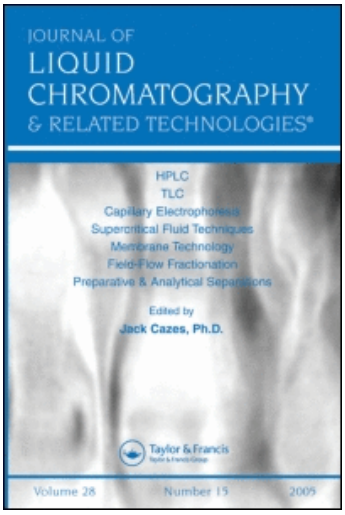

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Determination of Emtricitabine in Human Plasma using HPLC with Fluorometric Detection

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Abstract: A sensitive, specific, and simple high performance liquid chromatography assay with fluorometric detection for the determination of the antiretroviral agent emtricitabine in human plasma is described. Using 500 μL of plasma and Oasis MAX columns, the solid phase extraction (SPE) method results in a clean baseline and high extraction efficiencies (107%). An Atlantis dC18 analytical column is used along with a 15 min linear gradient elution of phosphate buffer and methanol to provide sharp peaks for emtricitabine at excitation and emission wavelengths of 244 and 356 nm, respectively. The method was validated over the range 0.01 to 5.0 mg/L and is accurate (average accuracy of five different concentrations ranged from 100% to 107%) and precise (inter- and intra-assay precision ranged from 0 to 3.7% and 1.8 to 5.3%, respectively). Participation in an external quality control program resulted in deviations for three different levels of less than 2.4% from the nominal concentrations. This method is suitable for use in clinical trials and therapeutic drug monitoring of HIV-infected patients.

Keywords: Emtricitabine, FTC, NRTI, HIV, Non-nucleoside reverse transcriptase inhibitor

INTRODUCTION

Emtricitabine (FTC) is a synthetic nucleoside analogue with activity against human immunodeficiency virus reverse transcriptase.[1] FTC is dosed once

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daily, without regard to food intake. A fixed dose combination of FTC, tenofovir DF, and efavirenz (Atripla) is available, and this once daily regimen is superior to zidovudine, lamivudine, and efavirenz,[2] and current guidelines for the management of HIV infection recommend the combination of FTC, tenofovir, DF, and efavirenz.[3]

After absorption, FTC is readily anabolized by cellular enzymes to form its monophosphate, diphosphate, and finally, triphosphate forms. The triphosphate form is the active intracellular moiety that inhibits reverse transcriptase.

Plasma levels may be important for patient care to check adherence to FTC, in patients with renal failure, to guide dosing in children, and for research purposes. Because there is no clear correlation observed between FTC plasma concentrations and virological response, measuring the intracellular FTC triphosphate levels might be more useful than measuring plasma levels. However, measurement of intracellular levels of FTC is technically difficult, time consuming, and needs expensive equipment. Several methods for the determination of FTC in human plasma have been reported.[4–6] These methods used HPLC-UV or LC-MS-MS and had several disadvantages.

Here, we report the development and complete validation of an HPLC method with fluorescence (FM) detection for the determination of FTC in human plasma.

EXPERIMENTAL

Chemicals

FTC was kindly provided by Gilead Sciences (Gilead Sciences, Foster City, Calif). Acetonitrile super gradient quality and methanol HPLC-grade were obtained from Labscan Analytical Sciences (Dublin, Ireland). Ultrapure water was produced on site using MilliQ Gradient (Millipore, Breda, The Netherlands). Drug free blank plasma was purchased from the regional blood bank. All other reagents were obtained from Merck (Darmstadt, Germany).

Chromatographic System and Conditions

The HPLC system consisted of a model P4000 solvent delivery pump, a model AS3000 autosampler, and a model FL3000 fluorescence detector set at excitation and emission wavelengths of 244 and 356 nm, respectively. All these instruments were from Thermo Finnigan (Breda, The Netherlands).

Separation was performed at 35°C on an analytical Atlantis dC18 column (150 × 4.6 mm) with a particle size of 5 μm (Waters, Etten-Leur, The Netherlands) equipped with an Atlantis dC18 guard column (20 × 4.6 mm)

with the same particle size. Chromquest software (Thermo Finnigan) was used to pilot the equipment and to process the data (i.e., peak height integration, external standard method: calculation log peak height versus log concentration with linear regression) throughout the method validation and sample analysis.

The mobile phase was composed of solution A (0.025 mol potassium phosphate and 200 μL of 85% (v/v) orthophosphoric acid/L ultrapure water, pH = 3.1), and B (methanol). The flow rate was 1.0 mL/min. Injection volume was 50 μL. The gradient program conditions are reported in Table 1.

Stock solution, Calibration Curve and Quality Control Samples

The stock solution of FTC was prepared as a 1 mg/mL concentration. Ten mg of FTC was dissolved in 10 mL ultrapure water and aliquots were stored at −40°C.

This stock solution was used to prepare six working solutions (0.01, 0.03, 0.10, 0.30, 1.0, and 5.0 mg/L) in drug free blank human plasma from the blood bank. Plasma quality control samples (QC samples) at 0.05, 0.5, and 4.0 mg/L were prepared from another 1 mg/mL stock solution.

Sample Preparation

Blood samples were collected in heparinized hard plastic tubes and were centrifuged at 2500 × g for 10 min at ambient temperature. The plasma was transferred to polypropylene tubes, and stored at −40°C for analysis.

On the day of analysis, patient samples, calibrators, and QC samples were brought to room temperature.

Solid-phase extraction (SPE) columns (1 cc, 30 mg Oasis MAX Waters) were placed on a vacuum elution manifold (Baker SPE 24G Column Processor) and rinsed with 1.0 mL of methanol, followed by 1.0 mL of 150 mM acetate buffer pH = 5. Next, 0.5 mL of this acetate buffer and 0.5 mL plasma samples were transferred onto the columns and were drawn into them by applying reduced pressure (flow rate <1 mL/min). The columns

Table 1. Gradient elution program

Time (min)	Flow (mL/min)	Solution A (%)	Solution B (%)
0	1.0	95	5
15	1.0	50	50
16	1.0	95	5
21	1.0	95	5

A: 0.025 mol KH_2PO_4 and 200 μl of 85% (v/v) orthophosphoric acid/L ultrapure water B: methanol.

were washed with 1.0 mL of 2% NH_3 in ultrapure water, followed by vacuum suction for 5 minutes. Elution of FTC was performed by using two volumes of 500 μL of methanol and reduced pressure (flow rate <1.0 mL/min). The eluent was evaporated to dryness under a nitrogen stream at 40°C. The residues were dissolved in 0.2 mL of mobile phase solution A and mixed on a vortex mixer for 20 seconds. The solutions were placed in autosampler vials and aliquots of 50 μL were injected onto the column.

Validation

Validation was performed according to guidelines of the food and drug administration (FDA).[7]

Specificity and Selectivity

Interference from endogenous compounds was investigated by analyzing blank plasma of six different individuals, who did not use FTC or any other drug. Possible interference by all antiretroviral drugs and regularly co-administered drugs in HIV patients was tested by analyzing samples that contained concentrations at therapeutic concentrations of each drug. No interference was allowed for any of the drugs.

Accuracy, Precision, Recovery, and Limits of Quantification

Five replicates of three different concentrations of QC samples were analyzed in three separate runs in order to determine the accuracy and precision. Samples for determining the lower and upper limit of quantification were assayed as five replicates in three separate runs. The percent of deviation from the nominal concentration and the relative standard deviation had to be less than 20%.

The efficiency of SPE (recovery) was determined at six different concentrations (5.0, 1.0, 0.30, 0.10, 0.03, and 0.01 mg/L). Average recovery of FTC was obtained by comparing responses with those obtained by direct injection of the same concentration of drug in mobile phase.

For the lower limit of quantification (LLQ) and higher limit of quantification (HLQ) the percent deviation from the nominal concentration and the relative standard deviation had to be less than 20%. Samples for determining the LLQ and HLQ were assayed as five replicates in three separate runs.

Stability

The stability during sample handling of FTC was verified, by testing the stability in plasma at room temperature, 4°C, and at -40°C during 6 days,

6 days, and 3 months, respectively, and subjecting samples to three freeze thaw cycles, all at different concentration levels in duplicate.

The stability of processed extracts in the autosampler was assessed after one week by reinjection of standards and three QC samples in duplicate. Stability of the standard solution in ultrapure water at −40°C was determined after 3 months, by reanalyzing aliquots of the stored stock solution in triplicate with aliquots of a fresh stock solution.

External Quality Control Samples

We participated in the 18th round of an international inter-laboratory proficiency testing program of the AIDS Clinical Trials Group.[8] Three plasma samples containing unknown amounts of FTC had to be analyzed.

RESULTS

Development Phase

A simple protein precipitation with perchloric acid, trichloric acid, methanol, or acetonitril resulted in chromatograms with many interfering peaks around the FTC peak. Since FTC is a polar component that tends to stay in the water phase, solid-phase instead of liquid-liquid extraction was chosen.

In search for a robust and simple manual solid-phase extraction, we chose a polymeric reversed phase sorbent. This sorbent is water wettable and, unlike silica based cartridges, reproducible results are obtained even when the cartridges run dry. Two cartridges were tested: Oasis HLB and Oasis Max, of which the latter gave the cleanest baseline and the best recovery (circa 100%). To improve flow during loading and to prevent blockages, plasma was diluted with the same amount of acetate buffer on top of the column.

FTC is extremely water soluble. Therefore, we chose a column (Atlantis), which is compatible with aqueous mobile phases that are necessary for retaining components like FTC.

Methanol and phosphate buffer were selected as mobile phase. The used gradient, combined with a column temperature of 35°C, provided the best separation of FTC and interfering plasma peaks. Different amounts of orthophosphoric acid that were added to the phosphate buffer were tested, because acetaminophen almost co-eluted with FTC. Two hundred μL of orthophosphoric acid added to 1 liter of phosphate buffer yielded the best separation between the two components.

Figure 1A, B, and C show chromatograms of extracted blank plasma, a standard plasma, and a HIV-patient plasma, respectively. Although a complete analysis took 21 min, only the first 10 min of the chromatograms are shown. The remaining time of the analysis is used for eluting plasma peaks and reequilibrating the column. The retention time for FTC was 9.6 min.

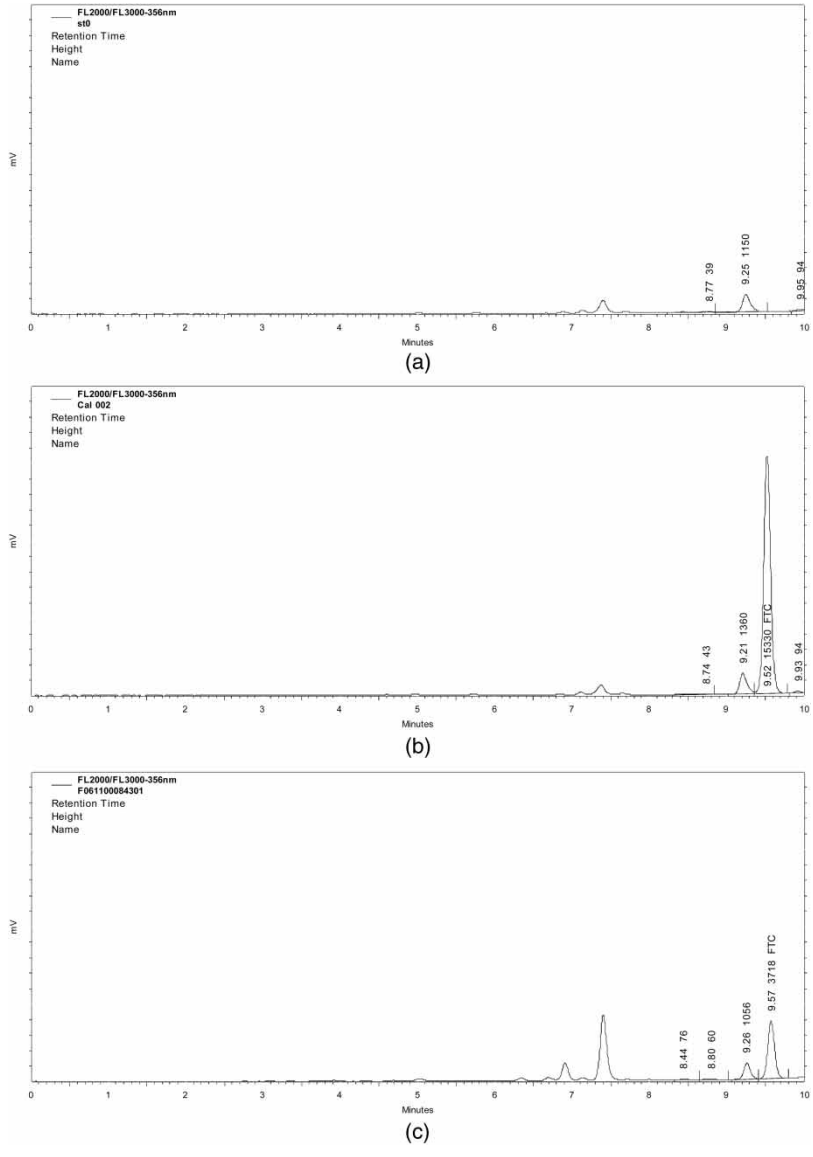

Figure 1. A: Blank human plasma. B: Human plasma containing 1 mg/L FTC. C: HIV-patient sample containing 0.27 mg/L FTC.

Specificity and Selectivity

The six blank plasma samples showed no peaks that coeluted with FTC. Potentially coadministered drugs tested (Table 2) had retention times that were different from FTC or were not detected with the described bioanalytical method.

Table 2. Comedicated drugs that did not interfere with the analytical method

Abacavir	Erythromycine	Nevirapine
Acenocoumarole	Ethambutol	Nitrazepam
Acetaminophen	Ethinylestradiol	Ofloxacine
Acetylsalicylic acid	Famotidine	Omeprazole
Acyclovir	Fenobarbital	Oxazepam
Amfotericine B	Fenytoine	Oxcarbazepine
Amitriptyline	Fluconazole	Paroxetine
Amoxicilline	Fluoxetine	Pentamidine
Amprenavir	Fluvoxamine	Pyramethamine
Atazanavir	Folic acid	Pyrazinamide
Atovaquone	Ganciclovir	Ranitidine
Azitromycine	Gemfibrozil	Rifabutin
Calcium folinate	Haloperidol	Rifampin
Carbamazepine	Hydrochlorothiazide	Ritonavir
Cetirizine	Hydrocortisone	Saquinavir
Claritromycine	Indinavir	Stavudine
Clindamycine	Isoniazide	Sulfamethoxazole
Clofazimine	Itraconazole	Sulfamethoxazole, N4 acetyl
Clomipramine	Ketoconazole	Sulfametrol
Caffeine	Lamivudine	Sulfametrol, N4 acetyl
Dapsone	Lidocaine	Temazepam
Dapsone, mono acetyl	Lopinavir	Tenofovir
Diclofenac	Methadone	Trimethoprim
Didanosine	Morphine	Valproic acid
Domperidone	Naproxen	Zalcitabine
Efavirenz	Nelfinavir	Zidovudine
Enalapril		

Accuracy, Precision, Recovery, LLQ and HLQ

The results of the determination of accuracy and precision of the assay are presented in Table 3. These results show that this method is accurate (average accuracy from 100 to 107%) and precise (inter-day coefficient of variation (C.V.) ranging from 0% to 3.7%, and intra-day C.V. from 1.8% to 5.3%).

The average recovery was 107% (S.D. 3.1%). The recovery was consistent across the concentration range.

The LLQ and HLQ were found to be 0.01 mg/L and 5 mg/L, respectively.

Stability

The results of the stability tests under various conditions are listed in Table 4. Under all conditions tested, FTC proved to be stable with a recovery of at least 96.4%.

Table 3. Accuracy and precision data

Concentration (mg/l)	Accuracy (%) (N = 15)	Intra-day precision (%) (N = 15)	Inter-day precision (%) (N = 15)
0.011	104	5.3	3.7
0.051	105	2.4	1.1
0.51	107	2.5	0
4.09	102	3.8	0
5.34	100	1.8	2.3

External Quality Control Samples

Participation in an inter-laboratory quality control program allowed us to evaluate the accuracy of our method. In this program, 20% limits around the nominal concentration of the drugs are considered to be appropriate thresholds for a satisfactory measurement. Three different samples containing 0.0675, 0.54, and 1.54 mg/L had to be analyzed. All 3 results obtained from the 18th round of this program deviated less than 2.4% from the nominal concentration.

DISCUSSION

This paper describes the development and extensive validation of a bioanalytical method for the determination of FTC in human plasma. When we started method development, only the assays of Rezk et al.,[4] Notari et al.,[5] and Gish et al.[6] were described.

In contrast with Rezk et al., we avoided the use of an ion-pair reagent for chromatographic separation, and used only methanol and phosphate buffer for the mobile phase. The slow equilibration of the column with ion-pair reagents can create problems if gradient elution is used. Retention times may be less

Table 4. Stability experiments

Concentration (mg/l)	Condition	Matrix	Time interval	Concentration found (mean % (SD))
0.05–4.33	20°C	Plasma	6 days	106.8 (3.8)
0.05–4.33	4°C	Plasma	6 days	101.4 (2.3)
0.05–4.33	Freeze at −40°C/thaw	Plasma	3 cycles	106.4 (6.3)
0.05–4.93	20°C	Extract	1 week	98.4 (3.1)
1.0	−40°C	Water	3 months	100 (1.8)
0.01–5.0	−40°C	Plasma	3 months	99.5 (1.4)

reproducible and baselines can be more erratic. Therefore, ion-pair HPLC in a gradient mode is usually not recommended.[9]

Validation of our assay included testing of all antiretroviral and other possible coadministered drugs in HIV patients for interference. Beside the currently described non-nucleoside reverse transcriptase inhibitors, the nucleoside reverse transcriptase inhibitors, and the protease inhibitors, we tested 64 comedications of which none showed any interference with the FTC peak. Rezk et al. only tested 7 possible comedications apart from the HIV-medication. Notari et al. only described testing all drugs employed in the treatment or prophylaxis of opportunistic infections without specifying which drugs were tested. Gish et al. did not describe having tested any possible coadministered drugs.

Contrary to the methods developed by Rezk et al. and Notari et al., who both used UV-detection, and Gish et al. who used MS-MS, we used fluorometric detection, which is more specific than UV-detection and less expensive and more available than MS.

The external standard method was used to quantify the FTC, with a calibration curve of six points. The correlation coefficients of the calibration curves were all higher than 0.99. Furthermore, the average recovery of FTC from plasma after SPE was 107% and was consistent across the concentration range of the calibration curve. Therefore, using an external standard method for this assay is allowed.

Recovery, specificity, accuracy, intra- and inter-assay precision, and lower limit of quantification have all been validated. The intra- and inter-day variability observed with this method was comparable with Rezk et al., but smaller than Notari et al., and Gish et al., especially for the low concentrations.

Finally, participation in an international inter-laboratory quality control program confirmed the accuracy of our method.

CONCLUSION

The HPLC-FM method reported here, for the quantitative determination of FTC in plasma, is accurate and precise. The solid phase extraction method provides good sample clean up and high recoveries. This method is suitable for use in clinical trials and therapeutic drug monitoring of HIV patients.

REFERENCES

1. EMEA. Emtriva; Summary of Product Characteristics. 2006.
2. Gallant, J.E.; DeJesus, E.; Arribas, J.R.; Pozniak, A.L.; Gazzard, B.; Campo, R.E.; Lu, B.; McColl, D.; Chuck, S.; Enejosa, J.; Toole, J.J.; Cheng, A.K.; Tenofovir, DF. Emtricitabine, and efavirenz vs. zidovudine, lamivudine, and efavirenz for HIV. N. Engl. J. Med. **2006**, *354* (3), 251–360.

3. Working Group of the Office of AIDS Research Advisory Council (OARAC), Guidelines for the Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents, version October 10, **2006**, 6 A.D.
4. Rezk, N.L.; Crutchley, R.D.; Kashuba, A.D. Simultaneous quantification of emtricitabine and tenofovir in human plasma using high performance liquid chromatography after solid phase extraction. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. **2005**, *822* (1–2), 201–208.
5. Notari, S.; Bocedi, A.; Ippolito, G.; Narciso, P.; Pucillo, L.P.; Tossini, G.; Donnorsdo, R.P.; Gasparrini, F.; Ascenzi, P. Simultaneous determination of 16 anti-HIV drugs in human plasma by high performance liquid chromatography. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. **2006**, *831* (1–2), 258–266.
6. Gish, R.G.; Leung, N.W.; Wright, T.L.; Trinh, H.; Lang, W.; Kessler, H.A.; Fang, L.; Wang, L.H.; Delehanty, J.; Rigney, E.; Mondou, E.; Snow, A.; Rousseau, F. Dose range study of pharmacokinetics, safety, and preliminary antiviral activity of emtricitabine in adults with hepatitis B virus infection. Antimicrob. Agents Chemother. **2002**, *46* (6), 1734–1740.
7. Anonymous. Guidance for Industry Bioanalytical Method Validation. 2001.
8. Holland, D.T.; Difrancesco, R.; Stone, J.; Hamzeh, F.; Connor, J.D.; Morse, G.D. Quality assurance program for clinical measurement of antiretrovirals: AIDS clinical trials group proficiency testing program for pediatric and adult pharmacology laboratories. Antimicrob. Agents Chemother. **2004**, *48* (3), 824–831.
9. Snyder, L.R.; Kirkland, J.J.; Glaajch, J.L. *Practical HPLC Method Development*, 2nd Ed.; Wiley: New York, 1997, Ch 7.

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