at the lowest level of QC concentration (20 ng/ml).

The limit of determination and quantification

The LOQ was 8.0 ng/ml for tenofovir and 3.0 ng/ml for emtricitabine with the signal noise ratio more than 3. The LLOQ for both analytes was determined as 10 ng/ml, which was the lowest concentration that could be measured with a R.S.D. within 20% and accuracy between 80 and 120%. The upper LOQ was 5,000 ng/ml.

Chromatogram for the QC samples 20, 200 and 2000 ng/ml are shown in Figures 4, 5 and 6, respectively.

Accuracy and precision

Results of the validation of the method are shown in Table 2. Intra- and inter-day period expressed as percent

Table 2. Summary of accuracy and precision (%) for method validation at low ,medium and high concentrations.

Analyte	Theoretical concentration (ng/ml)	Observed concentration (ng/ml)	Accuracy (%RE)	Intra-day precision (R.S.D%)	Inter-day precision (R.S.D%)
Tenofovir	20	22.1	110.5	9.6	9.3
	200	210.8	105.4	3.8	6.3
	2000	2122.0	106.1	2.4	5.4
Emtricitabine	20	22.1	110.5	7.1	13.4
	200	219.2	109.6	5.6	7.0
	2000	2152.0	107.6	2.6	5.6

R.S.D. were $\leq 6.3\%$ for tenofovir and $\leq 7.0\%$ for emtricitabine, whereas at the LOQ levels was $\leq 9.6\%$ for tenofovir and $\leq 13.4\%$ for emtricitabine, respectively. The accuracy in terms of %RE was within the range of 105.4 to 110.5% and 107.6 to 110.5% for tenofovir and emtricitabine, respectively.

Recovery

The mean recovery obtained by analyzing two pairs of samples has values of 80.6% for tenofovir (%R.S.D.:4.8%), 86.5% for emtricitabine (%R.S.D.:3.3%) and 81.7% for IS-lamivudine (%R.S.D.:3.5%), respectively.

Stability

The analytes stability was demonstrated in various conditions by the deviation of observed concentration from the nominal one in the range within $\pm 15\%$, as the requirements stipulated. The result revealed that tenofovir and emtricitabine were stable for at least 24 h in the auto sampler at room temperature. It was confirmed that repeated freeze and thawing (three cycles) of spiked plasma samples did not affect the stability. And in the long-term, stability results also indicated that both analytes, stored in a freezer at -20°C in matrix, remained stable for at least 2 months.

Analysis of samples from volunteers

Described method of this study was applied by analyzing plasma samples collected from 8 healthy volunteers (males). Volunteers were administrated with a single dose of tenofovir Viread[®] (300 mg) plus emtricitabine (200mg). The study was approved by the Ethics Committee on Human Research of Chongqing Medical University. Figure 7 was the chromatogram of the healthy volunteer sample.

The pharmacokinetics of tenofovir and emtricitabine in

the healthy population of western China *in vivo* was in accordance with two-compartment model. The main parameters of single administration of tenofovir were as follows: $t_{1/2}$ was 12.72 ± 2.83 h, t_{max} was 1.28 ± 0.29 h, C_{max} was 420.84 ± 96.71 ng/ml, AUC_{0-t} and $\text{AUC}_{0-\infty}$ were 3869.42 ± 962.85 and 4107.09 ± 974.82 $\mu\text{g}\cdot\text{h}\cdot\text{L}^{-1}$, respectively, and CL/F was 1.28 ± 0.28 $\text{L}\cdot\text{h}^{-1}\cdot\text{kg}^{-1}$. And the main parameters of single administration of emtricitabine were as follows: $t_{1/2}$ was 4.20 ± 0.71 h, t_{max} was 1.40 ± 0.21 h, C_{max} was 2143.97 ± 315.43 ng/ml, AUC_{0-t} and $\text{AUC}_{0-\infty}$ were 9972.97 ± 1571.03 and 10413.41 ± 1611.73 $\mu\text{g}\cdot\text{h}\cdot\text{L}^{-1}$, respectively, and CL/F was 0.39 ± 0.07 $\text{L}\cdot\text{h}^{-1}\cdot\text{kg}^{-1}$.

DISCUSSION

The combination of tenofovir and emtricitabine was supposed to be used as a daily oral preventative of HIV infection. In order to prove the hypothesis, some projects around the world are being implemented. It is necessary to establish a simple and reliable method for simultaneous determination of tenofovir and emtricitabine in human plasma.

In this study, we established a method for simultaneous determination of tenofovir and emtricitabine in plasma with HPLC-UV. The present assay was not only accurate and sensitive, but also reproducible and with lower cost for monitoring the plasma concentration of both tenofovir and emtricitabine in clinic. Compared with the other costly methods such as HPLC-MS and HPLC-MS/MS, the method of HPLC-UV cost a little longer time. However, the method of HPLC-UV was more economic and convenient for simultaneous determination of tenofovir and emtricitabine.

For extraction procedure in this study, SPE was chosen considering the high polarity of tenofovir. To get a high level of extraction efficiency, several columns were tried, such as OASIS MCX 1 CC/30 mg from waters, SampliQ C18 3 ml/200 mg from agilent, Strata-X 1 ml/30 mg from phenomenex and BOND ELUT-C18 3 ml/200 mg from VARIAN. Finally, BOND ELUT-C18 was more suitable for our aim analytes.

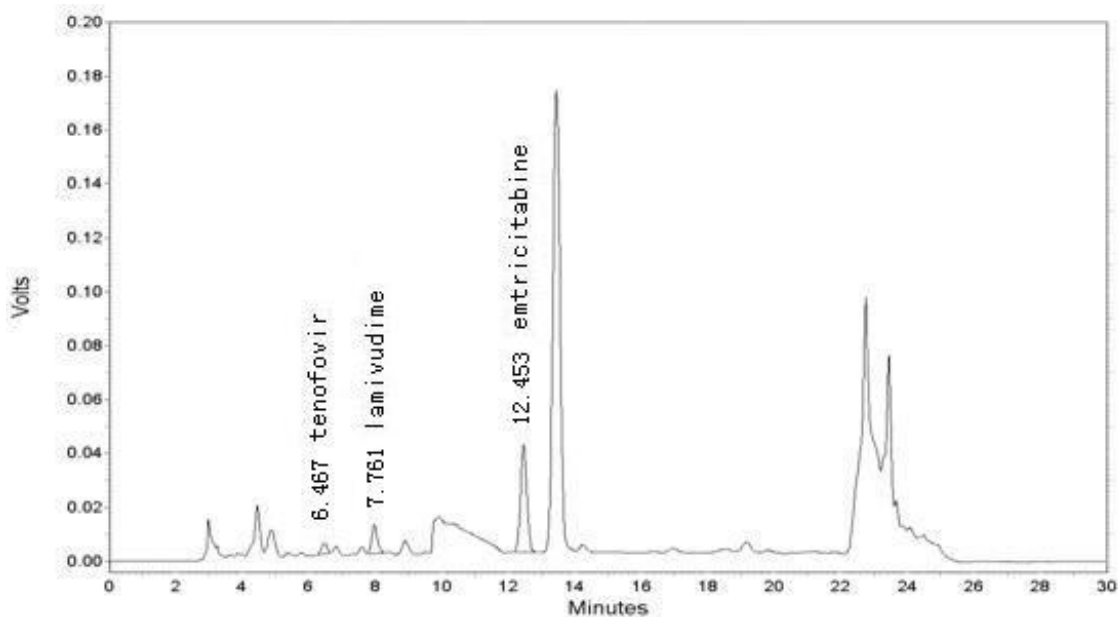


Figure 7. Chromatogram of a healthy volunteer sample taken 3 h after administration of 300 mg dose of VIREAD® and 200 mg dose of emtricitabine. The sample contained 1311.9 ng/ml of FTC and 195.5 ng/ml of TNF.

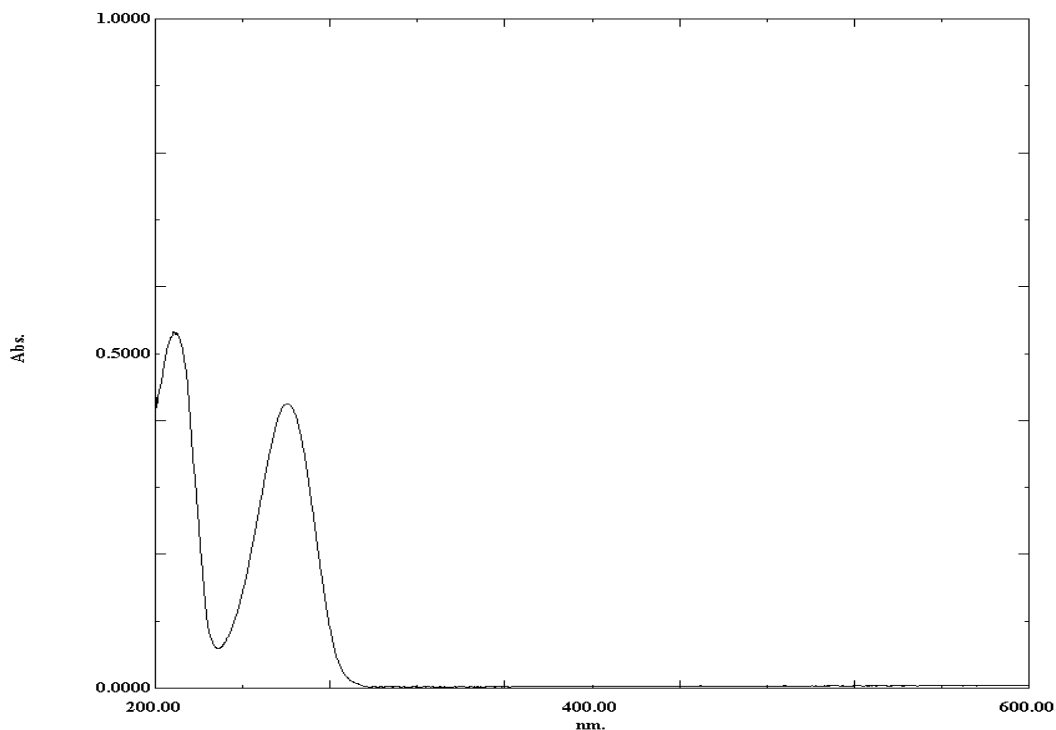


Figure 8. Result of tenofovir for scanned absorbance values at different wavelengths.

For the UV detection wavelength of the all the three drugs, we had scanned absorbance values at different wavelengths to ensure the best wavelength for

simultaneous detection of three drugs, and the result for scanning is shown in Figures 8, 9 and 10.

Compared with the SPE method reported by Rezk et al.

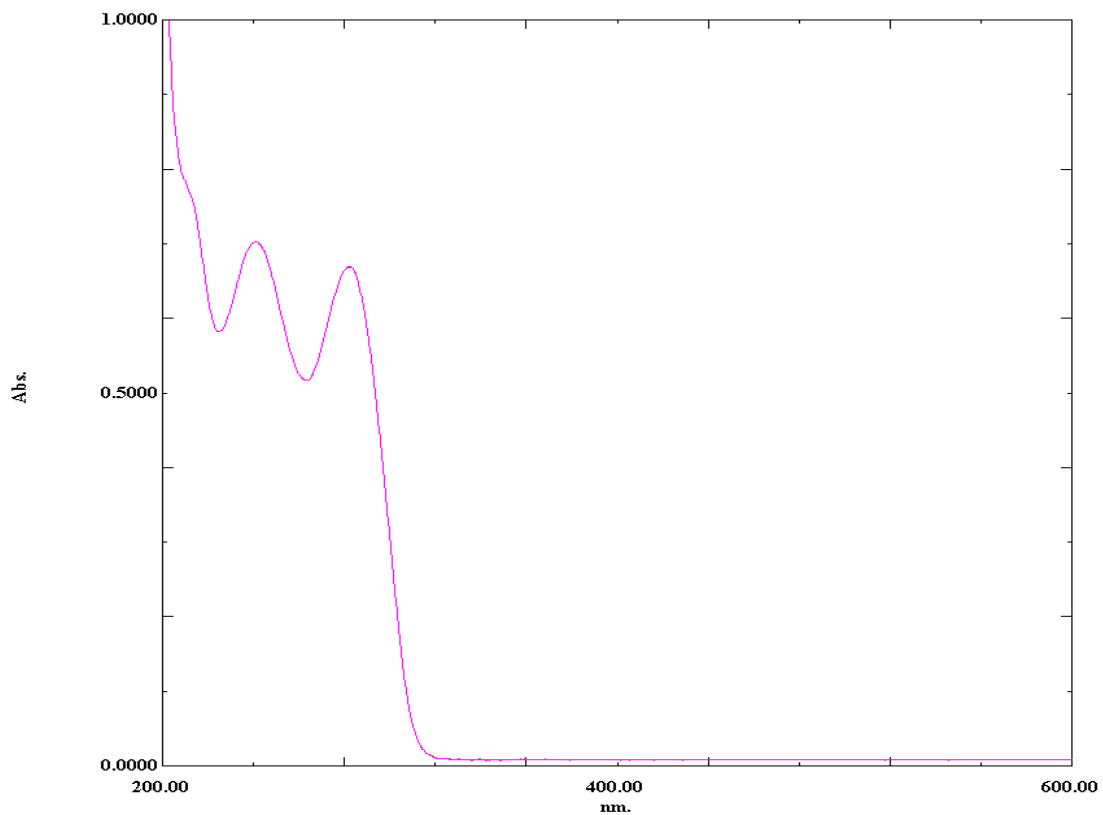


Figure 9. Result of emtricitabine for scanned absorbance values at different wavelengths.

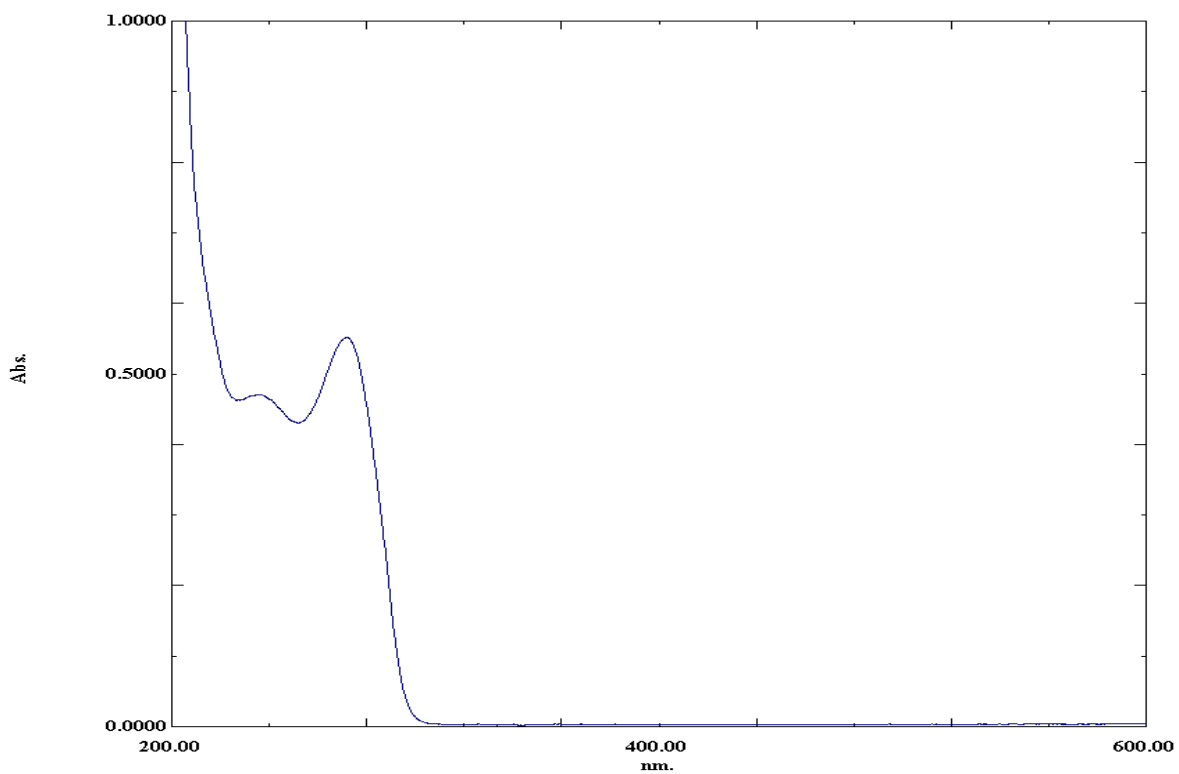


Figure 10. Result of lamivudine for scanned absorbance values at different wavelengths.

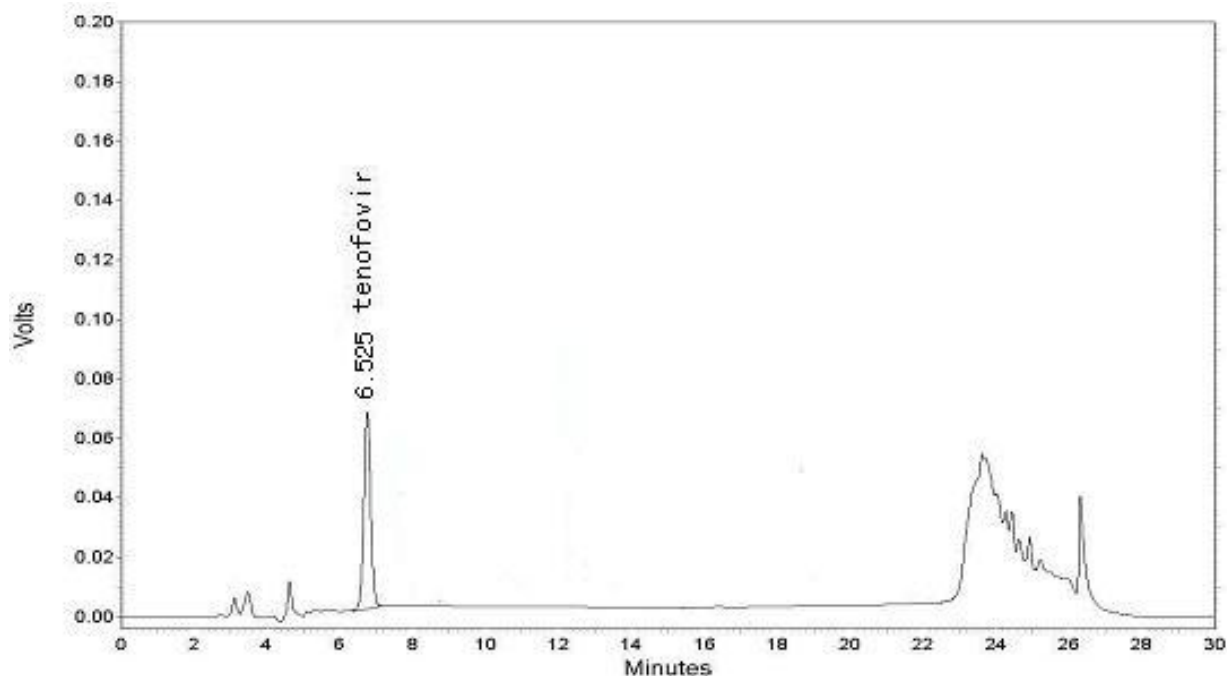


Figure 11. Chromatograms of standard tenofovir (2000 ng/ml).

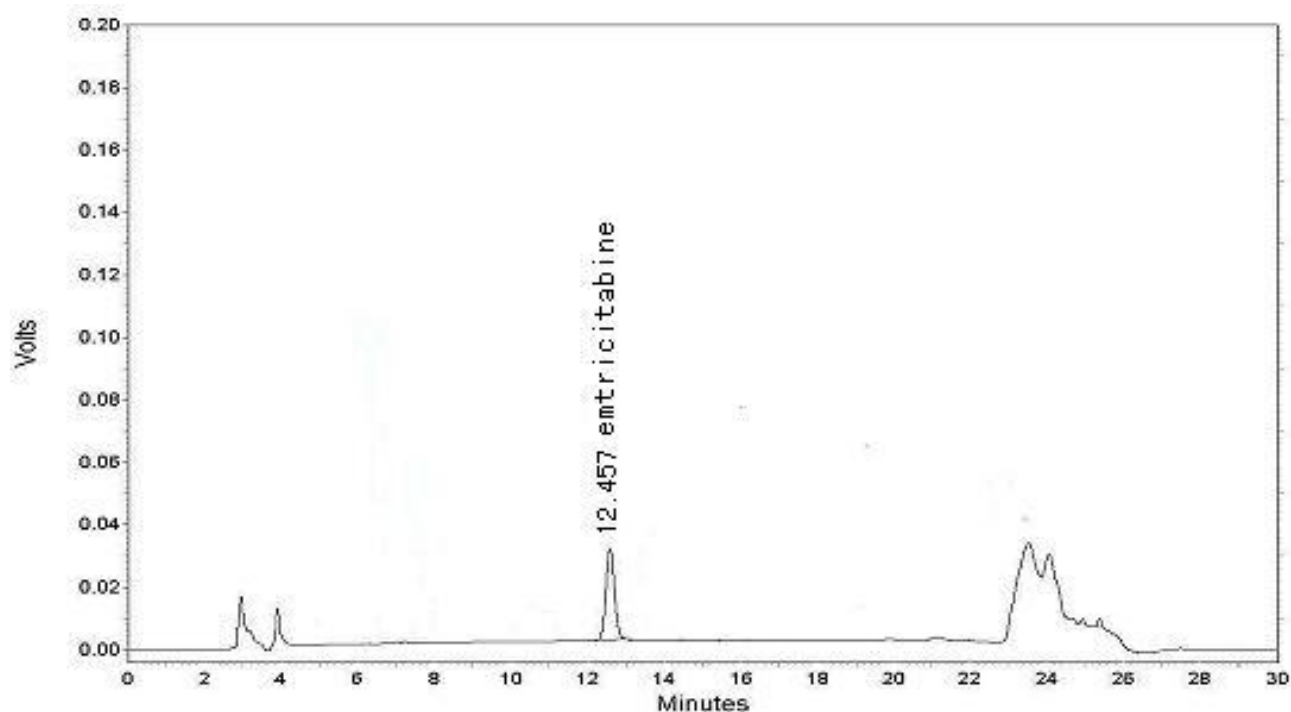


Figure 12. Chromatograms of standard emtricitabine (2000 ng/ml).

(2005), our SPE method showed relatively low recovery. However, in the method established by us, after once extracted, the column could be washed and activated by

methanol and water for three times. So, each column for SPE can be repeatedly used at least 3 times by a repeated validation experiment (% R.S.D < 15%) in our

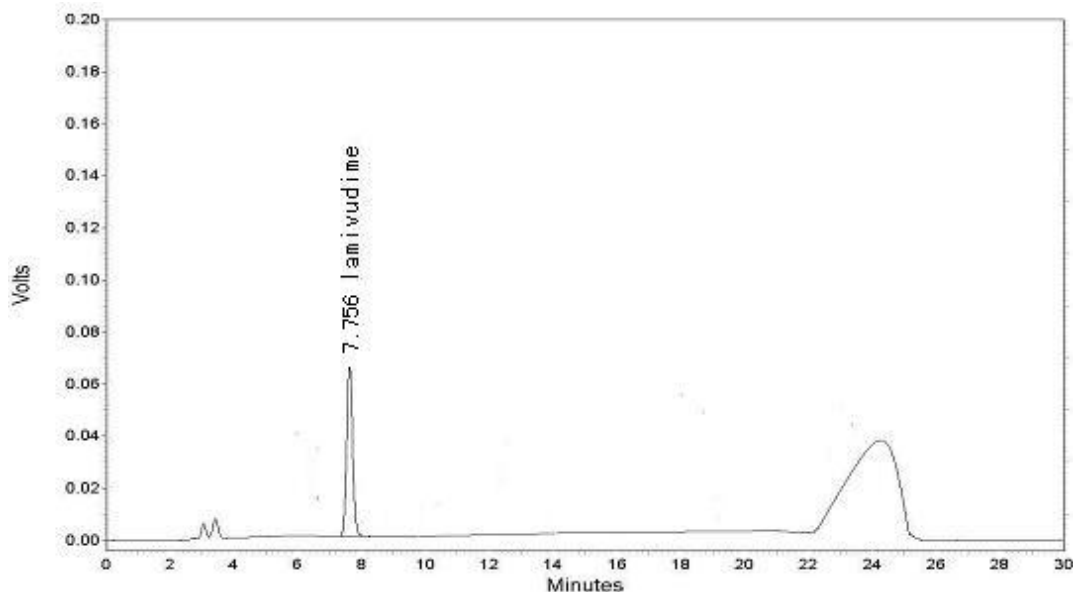


Figure 13. Chromatograms of standard lamivudine (2000 ng/ml).

study, in which spiked plasma replicates were extracted by one column 3 times successively at each three concentration levels (20, 200 and 200 ng/ml), respectively.

Studies showed that the pH of the mobile phase was found to be an extremely considerable factor for the separation of tenofovir with the endogenous substance and for the optimized shape of the peaks (Kuklennyik et al., 2009). For chromatographic conditions, potassium phosphate monobasic buffer with 0.08% of triethanolamine (pH 3.52) and methanol was chosen as the mobile phase A and B in our study. Under this pH of the buffer, the chromatographic peak tailing was minimized with an obviously extrusive separation for the aim analytes and the IS. The gradient condition in our paper makes it feasible for satisfactory separation of emtricitabine and tenofovir and their endogenous interference. Due to the HPLC instrument, wavelength for UV detection was chosen at 270 nm in order to detect tenofovir, emtricitabine and lamivudine, simultaneously.

Conclusion

The method of HPLC-UV established by us in this study is simple, reliable and economic for routinely monitor plasma concentrations of tenofovir and emtricitabine in Chinese population and it can be considered as a technical support for plasma concentration measure of tenofovir and emtricitabine of patients and volunteers especially in developing countries.

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