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On-line coupling of anion exchange and ion-pair chromatography for measurement of intracellular triphosphate metabolites of reverse transcriptase inhibitors

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ABSTRACT

We developed an automated on-line weak anion exchange (WAX) solid-phase extraction (SPE) method coupled with ion-pair (IP) chromatography–tandem mass spectrometry (MS/MS) detection for quantitatively measuring triphosphorylated metabolites of three reverse transcriptase inhibitors (RTI). The administered pro-drugs were Tenofovir disoproxil fumarate (TDF), Emtricitabine (FTC) and Lamivudine (3TC). Their intracellular metabolites Tenofovir-diphosphate (TFV-DP), Emtricitabine-triphosphate (FTC-TP), and Lamivudine-triphosphate (3TC-TP) were measured in peripheral blood mononuclear cells (PBMC). We coupled the WAX and IP chromatography systems using a combination of 6-port and 10-port switching valves, and we mixed the WAX elute with 1,5-dimethyl-hexyl-amine before IP chromatography separation. Multiple waste outlets allowed for eliminating potential matrix components interfering with MS/MS detection. Limits of detection were 9, 200 and 75 pg per sample for TFV-DP (448/176 *m*/*z*), FTC-TP (488/130 *m*/*z*) and 3TC-TP (468/119 *m*/*z*), respectively.

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1. Introduction

Tenofovir (TFV), Emtricitabine (FTC), and Lamivudine (3TC) are reverse transcriptase inhibitors (RTI) commonly used to treat patients infected with the human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2). These nucleoside/tide analogs act by blocking the reverse transcription process of the virus [1,2]. Their common structural feature is the lack of a 3′-hydroxyl group. After 5′-phosphorylation the active metabolites, Tenofovir-diphosphate (TFV-DP), Emtricitabine-triphosphate (FTC-TP) and Lamivudine-triphosphate (3TC-TP) (Fig. 1), are incorporated into the growing DNA chain where the lack of any 3′-hydroxyl group causes early termination of the newly formed DNA strand [1]. In order to be effective in blocking transcription, the 5′-triphosphates of FTC-TP and 3TC-TP and the 5′-diphosphate metabolite of TFV (TFV-DP) must reach a necessary concentration in susceptible target cells such as peripheral blood mononuclear cells (PBMC) [3,4].

The potent antiviral activity, favorable safety profiles, and long intracellular half-lives of FTC-TP, 3TC-TP and TFV-DP have spurred interest in evaluating their use as chemoprophylactic drugs for the prevention of HIV transmission, as either oral or topical pre-exposure prophylaxis (PrEP). Several studies in non-human primates have shown promise of daily and intermittent oral PrEP using TDF and FTC [5–7]. However, to fully understand the potential efficacy of daily or intermittent PrEP in preventing HIV transmission, a more detailed understanding of the relationship between drug pharmacokinetics and protection is required. The availability of robust, sensitive, and reproducible analytical methods to measure intracellular metabolite levels is therefore essential for the design of effective interventions with oral PrEP or topical microbicide gels.

The expected intracellular metabolite levels following the use of RTIs can be as low as 10–30 fmol/million cells [6,8]. Detecting nucleotide triphosphates at such low concentrations is an ana-

Abbreviations: WAX, weak anion exchange; IP, ion-pair; SPE, solid-phase extraction; HIV, human immunodeficiency virus; TFV, Tenofovir; FTC, Emtricitabine; 3TC, Lamivudine; PBMC, peripheral blood mononuclear cells; PrEP, pre-exposure prophylaxis; ESI, electrospray ionization; QC, quality control; HPLC, high-performance liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry.

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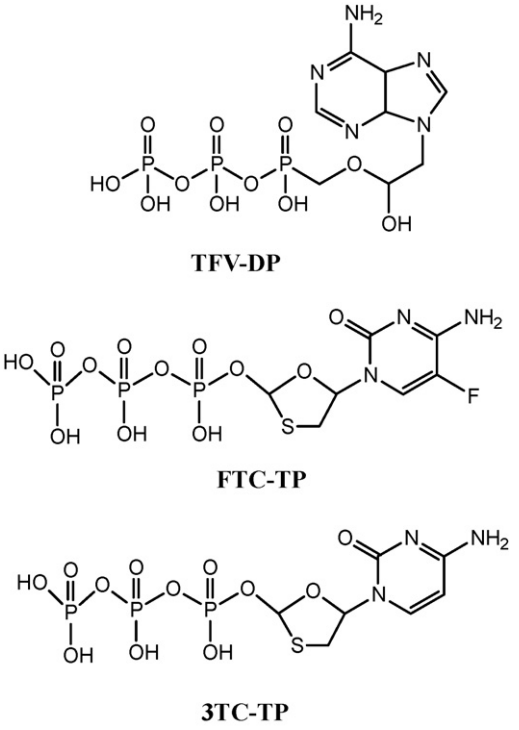

Fig. 1. Structure of Tenofovir-diphosphate (TFV-DP), Emtricitabine-triphosphate (FTC-TP), and Lamivudine-triphosphate (3TC-TP).

lytical challenge that can be circumvented by high-performance liquid chromatography (HPLC) and tandem mass spectrometry (MS/MS) with electrospray ionization (ESI). Most importantly, the effect of sample cleanup and the HPLC separation conditions on the sensitivity of the MS/MS detection must be carefully considered.

Generally, phosphate derivatives interact through strong secondary interactions with silica-based stationary phases which prevent their effective separation on regular reverse phase (RP) HPLC columns. One method of addressing this problem is enzymatic dephosphorylation, SPE, and analysis of the free nucleosides by RP HPLC; this multi-step approach can be very sensitive but it is labor-intensive [9,10]. Therefore, most published methods for measuring intracellular phosphate metabolites of RTIs use ion-pairing (IP) HPLC [8,11–16] or weak anion exchange (WAX) HPLC [17]. Recently the unique retention capability of porous graphite columns were used for separation of phosphate metabolites of fluorinated cytosine and uridine drug derivatives [18].

In the present work, we report an integrated method alternative which uses on-line HPLC column switching to eliminate the need for thorough sample cleanup. By combining 6-port and 10-port switching valves, we coupled WAX and IP chromatography together into one automated HPLC system. This approach took advantage of both the robustness and selectivity of WAX HPLC [17] and the MS/MS compatibility of IP HPLC [16]. The WAX column allows for concentration and separation from 50 to 200 μL sample injection volumes, therefore, evaporation to dryness or to small volume after cell lyses is not necessary. The concentration measurement of TFC-TP, 3TC-TP, and TFV-DP with the coupled WAX-IP HPLC system had three to four times better sensitivity than WAX HPLC alone and was less affected by interferences than IP HPLC alone. The MS/MS instrumentation allowed for simultaneous monitoring both in positive and negative ionization modes, with a method limit of detection (LoD) of 9 pg of TFV-DP, 200 pg of FTC-TP and 75 pg of 3TC-TP per sample.

2. Materials and methods

2.1. Standards and solutions

Methanol, acetonitrile, glacial acetic acid, formic acid, and ammonium hydroxide were all HPLC-grade. 1,5-Dimethyl-hexylamine (DMHA, 99%) was purchased from Aldrich (Milwaukee, WI). Triphosphate derivatives (Fig. 1) of 3TC, 1-(2*R*,5*S*)-[2-(hydroxymethyl)-1,3-oxathyolan-5-yl]cytosine (3TC-TP), and FTC,5-fluoro-1-(2*R*,5*S*)-[2-(hydroxymethyl)-1,3-oxathyolan-5-yl]cytosine (FTC-TP), and the diphosphate derivative of TFV, (*R*)-9-(2-phosphonylmethoxypropyl)adenine (TFV-DP) were donated by Gilead Sciences (Foster City, CA). Isotopically labeled TFV-DP-[adenine-$^{13}C_5$] ([$^{13}C_5$]-TFV-DP) was purchased from Moravek Biochemicals (Brea, CA).

We prepared native standard solutions by diluting ~50 μg/mL solutions to create a 500-ng/mL stock solution in 50% methanol/water. This intermediate stock solution was then serially diluted with 50% methanol/water such that a 20-μL spike contained 2–3000 pg of FTC-TP, 3TC-TP and TFV-DP calibration standards. We prepared the internal standards by weighing neat solid compounds, dissolving, and diluting in 50% methanol to provide working stock solutions at a concentration such that a 20-μL spike provided 2000 pg of the [$^{13}C_5$]-TFV-DP internal standard per sample.

2.2. Preparation of human PBMC

Buffy coat PBMC collections were purchased from Interstate Blood Bank Inc. (Memphis, TN). The PBMC cells were washed with 0.9% NaCl, gently centrifuged, and separated. This step was repeated three times. After the final wash cycle, we added 0.15 mL of ice-cold 80% methanol for each 1 million cell pellet. After lysis the methanol-cell suspension was divided into four fractions, three of which were spiked such that they contained 125, 1250, or 2500 pg of analytes in a 150-μL aliquot, to provide low-, medium-, and high-concentration quality controls (QCs), respectively. The fourth fraction was used as a blank QC. The spiked methanol-cell suspensions were dispensed into 2 mL cryovials and stored at −80 °C until use.

2.3. Preparation of macaque PBMCs

We administered a single dose of Emtricitabine (20 mg/kg of animal weight) and Tenofovir (22 mg/kg) by oral gavage to five Indian Rhesus macaques. These doses are comparable to the dosing currently used in humans [5]. The Internal Animal Care and Use Committee (IACUC) of the Centers for Disease Control and Prevention (CDC) approved all these procedures. Blood (Vacutainer CPT) was collected from each animal at baseline and after 2, 4, 5, and 24 h after dosing. The collected tubes were mixed gently by inverting the tube several times, and centrifuged at room temperature for 30 min at 1600 × *g* to separate the PBMCs. After washing with 0.9% NaCl and lysing the red blood cells with 5 mL of lysis buffer for 5 min (41.3 mg ammonium chloride, 0.005 mg potassium bicarbonate, 0.185 mg tetrasodium EDTA) cells were counted using a Guava Cell counter with CytoSoft Data Acquisition and Analysis Software version 6.0.2 (Millipore, Billerica, MA). One mL of ice-cold 80% methanol in water and 20 μL of internal standard (2000 pg per sample) were then added to each specimen (median = 5.7×10^6 PBMCs, range = $2.5–9.5 \times 10^6$). FTC-TP and TFV-DP were extracted from the lysed cells overnight at −80 °C followed by centrifugation at 18,000 × *g* for 15 min at 4 °C to remove cellular debris. Supernatants were then transferred to a new tube and stored at −80 °C until evaporation.

2.4. Preparing calibration standards and quality controls

With each batch of unknowns an additional set of tubes containing 1 mL of 80% methanol, 20 μL of internal standard, and 50 μL of 0.9% NaCl was made and used to prepare calibration standards and QCs. Calibration standards contained an additional 20 μL of the standard spike solution (without PBMC). QC samples were prepared by adding 150 μL of the spiked low-, medium-, and high-concentration QC cell suspensions (1–2 million cells).

2.5. Evaporation and reconstitution

The calibration standards, unknown cell extracts, and QCs were placed into a 36 °C vacuum centrifuge for 45 min. After the evaporation, approximately 50–100 μL remained. The content of each well was diluted with 50 μL of 20 mM ammonium acetate, pH 7, and transferred into a 300-μL conical bottom 96-well plate. The plate was centrifuged to settle the particles and heat sealed with aluminum foil.

2.6. Design of experiments (DoE)

To evaluate the main parameter effects that determined the performance of the WAX only, IP only and WAX-IP on-line coupled HPLC approaches, experimental runs were designed using the JMP 7.0.2 Statistical Discovery software (SAS Institute Inc., Cary, NC). The Fractional Factorial DoE-Screening Designs included one center point with one or two pairs of duplicates. Evaluation of main effects (Effect Screening) was performed based on deviation from mean response (Contrast) for each parameter, expressed in percentage of the best response. The WAX and IP HPLC methods were evaluated separately varying three parameters with two duplicates (15 experiments total), and the combined WAX-IP on-line HPLC method was tested varying four parameters with one duplicate (18 experiments total). Each 100 μL injection contained 6 ng [$^{13}C_5$]-TFV-DP internal standard, 6 ng FTC-TP, 2 ng TFV-DP and 1.5 ng 3TC-TP. We tested the effect of the cellular extract (present or not present) and eluent parameters on the internal standard peak area and the (analyte peak area)/([$^{13}C_5$]-TFV-DP peak area) ratios (shown in percent of the maximum or target values, respectively) (Table 1).

2.7. Column-switching system

The chromatography system (Fig. 2) contained an autosampler (Agilent 1200, Wilmington, DE), a 6-port valve (FCV-20A, Shimadzu, Columbia, MD), a 10-port valve (Valco Instruments, Canada), a divert valve, and six single-solvent-line pumps (Shimadzu, Columbia, MD), labeled Pumps A–F in Fig. 2. The Analyst MS workstation controlled all components except for Pumps E and F, which were run independently.

The following eluents were used (Fig. 2): Pump A: 20 mM ammonium acetate in water, pH 7; Pump B: 10 mM ammonium dihydrogen phosphate in 50% methanol/water, pH 7; Pump C: 20 mM DMHA and 6 mM ammonium formate in 20% acetonitrile/water, pH 10; Pump D: mix of 25 mM DMHA and 6 mM ammonium formate, pH 10 in 50% acetonitrile; Pump E: mix of 0.1% ammonium hydroxide and 2 mM ammonium formate in water; and Pump F: 0.1% ammonium hydroxide in 50% acetonitrile.

Three columns were built into the column-switching system (Fig. 2): WAX-trap: 10 mm × 2.1 mm, 3-μm particle size Biomax AX (Thermo, USA); IP-trap: two 10 mm × 2.1 mm, 3-μm particle size Persuit C18 pre-columns in tandem; and analytical column: 50 mm × 2.1 mm, 3-μm particle size Persuit C18 (Varian, Palo Alto, CA).

Each sample run could be divided into four periods (Figs. 1 and 2): Period A: A 50–200-μL cell extract was injected into a 0.3-mL flow of Pump A, and the analytes were retained on the WAX-trap while the matrix breakthrough was led to Waste Port I; Period B: At 4 min, the 6-port valve was in its alternate position and the WAX-trap was eluted by 0.1-mL/min flow of Pump E; from 4 to 6 min the eluent leaving the WAX-trap was directed to Waste Port II; Period C: At 6 min, with the turn of the 10-port valve, the WAX elute started to mix with a 0.2-mL/min flow of Pump C, and the IP-trap retained the analytes while the breakthrough went to Waste Port II; Period D: At 9 min, we stopped the T-mixing by turning the 10-port valve back to its original position, and from 9 to 18 min, the ion-pair band was transferred to the analytical column by a 0.1-mL 0–100% Eluent D/C gradient. The divert valve sent Pump C/D flow to the MS instrument only from 12 to 18 min, which is when the analytes of interest eluted. Any other time during the run, the MS interface was washed with Pump F flow.

During Periods C and D, the injector lines and the WAX-trap were regenerated with 40% B/A from 5 to 13 min, and equilibrated back to 0% B from 13 to 18 min. The total sample run time was 18 min.

2.8. Mass spectrometry

The mass spectrometer was a 4000 QTrap (Applied Biosystems, Foster City, CA) operated in alternate negative and positive ESI

Table 1
Comparison of the different high-performance liquid chromatography–tandem mass spectrometry approaches on the bases of parameter effect contrasts expressed in percent of maximum or target values.

Method[a]	Parameter, range	[$^{13}C_5$]-TFV-DP peak area		Analyte/[$^{13}C_5$]-TFV-DP peak area ratio, contrast%[b]		
		Max.[a]	Contrast%[c]	FTC-TP[d]	TFV-DP[d]	3TC-TP[d]
WAX only method	PBMC extract in sample, present or not present	1.14E5	−17	58	5	270
	Acetate in WAX loading buffer (Eluent A), 10–30 mM		12	−23	1	99
	Formate in WAX elution buffer (Eluent E), 1–10 mM		−16	3	1	175
IP only method	PBMC extract in sample, present or not present	2.98E5	−28	13	2	−32
	Formate in IP loading buffer (Eluent C), 1–10 mM		−8	−7	1	23
	DMHA in IP elution buffer (Eluent D), 15–25 nM		−2	−3	<1	17
WAX-IP on-line method	PBMC extract in sample, present or not present	1.44E6	−4	−9	<1	5
	Formate in IP loading buffer (Eluent C), 6–18 mM		5	−2	<1	2
	10–35 acetonitrile% gradient time 3–9 min		10	3	<1	2
	WAX elute/IP loading buffer flow rate ratio 1:1 to 1:5		2	−2	<1	3

[a] Maximum peak area for [$^{13}C_5$]-TFV-DP internal standard with 453/181 *m/z*.
[b] Contrast in % of the target area ratio FTC-TP: 1.09, TFV-DP: 0.33 and 3TC-TP: 0.18.
[c] Contrast in % of maximum 453/181 *m/z* peak area response for the experiment.
[d] Positive ion MRM transition in table: FTC-TP: 488/130, TFV-DP: 448/176 *m/z*, and 3TC-TP: 470/119 *m/z*.

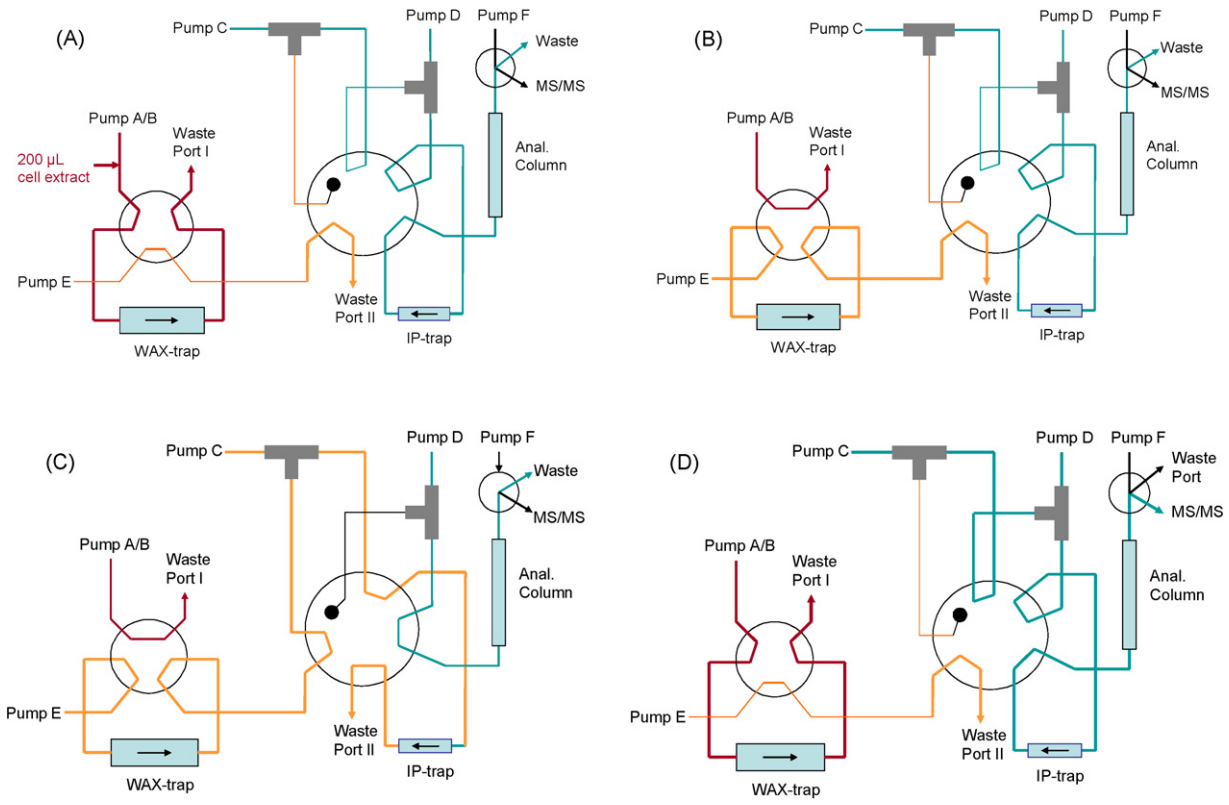

Fig. 2. Schematic diagram of weak anion exchange (WAX) and ion-pair (IP) chromatography column coupling at different time intervals: (A) 0–4 min; (B) 4–6 min; (C) 6–9 min; (D) 9–18 min.

modes. The declustering potential and collision energies were optimized for each molecular ion/fragment pair chosen for multiple reaction monitoring (MRM). The ions monitored for negative MRM (m/z) were FTC-TP: 486/159, 3TC-TP: 468/159, TFV-DP: 446/177 and 446/159, [$^{13}C_5$]-TFV-DP: 451/177 and 451/159. The ions monitored for positive MRM (m/z) were FTC-TP: 488/130, 3TC-TP: 470/119, TFV-DP: 448/176 and 448/270, [$^{13}C_5$]-TFV-DP: 453/181 and 453/275. We used 50 ms dwell times resulting in a 2.2-s cycle time.

2.9. Methods of validation

Concentrations were calculated for each positive and negative MRM transitions then an average concentration was calculated for each analyte. To assess the accuracy and precision of the final method, we measured quintuplicate spiked blank human PBMC extracts. The LoD and lower limit of quantitation (LLoQ) were calculated as 3 times and 10 times the standard deviation at 0 ng/mL concentration, from the intercept of a concentration versus standard deviation plot [19]. This definition of LoD is preferable when there is no identifiable peak in the blank response chromatogram and usually more conservative than LLoQs determined by using the FDA recommended definition of 5 times the response comparing the blank response [20]. The calibration standard accuracies were calculated from the average measured concentration divided by the expected concentration. Method precision was characterized by intra- and inter-assay coefficient of variation (CV) calculated from 36 replicate measurements of quality controls in 6 independent runs, i.e. intra-assay CVs were calculated from 6 replicate extractions using same calibration curve, and inter-assay CVs were calculated from 6 independent intra-assay mean values on 6 different days using 6 independent daily calibration curves.

3. Results and discussion

3.1. Comparing negative and positive ESI modes

An important consideration in the HPLC–MS coupling is the selection between negative or positive ESI modes. Since nucleotide phosphates are negatively charged in aqueous solution the obvious choice is negative ESI detection mode [21,22]. Generally, conditions that favor negative or positive ion formation in solution also favor negative ionization in the LC–MS interface [23]. However, RTI phosphate metabolites were found to ionize well in positive ionization mode. In fact, the higher the ammonium buffer pH the more effective was the positive ionization [16,24,25]. This apparent contradiction is not surprising in view of the gas-phase basicity (proton affinity) of purine and pyrimidine derivatives versus ammonia and alkyl amines [26–30]. MS ionization is determined not by the ionization state in solution but by the delicate balance of the relative gas-phase basicity and surface activity of the analytes and all other electrolytes present during elution. Ammonia has a higher pK_a in aqueous solution but less basicity (196 kcal/mol) than purine (215–217 kcal/mol) or pyrimidine derivatives (201–206 kcal/mol) in gas phase, allowing for proton transfer from ammonium ions to purine and pyrimidine bases [26,27]. Molecular modeling studies suggests that the proton affinity of nucleotide bases can be enhanced by intra-molecular H-bonding between the base and the 5′-phosphate group [30].

The applicability of alkyl amine IP reagents for MS detection is also determined by their gas-phase basicity. Primary amines, such as 1,5-DMHA, have less gas-phase basicity (210–212 kcal/mol), than secondary and tertiary amines (221–223 kcal/mol) [26,27], such as *N*,*N*-DMHA. Therefore, 1,5-DMHA is able to transfer a proton to purine and pyrimidine bases in gas phase while *N*,*N*-DMHA is not. These relative gas-phase basicities explain our observations

that during WAX HPLC elution with 0.1% NH_4OH eluent, FTC-TP, TFV-DP and 3TC-TP ionized with similar efficiency in negative and positive ESI modes. Relative to WAX HPLC elution conditions, during IP HPLC elution even with relatively low (<1 mM) concentration of *N*,*N*-DMHA (2 mM ammonium phosphate buffer, pH 7) negative ionization was enhanced 4–5 times while positive ionization was suppressed 2–3 times. On the contrary, during IP HPLC elution even with relatively high (20 mM) 1,5-DMHA (6 mM ammonium formate buffer, pH 10) both negative and positive ESI signals were enhanced about three times compared to signals observed with WAX HPLC conditions.

3.2. Comparing WAX and IP chromatography

Both WAX and IP HPLC separations occur through ionic interaction between alkyl amine bases and the triphosphorylated analytes. In the case of IP HPLC, the alkyl amine base is present as a mobile phase additive, while in WAX HPLC it is immobilized on the stationary phase. In both HPLC approaches, the chromatographic retention and adequate separation from the endogenous cellular components require careful adjustment of the activity of the cationic alkyl amine base and the buffer anion.

We tested the effect of the cellular extract (present or not present) and eluent parameters on the internal standard peak area and the (analyte peak area)/([$^{13}C_5$]-TFV-DP peak area) ratios (shown in percent of the maximum or target values, respectively) (Table 1).

Increasing acetate concentration had a positive effect on the WAX HPLC–MS/MS peak intensities (12%). The presence of the cellular extract had a stronger negative effect on the IP HPLC–MS/MS (−28%) than on the WAX HPLC–MS/MS (−17%) performance. Interestingly, increasing the formate concentration negatively affected both WAX and IP HPLC–MS/MS signal intensities (−16% and −8%). 5–10 mM formate also lead to the broadening of the WAX HPLC peak and 12–18 mM formate caused splitting of a IP HPLC peak. This indicates that formate anions at above optimal concentration may cause the desalting of the analytes leading to increased partitioning on the RP stationary phases.

The much higher deviation of the area ratios from target values for WAX HPLC (FTC-TP: 58% and 3TC-TP: 270%) relative to IP HPLC (FTC-TP: 13% and 3TC-TP: 32%) shows that the FTC-TP and the 3TC-TP MRM transitions contain significant interferences. In other words, the WAX HPLC–MS/MS method is more robust but less selective then the IP HPLC–MS/MS method.

3.3. Optimizing the WAX-IP coupled HPLC system

Using the HPLC solvents that led to the best responses in the above screening test, four experiments were designed to determine the optimum programming for the automated column-switching system (Fig. 2).

In the first experiment, we determined the timing of the 6-port valve. We connected the MS/MS detector to Waste Port I on the 6-port valve (Fig. 2) and established that the WAX-trap retained the analytes for at least 4 min with 0.3-mL/min 30 mM ammonium acetate flow.

In the second experiment, we determined the timing of the 10-port valve. We connected the MS/MS detector to the line between the 6-port valve and the mixing-T (Fig. 2). From the WAX chromatogram (Supplementary Information), we determined that with 0.1-mL flow of 0.1% ammonium hydroxide and 1 mM ammonium formate, the analytes eluted from the WAX-trap between 6 and 9 min, thus defining when the 10-port valve had to be in the alternate position. As a precaution, this experiment was repeated before every batch run. Typically, the WAX chromatography peaks remained in the same time window up to 100 injections. Timing

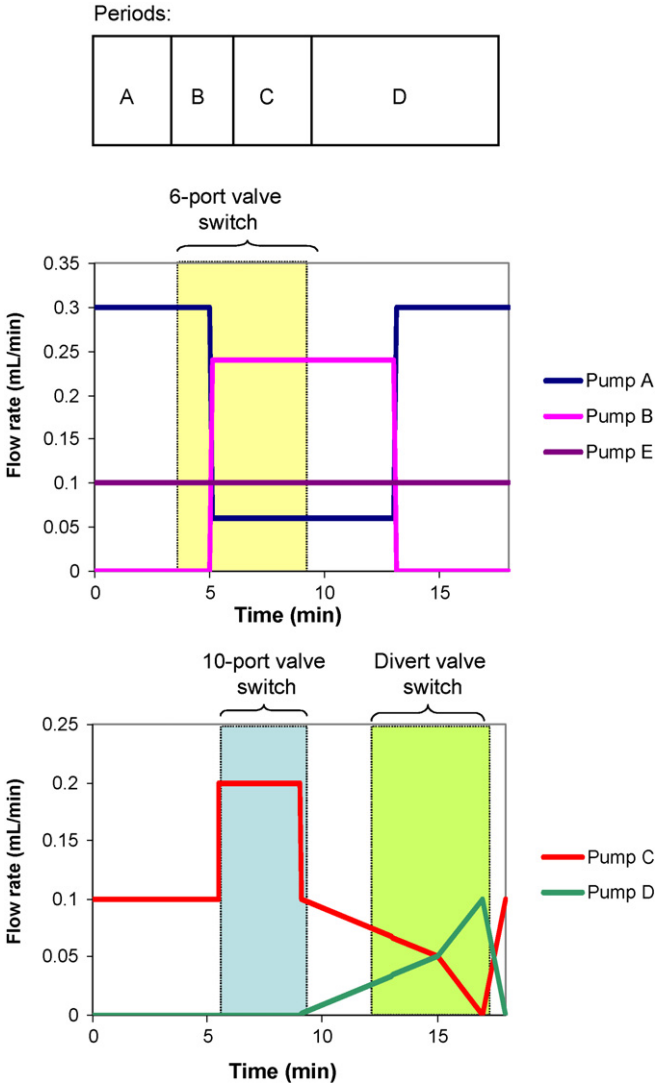

Fig. 3. Graphical representation of pump flow rates and valve positions during the time course of the column-switching chromatography run. Periods A, B, C, and D correspond with Fig. 2.

adjustment was not necessary as long as the run started with a new WAX-trap, fresh ammonium formate buffer, and the purging of Pump E.

In the third experiment, we determined the (WAX elute)/(IP reagent) mixing ratio (Eluent E/C). The MS/MS detector was connected to the line between the 10-port valve and the analytical column. After a few experiments, we determined that the 0.1-mL/min flow of the WAX elute (Eluent E) had to be mixed with at least 0.2 mL/min flow of 20 mM 1,5-DMHA and 6 mM ammonium formate in 20% acetonitrile (Eluent C), achieving retention on the IP-trap for at least 4 min. Because the flow went through only the IP-trap, the liquid pressure stayed under 1000 psi in spite of the increased flow rate (0.3 mL). After the mixing period, the analytical separation continued with 0.1 mL/min flow of the acetonitrile gradient from Pumps C and D.

In the fourth experiment, we determined the timing of the divert valve. With the analytical column attached without the divert valve, the analytes eluted from the column around 15 min. Therefore, the IP HPLC elute was sent to the MS interface only for this period to minimize the possible accumulation of the 1,5-DMHA IP reagent in the LC–MS interface. The final optimized valve switching and solvent gradient program is shown in Fig. 3.

3.4. Minimizing carryover

Carryover often occurs when phosphorylated analytes are analyzed, especially if the application requires a wide linear calibration range. Cross-contamination can result from binding to the stainless steel parts of the injector lines or from the chromatography columns because of the tendency for strong secondary interaction with silica-based stationary phases. Under these conditions, part of the sample can remain near the front end of the column and elute only with the next injection. To minimize carryover, we took preventative measures to address these problems. The outside of the stainless steel injector needle was washed with 1% ammonium hydroxide in 50% methanol. We regenerated the stainless steel injector lines and the WAX-trap using 10 mM ammonium phosphate in 50% methanol (Eluent B). In spite of these measures, we still found ~1% cross-contamination from injection to injection.

3.5. Removing interferences

Our column-switching system allowed for eliminating matrix components at three different waste ports (Fig. 2). The WAX breakthrough and wash were discarded through Waste Port I (Period 1). Other matrix components which followed the elution of the analyte bands on the WAX-trap were eliminated through Waste Port II (Period 2). The matrix components eluting from the IP-trap before the analytes were also removed through Waste Port II (Period 3) and the divert valve, the IP HPLC flow was injected into the MS interface for only the duration of the analyte bands (Period 4). Because of all these automated cleaning steps, the MS interface required minimal maintenance. Because the WAX-trap and the IP-trap were relatively inexpensive pre-columns, they were replaced after injecting 100 cell extracts. Between batches, we regenerated the analytical column by washing with 0.1% formic acid in 50% acetonitrile (Eluent F).

3.6. Evaluating WAX-IP HPLC–MS/MS method performance

After achieving stable method performance with the column-switching system, we performed a ruggedness test by varying four parameters of the WAX-IP HPLC system: presence or absence of the PBMC extract, 6–18 mM formate concentration in IP loading buffer (Eluent C), acetonitrile gradient time (from 10% to 35% increase in 3–9 min) and the (WAX elute)/(IP loading buffer) flow ratio. Comparison of the magnitude of the parameter effects with those for the WAX HPLC only and the IP HPLC only experiments in acetonitrile gradient time had only a 10% effect on peak intensities and minimal effect on area ratios. Therefore, the on-line coupling of the WAX and IP HPLC methods gave a more robust, more sensitive, and more selective HPLC–MS/MS performance.

3.7. Method validation

Generally, at low pg levels, method LoDs and LLoQs are strongly affected by the reproducibility of the LC–MS/MS method, baseline noise levels, and the relative peak size of co-eluting matrix components. Despite of the multi-step sample preparation process, which included cell lyses, partial evaporation, reconstitution, and multiple sample transfers, the internal standard signal intensity remained within ±10% throughout each 50–100 sample runs including standards, QCs, and unknowns, indicating good internal consistency for the entire sample preparation and quantification process (Supplementary Information). We determined calibration accuracies based on the calculated and expected concentrations for spiked methanol-cell suspensions at the LLoQ, 200, 1000, and 3000 pg per sample quantified with calibration standards that were prepared without cell extracts present. These calibration accuracies measured in spiked suspensions were in the 92–112% range (Table 2), indicating no significant calibration bias above the LLoQ.

Table 2
Limit of detection (LoD), lower limit of quantification (LLoQ) and accuracy% (±S.D.) of measurement of LLoQ amount, 200, 1000, and 3000 pg analytes spiked into human peripheral blood mononuclear cell extracts.

Analyte	LoD (pg)	LLoQ (pg)	Accuracy% at pg/sample			
			LLoQ	200	1000	3000
FTC-TP	200	660	109 ± 12	103 ± 32	93 ± 11	99 ± 12
3TC-TP	75	250	87 ± 22	92 ± 18	94 ± 10	98 ± 8
TFV-DP	9	32	112 ± 10	95 ± 8	95 ± 15	100 ± 2

Table 3
Intra-assay (and inter-assay) coefficient of variation (CV%) calculated for quality control materials from 36 individual measurements acquired in 6 independent runs.

pg spike in quality control methanol-cell suspensions	FTC-TP (%)	3TC-TP (%)	TFV-DP (%)
125	–	20 (9)	15 (8)
1250	17 (11)	13 (8)	12 (6)
2500	17 (14)	14 (11)	10 (5)

We determined intra- and inter-day method precisions using 36 separate preparations of QCs (1–2 million human PBMC each) fortified with 125, 1250, and 2500 pg/sample (Table 3) analyzed in 6 independent runs (6 QCs per each run) over a period of 3 months and stored at −80 °C. The intra-day (and inter-day) coefficient of variation (CV%) was in the 10–17% (5–14%) range at concentrations of 1250 and 2500 pg/sample and was in the 15–20% (and 8–9%) range at the 125 pg/sample concentration level (Table 3).

LoDs in Table 2 were obtained using human PBMC samples spiked with low concentration standards and calculated using calibration standards containing reagent blanks only; in the positive ion mode these LoDs were 75, 200, and 9 pg/sample for 3TC-TP (468/119 *m*/*z*), FTC-TP (488/130 *m*/*z*) and TFV-DP (448/176 *m*/*z*), respectively. Fig. 4 compares chromatograms of sample extracts from typical dosed and control macaques and from calibration standards near the lower limit of quantification concentration. The higher LoD for FTC-TP reflected the presence of interferences in the human PBMC extract that gave the same MRM peak at the same retention time as FTC-TP. Similar low pg amount of interferences were also found in some PBMC extract from non dosed control macaques but not all. Therefore, for the measurements of macaque samples we used calibration standards spiked into reagent blanks that contained the same additives and went through the same treatment as all other unknowns and QCs.

Because the number of viable cells collected from each macaque varied, for practical reasons we defined the LoD in pg/sample. Only measured amounts per sample above the LoD were reported in pg/million cells. Typically using 5 million cells, our LLoQ was 50, 130, and 6 pg/million cells (106, 267 and 13 fmol/million cells), for 3TC-TP, FTC-TP and TFV-DP, respectively.

3.8. Analysis of macaque samples

We evaluated the kinetics of intracellular FTC-TP and TFV-DP in macaque PBMCs following administration of human bioequivalent doses of Emtricitabine or Tenofovir orally. Fig. 5 shows the mean FTC-TP and TFV-DP levels measured during 22 h. Mean FTC-TP levels at the 2 h mark were 529 pg/million cells (1087 fmol/million cells) and were comparable to the mean levels observed at 2 h in humans receiving a therapeutic (200 mg qd) dose of FTC (487 pg or 1000 fmol/million cells) [31]. FTC-TP levels at 22 h (376 pg or 773 fmol/million cells) were also comparable to those observed in humans [31] suggestive of a similar intracellular drug half-life of FTC-TP in human and macaque PBMCs. The kinetics of TFV-DP

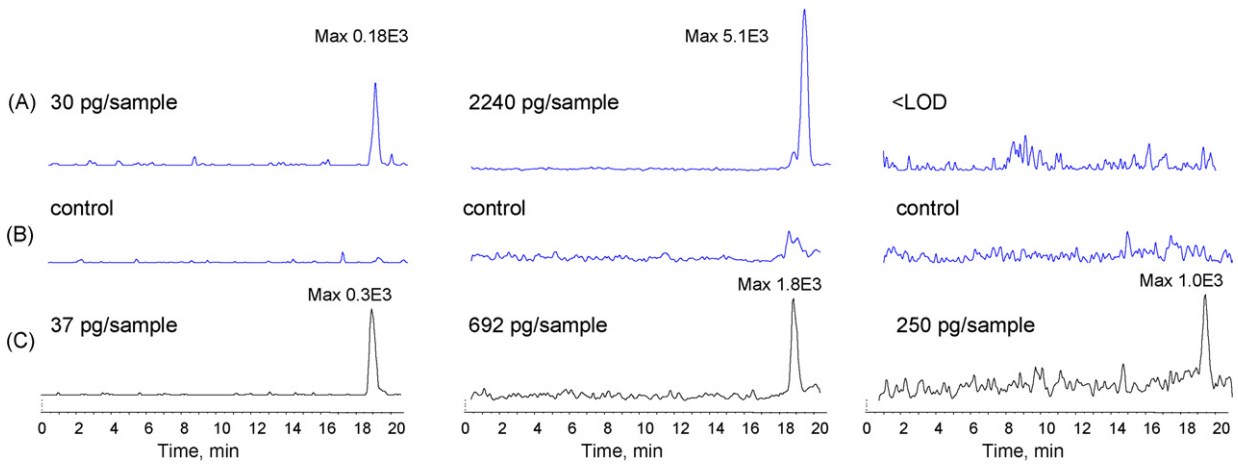

Fig. 4. Comparison of chromatograms for TFV-DP (left), FTC-TP (middle) and 3TC-TP (right). (A) Extract from a typical dosed macaque, (B) extract from control macaque and (C) calibration standard used near the lower limit of quantification concentration.

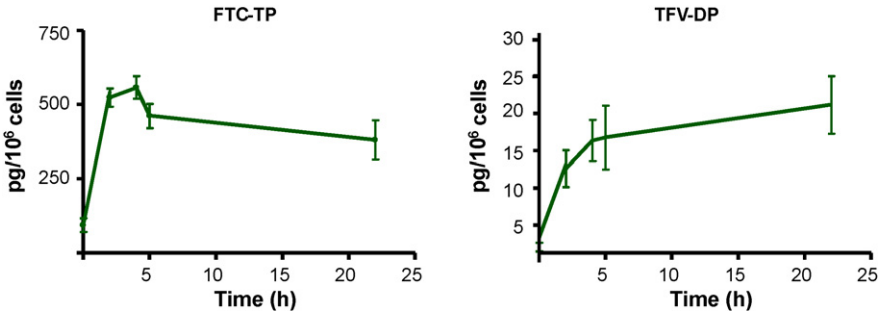

Fig. 5. Intracellular FTC-TP and TFV-DP levels measured in Rhesus PBMCs following administration of a single dose of Emtricitabine (20 mg/kg) or Tenofovir (22 mg/kg) orally. Data reflected the mean ± S.D. levels observed in longitudinal specimens collected from five macaques.

were different than those observed for FTC-TP. Levels of TFV-DP increased over time and reached concentrations at 22 h (20.3 pg or 45.4 fmol/million cells) within the range observed at 24 h in humans (18–44.7 pg or 40–100 fmol/million cells) [6] (Fig. 5). These kinetics also suggest that in Rhesus PBMCs, the intracellular half-life of TDF-DP is longer than that of FTC-TP, similar to what has been observed in humans.

4. Conclusions

We have implemented a unique 10-port valve design combined with mixing-Ts. This combination allows coupling a WAX extraction with gradient IP HPLC analytical separation. The 10-port valve connects the two solvent systems only for the duration of the analyte elution from the WAX column. Before and after analyte transfer from the WAX to IP HPLC systems, the two chromatography systems run separately, allowing the regeneration of the WAX extraction system, while the IP HPLC analytical system can be run with mobile phase conditions optimal for MS/MS detection. The multiple waste outlets in the design allow for eliminating potential matrix interferences, providing robust sensitivity and reproducibility.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2009.09.007.

References

[1] E. De Clercq, J. Clin. Virol. 30 (2004) 115.
[2] M.J. Otto, Curr. Opin. Pharmacol. 4 (2004) 431.
[3] R.H. Florese, K.K.A. Van Rompay, K. Aldrich, D.N. Forthal, G. Landucci, M. Mahalanabis, N. Haigwood, D. Venzon, V.S. Kalyanaraman, M.L. Marthas, M. Robert-Guroff, J. Immun. 177 (2006) 4028.
[4] M. Gotte, Expert Rev. Anti. Infect. Ther. 2 (2004) 707.
[5] J.G. Garcia-Lerma, R.A. Otten, S.H. Qari, E. Jackson, M.E. Cong, S. Masciotra, W. Luo, C. Kim, D.R. Adams, M. Monsour, J. Lipscomb, J.A. Johnson, D. Delinsky, R.F. Schinazi, R. Janssen, T.M. Folks, W. Heneine, PLoS Med. 5 (2008) 291.
[6] T. Hawkins, W. Veikley, R.L. St Claire, B. Guyer, N. Clark, B.P. Kearney, J. Acquir. Immune Defic. Syndr. 39 (2005) 406.
[7] S. Subbarao, R.A. Otten, A. Ramos, C. Kim, E. Jackson, M. Monsour, D.R. Adams, S. Bashirian, J. Johnson, V. Soriano, A. Rendon, M.G. Hudgens, S. Butera, R. Janssen, L. Paxton, A.E. Greenberg, T.M. Folks, J. Infect. Dis. 194 (2006) 904.
[8] A. Pruvost, E. Negredo, H. Benech, F. Theodoro, J. Puig, E. Grau, E. Garcia, J. Molto, J. Grassi, B. Clotet, Antimicrob. Agents Chemother. 49 (2005) 1907.
[9] T. King, L. Bushman, J. Kiser, P.L. Anderson, M. Ray, T. Delahunty, C. Fletcher, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 843 (2006) 147.
[10] T. King, L. Bushman, P.L. Anderson, T. Delahunty, M. Ray, C. Fletcher, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 831 (2006) 248.
[11] S. Compain, D. Schlemmer, M. Levi, A. Pruvost, C. Goujard, J. Grassi, H. Benech, J. Mass Spectrom. 40 (2005) 9.
[12] F. Becher, R. Landman, S. Mboup, C.N.T. Kane, A. Canestri, F. Liegeois, M. Vray, M.H. Prevot, G. Leleu, H. Benech, AIDS 18 (2004) 181.
[13] R.L. St Claire, Rapid Commun. Mass Spectrom. 14 (2000) 1625.
[14] F. Becher, A. Pruvost, C. Goujard, C. Guerreiro, J.F. Delfraissy, J. Grassi, H. Benech, Rapid Commun. Mass Spectrom. 16 (2002) 555.
[15] D.V. McCalley, J. Chromatogr. A 1075 (2005) 57.
[16] A. Pruvost, F. Theodoro, L. Agrofoglio, E. Negred, H. Benech, J. Mass Spectrom. 43 (2008) 224.
[17] S.A. Veltkamp, M.J.X. Hillebrand, H. Rosing, R.S. Jansen, E.R. Wickremsinhe, E.J. Perkins, J.H.M. Schellens, J.H. Beijnen, J. Mass Spectrom. 41 (2006) 1633.
[18] R.S. Jansen, H. Rosing, J.H.M. Schellens, J.H. Beijnen, J. Chromatogr. A 1216 (2009) 3168.
[19] J.K. Taylor, Quality Assurance of Chemical Measurements, Lewis Publishers, Chelsea, MI, 1987.
[20] Food and Drug Administration, Bioanalytical Method Validation, 2001.
[21] G. Hennere, F. Becher, A. Pruvost, C. Goujard, J. Grassi, H. Benech, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 789 (2003) 273.
[22] A. Pruvost, F. Becher, P. Bardouille, C. Guerrero, C. Creminon, J.F. Delfraissy, C. Goujard, J. Grassi, H. Benech, Rapid Commun. Mass Spectrom. 15 (2001) 1401.

[23] R.F. Straub, R.D. Voyksner, J. Am. Soc. Mass Spectrom. 4 (1993) 578.
[24] R.L. Claire, Rapid Commun. Mass Spectrom. 14 (2000) 1625.
[25] G. Shi, J.T. Wu, Y. Li, R. Geleziunas, K. Gallagher, T. Emm, T. Olah, S. Unger, Rapid Commun. Mass Spectrom. 16 (2002) 1092.
[26] T.Y. Yen, M.J. Charles, R.D. Voyksner, J. Am. Soc. Mass Spectrom. 7 (1996) 1106.
[27] M.G. Ikonomou, A.T. Blades, P. Kebarle, Anal. Chem. 62 (1990) 957.
[28] M.M. Bursey, C.E. Parker, R.W. Smith, S.J. Gaskell, Anal. Chem. 57 (1985) 2597.
[29] A. Bagag, A. Giuliani, O. Laprevote, Int. J. Mass Spectrom. 264 (2007) 1.
[30] K.B. Green-Church, P.A. Limbach, J. Am. Soc. Mass Spectrom. 11 (2000) 24.
[31] L.H. Wang, J. Begley, R.L. St Claire, J. Harris, C. Wakeford, F.S. Rousseau, AIDS Res. Hum. Retroviruses 20 (2004) 1173.