Analytical methods to determine the degree of drug association for complex injectable drug-combination particles containing multiple drug substances

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A thesis

submitted in partial fulfillment of the

requirements for the degree of

Master of Science

University of Washington

2024

Committee:

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Program Authorized to Offer Degree:

Pharmaceutics

University of Washington

Abstract

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The aim of this research is to develop and characterize complex injectable products composed of multiple drug substances targeted to human immunodeficiency virus (HIV). Some of these product candidates are composed of current HIV drug substances that exhibit desperate water solubilities. For example, lopinavir (LPV), ritonavir (RTV) and dolutegravir (DTG) are water insoluble while tenofovir (TFV) and lamivudine (3TC) are water soluble. Water insoluble LPV/RTV and water soluble TFV (or TDF) prodrugs are given orally to provide sustained viral suppression as a one-pill-a-day treatment in people living with HIV (PLWH). Recently DTG, 3TC and TFV, referred to as TLD one-pill-a day, has been widely used in the low-to-middle income countries with the goal to bring down HIV infections worldwide. Unfortunately, PLWH express pill fatigue and missing a daily

dose may lead to virus rebound and disease progression. In addition, uncontrolled virus levels in PLWH may relate to a continued increase in the number of people with HIV. This laboratory is developing a complex injectable LPV/RTV/TFV in a drug-combination nanoparticle (DcNP) product candidate, referred to as targeted long-acting combination antiretroviral therapy 101 (TLC-ART 101) that is currently under clinical testing. A next generation product, TLC-ART 301 composed of DTG, 3TC and TFV (or TLD) is currently under preclinical development.

As DcNP enabled TLC-ART 101 and TLC-ART 301 products containing 3 drug substances assembled to associate with DcNP particles, a key performance characteristic is the degree of drug substance association to the DcNP product in the suspension. The initial approach is based on dialyzing the unbound 3 drug substances under sink-condition, to enable diffusion of free drug through a semi-permeable membrane across a large volume of solvent. This degree or percentage of DcNP association for each drug is based on the fraction of drug retained within the dialysis chamber and to account for dialysate (due to large volume and analytical assay sensitivity). With the development of a combination of increasing analytical assay and volume reduction dialysate (thus increasing drug concentrations amenable for drug analysis), this thesis research was able to determine the percentage of drug association to DcNP based on a mass-balance approach. We found that comparing the association efficiency (AE) analysis for the 3 drug substances based with retentate only, or with a mass-balance approach was well-correlated.

In addition, under the sink conditions, only one of the two lipid excipients, sodium; [(2R)-2,3-di(octadecanoyloxy)propyl] 2- (2-methoxyethoxycarbonylamino)ethyl phosphate (mPEG-$_{2000}$ DSPE), but not 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) are found in dialysate; suggesting that some of the water soluble drug TFV may have remained bound to mPEG-$_{2000}$ DSPE and TFV-mPEG$_{2000}$-DSPE or that micelles may have also contributed to apparently over 90% of the TFV behaving as a DcNP bound drug; reported in primates after TLC-ART 101 dosing via IV as well as SC route.

The method of AE estimate – both retentate and mass-balance approaches and correlation, for the 3 drug substances in the TLC-ART 101, is demonstrated to extend to the TLC-ART 301 product composed of DTG, 3TC and TFV.

Collectively, this thesis research was able to verify the analytical methods to characterize the degree of drug association for both abbreviated retentate as well as mass-balance approach for a complex injectable drug-combination formulated in drug-combination nanoparticle product. Having the validated method may help accelerate development of the drug-combination injectable nanoparticles, intended to provide long-acting and longer lasting viral suppression in people living with HIV.

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1 Introduction:

A combination of anti-HIV drugs is given orally to provide sustained viral suppression as a one-pill-a-day treatment for people living with HIV (PLWH)[1]. Unfortunately, PLWH express pill fatigue and missing a daily dose may lead to virus rebound and disease progression. In addition, uncontrolled virus levels in PLWH may relate to a continued increase in the number of people with HIV.[2–4] Long-acting (LA) injectable nano-drug delivery systems, using nano formulations containing small-molecule antiretroviral drugs, has brought a new light to the treatment of AIDS.[5,6] These new technologies offer the benefit of less frequent dosing intervals while ensuring more stable drug concentrations, improving antiviral effectiveness, and lowering the chances of developing drug resistance.[7,8] Studies have shown that long-acting formulations like cabotegravir and rilpivirine, when administered as injections, maintain effective drug levels for extended periods, often up to 8 weeks or more.[1] Clinical trials also reported viral suppression rates of over 90% at 48 weeks when using these long-acting formulations, which is comparable to or even better than daily oral regimens.[9] These formulations are designed to precisely control the drug's release at the injection site or to regulate its distribution within the body. For the application of nano-drugs, the quantification of its physical and chemical characteristics is especially important.[10]

1.1 Class I LA Products and Mechanism

In our point of view, there are two classes of LA product.[11] We suggest classifying LA products with the key feature of drug release from the injected formulations the primary factor influencing their extended pharmacokinetics (PK) as Class I drug product. At the injection site, this solution precipitates, creating a controlled release mechanism that maintains prolonged drug levels in the plasma. For example, Cabenuva by ViiV Healthcare and Sunlenca by Gilead Sciences are two innovative LA HIV treatments that the FDA has approved: Cabenuva is composed of two antiviral agents prepared for intramuscular injection: cabotegravir, formulated as nano-sized crystals, and rilpivirine, encapsulated within a polymeric framework.[12,13] In the ATLAS and FLAIR studies, over 90% of participants who switched to Cabenuva maintained viral suppression (HIV-1 RNA <50 copies/mL) through Week 48.[14,15] The HIV-1 capsid inhibitor lenacapavir, dissolved in polyethyleneglycol300, is to make Sunlenca designed for patients with multi-drug-resistant HIV.[16,17] After 52 weeks, 81% of participants receiving Sunlenca achieved virologic suppression (HIV-1 RNA <50 copies/mL). The study reported that resistance to Sunlenca occurred in a minority of participants (19%), showing a low tolerance.[18,19] They show the potential of the Class I LA products.

1.2 Class II LA Products and Mechanism

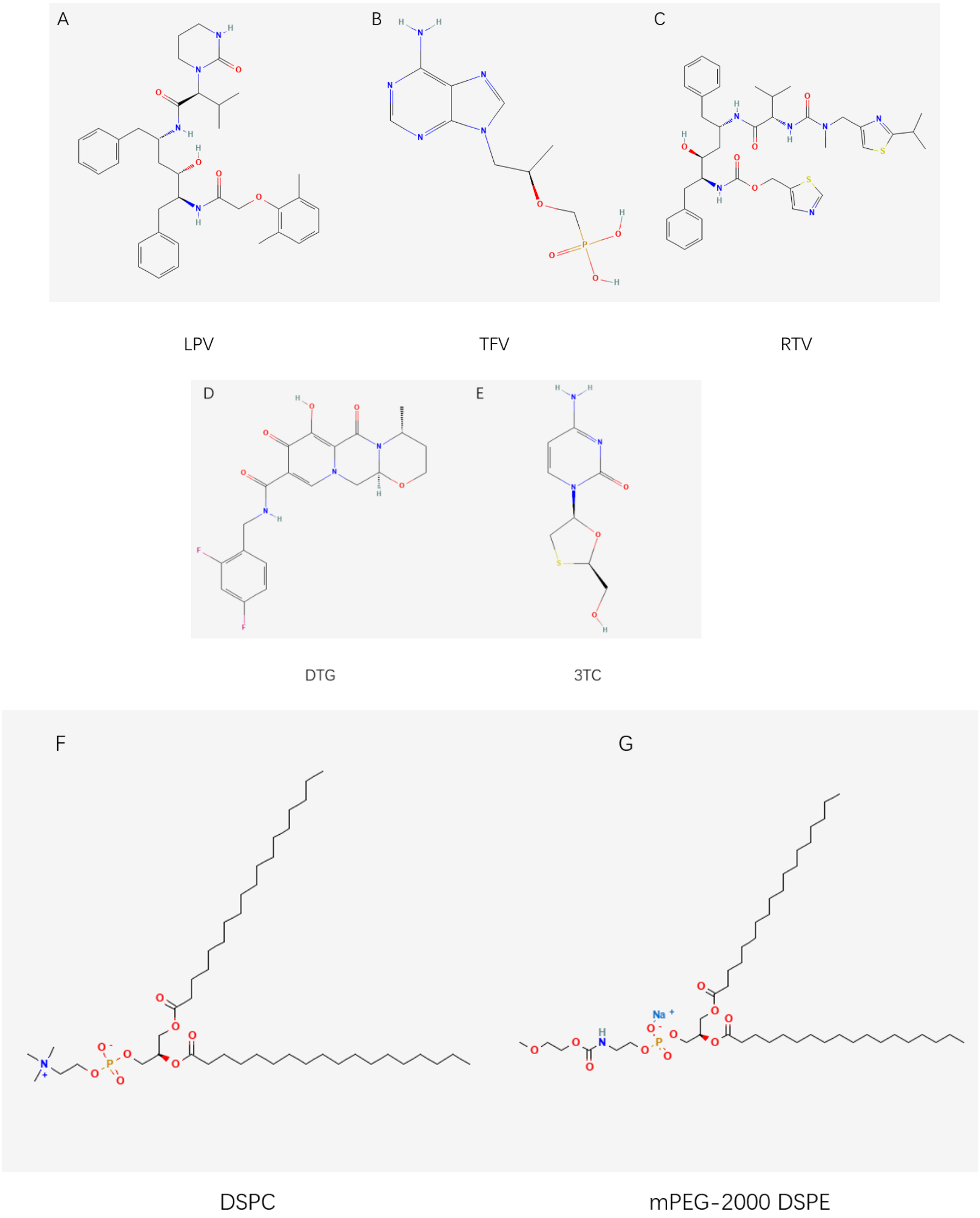
Lymph nodes or lymphatic tissues can be the focal points through a different mechanism for LA or targeted delivery products, where the dose extends beyond the injection site.[20] We suggested classify LA formulations using this mechanism as Class II LA products. An extended pathway is taken via the lymphatic system to eventually enter the blood. Subcutaneously administered large molecules like monoclonal antibodies, interferon-gamma, or lipid-based nanocarriers may be included in this class. The mechanism of Class II delivery system involves multiple ADME processes where the pharmacokinetic rate-limiting step includes the stable carrier-particle-drug being taken up by selective tissues and cells, such as lymph nodes and lymph cells, as opposed to blood vessels. This process leads to reduced metabolic and renal clearance, along with other specific mechanisms like recycling. **Table 1** compares the differences between Class I and Class II LA products.[11]

Table 1. Comparation of the Class I and Class II LA product.

	Class I	Class II
Mode and Route of Drug Delivery Systems (DDS)	Typically, IM or SC	Typically, SC
Drug release at injection site	Substantial drug localization at injection site	Substantial drug distribution beyond injection site
Injection site drug product retention	Significant duration ($T_{1/2}$~1-3 months)	No significant duration ($T_{1/2}$ ~ 1-3 days). Drug product or DDS distributed readily from injection site
Mechanism of LA	Absorption: Sustained/controlled release at the injection site	Multiple ADME: stable carrier-particle-drug taken up to select tissues and cells
Example of LA products	LA-progesterone; LA-paliperidone; LA-risperidone. **HIV:** LA-cabotegravir, LA-rilpivirine, LA-lenacapavir	LA-mAb, IFN-gamma; liposomes. **HIV:** TLC-ART 101 and 301

1.3 TLC-ART 101 and 301 Overview

Our laboratory has been focused on the drug-combination nanoparticles (DcNP) technology to invent and investigate the preclinical Class II products.[5,21–24] A flexible drug



LPV TFV RTV

DTG 3TC

DSPC mPEG-2000 DSPE

Figure 1. APIs and excipients of TLC-ART 101 and 301. A, LPV; B, TFV; C, RTV; D, DTG; E, 3TC; F, DSPC; G, mPEG-$_{2000}$ DSPE. TLC-ART 101 is composed of LPV, TFV, RTV, DSPE and mPEG-$_{2000}$ DSPC. TLC-ART 301 is composed of TFV, 3TC, DTG, DSPC and mPEG-$_{2000}$ DSPE.

delivery system as it is, DcNP can hold multiple drugs with varying physiochemical properties in a straightforward and scalable way. We have been working on the targeted LA combination antiretroviral therapy 101 (TLC-ART 101), which is a drug-combination nanoparticle (DcNP) product currently in Phase 1 human trials (NCT05850728).[25] TLC-ART 101 is a lipid nanoparticle composed of 3 active pharmaceutical ingredients (API, also referred to as drug in this thesis) and 2 lipids. The three drugs are tenofovir (TFV), lopinavir (LPV) and ritonavir (RTV). TFV is nucleoside reverse transcriptase inhibitor (NRTI). It is hydrophilic, with LogP of -1.6 and water solubility of 13.4 mg/mL. LPV and RTV are protease inhibitors. They are hydrophobic and water insoluble. LPV's and RTV's LogP are 3.91 and 4.24 respectively. The lipids are 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) and sodium; [(2R)-2,3-di(octadecanoyloxy)propyl] 2-(2-methoxyethoxycarbonylamino) ethyl phosphate (mPEG-$_{2000}$ DSPE), which are biocompatible and help the DcNP to stabilize. As a Class II product, TLC-ART 101 lipid nanoparticles would be selectively and quickly absorbed by the lymphatic vessels instead of blood vessels after SC injection.[11] In addition, we are now working on a new formulation called TLC-ART 301, which has been improved from TLC-ART 101. In TLC-ART 301, we used a different 3-drug combination that includes TFV, lamivudine (3TC) and dolutegravir (DTG). Given orally, the three drugs, referred to as TLD one-pill-a-day, have been widely used in the low-to-middle income countries with the goal to bring down HIV infection worldwide. The National AIDS Control Organization (NACO) of India recommended them as the first line treatment for AIDS.[26] 3TC is a NRTI. It has a LogP of -1.4 and is water soluble. DTG is an HIV-1 integrase inhibitor. It has a LogP of 2.2 and is slightly water soluble. **Figure 1** shows the structures of the APIs and excipients of TLC-ART 101 and 301.

1.4 Investigation of Drug Association Efficiency (AE) of TLC-ART

After the manufacture of TLC-ART, we aim to determine the AE of each drug molecule to evaluate the quality of the suspension. AE represents the percentage of drug molecules that are associated with the DcNP rather than being "free" (i.e., not associated with the DcNP). Our laboratory used two methods to measure AE: *in vitro* dialysis method and *in vivo* PK method.[5]

The *in vitro* dialysis method involves separating molecules in a solution by their rates of diffusion through a semipermeable membrane, like dialysis tubing. We assume that drugs associated with the lipid nanoparticle cannot pass through the dialysis tubing, while free drugs can. When equilibrium is reached, we assume all free drugs have diffused into the dialysate, given the volume of the dialysate is two hundred times larger than the volume inside the dialysis tubing (sink condition). Thus, any drugs measured inside the tubing are considered bound to DcNP. AE is determined by comparing the drug amount before and after dialysis in the retentate. The concept of AE by dialysis method of TLC-ART is shown in **figure 2**.

The *in vivo* experiment was performed in non-human primates by injecting the TLC-ART SC and evaluating plasma PK time course to determine the AUC (area under the curve) of each drug, assuming that free drugs are rapidly cleared from the body, while drugs associated with lipid nanoparticles are retained longer.

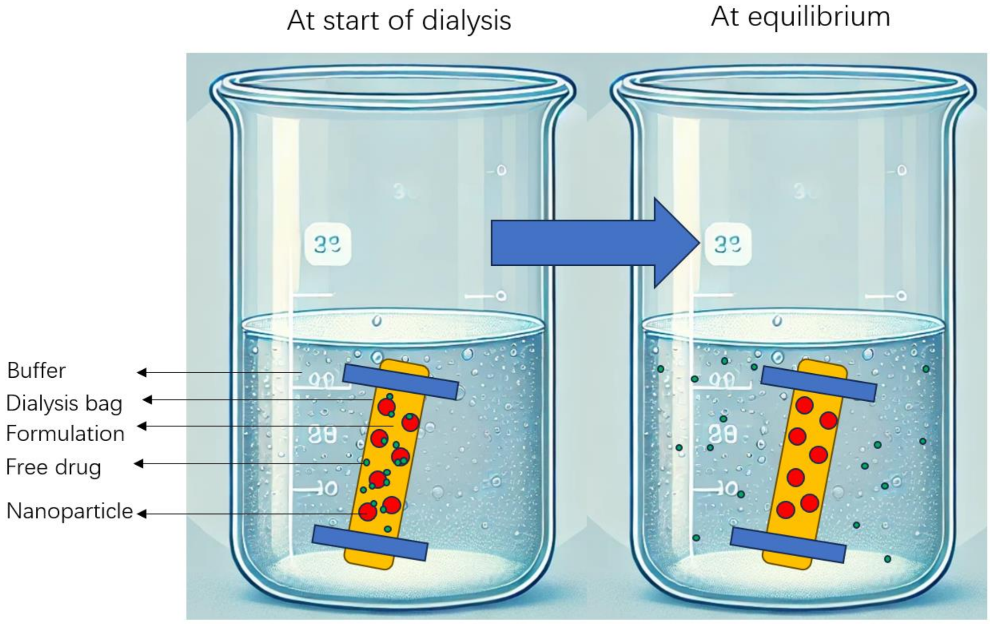


Figure 2. The concept of dialysis method for the measurement of AE of TLC-ART. At equilibrium, free drugs with a small molecular weight perfused from the dialysis tubing while the large lipid nanoparticles remain inside the bag. The cartoon of the beakers was generated by ChatGPT.

This allowed for the calculation of the maximum fraction dissociated, by comparing the AUC of the free drug with that of the lipid nanoparticle. The minimum AE could then be determined by subtracting the maximum fraction dissociated from one.[5]

There was, however, a discrepancy between the AE measurements obtained from these two methods. The *in vivo* method showed a minimum AE of 99.4% for TFV, while the *in vitro* method indicated only 15%.[5] To address this, we reviewed our *in vitro* methodology. One issue was that we only measured the concentration within the dialysis tubing (the original formulation and the retentate), not accounting for the mass balance by considering the dialysate. Another possibility was that high AUC measurements *in vivo* might be due to TFV forming micelles with lipid molecules, which could pass through the dialysis membrane *in vitro* due to their small size. The dialysis membrane we used had a molecular weight cut off (MWCO) of 6-8 kDa, which meant that molecules with molecular weight (MW) smaller than 6-8 kDa could perfuse freely from the tubing. Since mPEG-$_{2000}$ DSPE had an average MW of 2000 and the MW of TFV is 287.2, free mPEG-$_{2000}$ DSPE and small micelles of TFV and mPEG-$_{2000}$ DSPE could freely perfuse from the dialysis tubing.

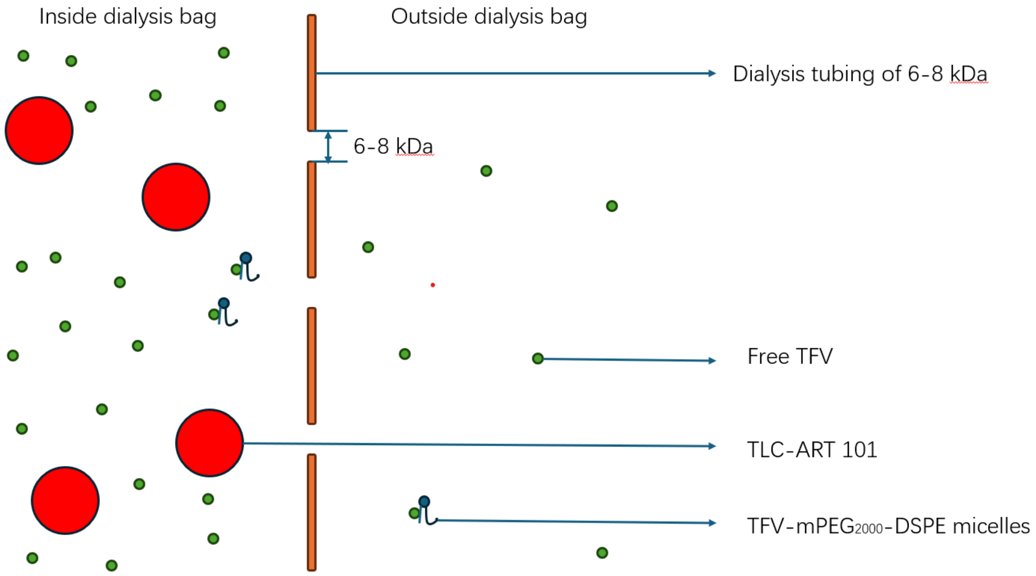


Figure 3. Display of hypothesis is that TFV may combine with mPEG$_{2000}$-DSPE to form micelles and cause the seemingly low AE *in vitro*.

In this thesis, we will explore this discrepancy and examine the interactions within the system by verifying the mass balance. We will measure the concentrations of both drugs and lipids in the retentate and the dialysate. The mass balance will be assessed by calculating recovery, which compares the total amount of a compound after dialysis to the amount before dialysis. AE will be reconsidered based on this mass balance and compared to the previous AE determined only from the inside of the dialysis membrane. Additionally, we will investigate the presence of lipids in the dialysate to determine if they can pass through the dialysis tubing, indicating the potential existence of micelles.

To measure the concentration of drugs in the dialysate and the retentate, we use the method of reversed-phase high-performance liquid chromatography (RP-HPLC). It is one of the most widely used chromatographic techniques in analytical chemistry. It is employed for the separation, identification, and quantification of components in a mixture, particularly for non-volatile and thermally labile compounds. RP-HPLC is characterized using a non-polar stationary phase and a polar mobile phase, which differentiates it from other types of liquid chromatography.[27]

However, lipid cannot be detected by RP-HPLC because it does not absorb UV. To measure the concentration of lipids, we used a charged aerosol detector (CAD) combined with HPLC. CAD is a type of universal detector used in liquid chromatography. It is based on the transformation of analytes into charged aerosol particles, which are then detected by measuring the total charge.[28]

In this thesis, we will characterize the association and disassociation of LA drug products of Class II. Our first aim is to check the mass balance of the dialysis system. Our second aim is to measure the AE to solve the contradiction between *in vitro* and *in vivo* AE of TFV. The results are important for the safety and potency of the TLC-ART formulations.

2 Materials and methods

2.1 Dialysis of TLC-ART

2.1.1 Chemicals

Sodium chloride, CAS # 7647-14-5, EMD product # SX0420-3 or equivalent

Sodium bicarbonate, CAS # 144-55-8, EMD product # SX0320-1 or equivalent

2.1.2 Equipment

Dialysis Tubing, 6-8 kDa MWCO, 10.0 mm flat width, Spectrum Labs product # 132645.

Dialysis clips, Spectrum Spectra/Por Standard 35.0 mm # 132736 or equivalent.

1.0 mL pipette tips, Gilson PIPETMAN EXPERT ED1000ST XL Sterilized Filter Tips or equivalent.

Sterile Fisherbrand Octagon Spinbar Magnetic stir bar, 1.0" length ×0.375" diam. or equivalent (1 per sample).

Scissors**,** 1.0 mL pipette**,** analytical balance**,** 400 mL sterile glass beakers (1 per sample)**,** 4.0"×4.0" sterile aluminum foil sheets**,** graduated cylinder**,** magnetic stir plate (1 per sample)

2.1.3 Methods

0.9% NaCl with 20 mM NaHCO3 buffer was prepared. 200 mL of buffer was aliquoted into a 400 mL beaker with a stir bar added. This process was repeated for each sample with duplicates.

Dialysis tubes were cut to a length of 10.0 cm each. The tubing and dialysis clips were placed in a beaker of dialysis buffer and allowed 10 minutes to equilibrate. During the equilibration, it was stirred at 60 resolutions per minute (rpm). After equilibration, buffer was squeezed out of the tubing as much as possible. One end of the tubing was folded over twice (about 1.0 cm per fold) and clipped. As much buffer as possible was removed from the clipped tube using a pipette. 1 mL of sample was loaded. The remaining formulation in the pipette was measured. The other end was carefully folded and clipped, removing as much air as possible without allowing the sample to run up the sides. The openings of the tubing were washed with DI water to avoid contamination. The sample was placed in a beaker with 200 mL of dialysis buffer and covered with a sterile foil sheet to prevent evaporation. This process was repeated with the remaining samples, with samples being prepared in duplicate. The beakers were placed on a magnetic stir plate and stirred at 60 rpm. All tubes were fully submerged to ensure that the buffer was circulating well around all samples. The dialysis lasted for 4 hours. After dialysis, the retentate was gathered and its weight was measured. Dialysate was also gathered.

2.2 Quantification of LPV, RTV, and TFV in TLC-ART 101 by RP-HPLC

2.2.1 Chemicals

Acetonitrile (ACN), HPLC grade, ≥99.9%, Fisher Optima Cat. # A996-4 or equivalent

Milli-Q or HPLC Grade water, J.T. Baker Cat. # 4218-03 or equivalent

Trifluoroacetic acid (TFA), LC/MS grade, Thermo Scientific Cat# 28904 or equivalent

Ammonium Hydroxide (14.8N), Fisher Cat. # A669S or equivalent

Sodium Bicarbonate, Fisher Cat. # S233-500 or equivalent

Sodium Chloride, Fisher Cat. # S271-500 or equivalent

1N Sodium Hydroxide, Acros Cat. # 12426-0010 or equivalent

Lopinavir, Mylan Cat# 100502231

Ritonavir, Mylan Cat# 100501905

Tenofovir Fumarate (TCF) II, Mylan Cat# 40052144

Ethanol

2.2.2 Equipment

Dialysis Tubing, 6-8 kDa MWCO, 10.0mm flat width, Spectrum Laboratorys Cat. # 132645

Dialysis clips, Spectrum Spectra/Por Standard 35.0 mm # 132736 or equivalent

Agilent 1260 HPLC, which consists of the following sub-systems

Quaternary Pump, part # G1311B or equivalent

Autosampler, part #G1329B or equivalent

Autosampler Thermostat, part #G1330B or equivalent

Thermostatted Column Compartment, part #G1316A or equivalent

Agilent ChemStation software, version C.01.05, or equivalent

XBridge BEH C18 130Å 4.6 ×250 mm, 5 µm, Waters, Cat# 186003117

XBridge BEH C18 VanGuard Cartridge, 130Å, 5 µm, 3.9 mm ×5 mm, Waters, Cat# 186007771

VanGuard Cartridge Holder, Waters, Cat# 186007949

Positive Displacement Pipets, various volumes from 1-1000 µL.

Sample Concentrator with regulated nitrogen supply, Techne DRI-BLOCK DB-3A heat block and Sample Concentrator Cat. #1100500 or equivalent

General Class A laboratory glassware, vortexer, sonicator, analytical balance with readability of 0.0001 g

Freeze drier, Nitrogen drier.

2.2.3 Methods

Mobile phase A was prepared by mixing 1 L of Mili-Q water with 1 mL of trifluoroacetic acid, and mobile phase B was prepared by mixing 1 L of acetonitrile with 0.75 mL of trifluoroacetic acid.

To prepare LR standard stocks, a total of 200.0 mg of LPV and 60.0 mg of RTV were measured and 20 mL acetonitrile was added to the to dissolve. 2.0 mg of LPV and 0.6 mg of RTV (200 µL) of the stock solution were aliquoted into a series of vials. The vials were dried at 60°C under N_2, capped, and stored in a freezer at -20 °C. To prepare T standard stock, a total of 120.0 mg of TFV was measured and 6 mL water, 14 mL acetonitrile, and 40 µL ammonium hydroxide were added to dissolve. 1.2 mg of TFV (200 µL) of the stock solution was aliquoted into a series of vials. The vials were dried at 60°C under N_2, and stored in a freezer at -20 °C. To prepare TLR working standard sample set, one vial of LR Stock was removed, and 1.0 mL of Mobile Phase B was added. One vial of TLC-ART101 API Standard T Stock was removed, and 1.0 mL of Mobile Phase A was added. These solutions were prepared fresh daily. TLR working standards were prepared at properly spaced levels according to **table 2** by diluting with 70:30 Mobile Phase A: Mobile Phase B and mixed well.

A positive displacement pipet was used for pipetting viscous samples such as formulated TLC-ART 101. The outside of the pipet tip was wiped with a Kimwipe before the sample was added to the diluent.

The test samples were diluted to a 1:50 dilution by adding 20 µL of the test sample into 980 µL of a 70:30 solution of Mobile Phase A: Mobile Phase B. The samples were centrifuged at 14,000 rpm for 10 minutes. For each preparation, at least 500 µL of the supernatant was transferred to an HPLC autosampler vial. Those auto-samplers and standard samples were then loaded into the HPLC-UV system. The dialysate was centrifuged at 14,000 rpm and was directly placed into the autosampler for analysis. The HPLC-UV system is set according to **table 3**.

Table 2. Preparation of TLC-ART 301 Standards.

Label	Stock Standard T (µL)	Stock Standard LR (µL)	70:30 Mobile Phase A: B (µL)	Final concentration of drugs (µg / 20 µL injection)		
				LPV	RTV	TTF
Standard 1	250	250	500	10	3.0	6.0
Standard 2	200	200	600	8.0	2.4	4.8
Standard 3	150	150	700	6.0	1.8	3.6
Standard 4	100	100	800	4.0	1.2	2.4
Standard 5	50	50	900	2.0	0.60	1.2
Standard 6	37.5	37.5	925	1.5	0.45	0.90
Standard 7	18.75	18.75	962.5	0.75	0.23	0.45
Blank	N/A	N/A	1000	N/A	N/A	N/A

Table 3. HPLC-UV Running Conditions for TLC-ART101 Assay.

Parameter	Setting

Channel A	0.1% Trifluoroacetic Acid in Water			
Channel B	0.75% Trifluoroacetic Acid in Acetonitrile			
Flow Rate	1.0 mL/min			
Guard Column	XBridge BEH C18 VanGuard Cartridge, 130Å, 5 µm, 3.9 mm X 5 mm, Waters Cat# 186007771			
Column	XBridge BEH C18 130Å 4.6 x 250mm, 5 µm, Waters Cat# 186003117			
Gradient	Time	%B		
	0.0	0.0		
	7.5	55.0		
	22.5	62.5		
	23.5	0.0		
	30.0	0.0		
Run Time	30 min			
Column Temperature	Ambient			
Autosampler Temperature	Ambient			
Injection Volume	10 µL			
Detecting Wavelength	Wavelength	Bandwidth	Reference	Bandwidth
Signal A: LPV	220 nm	4 nm	360 nm	100 nm
Signal B: RTV	240 nm	4 nm	360 nm	100 nm
Signal C: TFV	260 nm	4 nm	360 nm	100 nm

2.3 Quantification of 3TC, DTG, and TFV in TLC-ART 301 by RP-HPLC

2.3.1 Chemicals

ACN, HPLC grade, FisherScientific or equivalent

Tetrabutylammonium phosphate monobasic, Sigma Aldrich or equivalent

Methanol HPLC grade, Fisher Scientific or equivalent

Ammonium phosphate monobasic, Sigma or equivalent

DTG powder, 3TC powder, TFV powder, TLC-ART 301 suspension, DI water

2.3.2 Equipment

ThermoFisher 2 mL glass autosampler vials, 12 mm ×32 mm, catalog no. C4011-59

ThermoFisher snap it cap, catalog no. C4011-5

Axygen 1.5 mL microtubes, clear, homo-polymer, boil-proof, REF MCT-150-C

Fisher VortexGenie catalog no. 12-812, or equivalent

Eppendorf 120 V desktop centrifuge 22331, or equivalent

Fisher Scientific Wheaton Glass 20 mL scintillation vial

Shimadzu CBM-20A Communications Bus Module

Shimadzu LC-20AD Liquid Chromatograph (X2)
Shimadzu DGU-20ASR Degassing Unit
Shimadzu SIL-20A HT Autosampler
Shimadzu 20AV UV-Vis Detector
Mettler Toledo MS4002TS Precision Balance, or equivalent
Sterile spatulas for weighing powders
Macherey Nagel Polartec C18, 100 ×3 mm, 3 µm particle size column, REF 760476.3
Elmasonic Sonicator, E100H

2.3.3 Methods

Mobile phase A was prepared by mixing 1.25 mM tetrabutylammonium perchlorate in 20% acetonitrile and 20 mM ammonium phosphate buffer, and mobile phase B was prepared by mixing 85% acetonitrile and 15% methanol. Standard samples of the drugs were prepared.

1 mg of DTG, 3TC and TFV powder were put into a 20 mL glass scintillation vial. 10 mL of diluent made of 50% mobile phase A and 50% mobile phase B was added to the scintillation vial. The mixture was briefly vortexed to mix and then sonicated for 10 minutes to fully dissolve. The standard curve was prepared according to **table 4**.

For pipetting viscous solutions like TLC-ART 301 suspension, a Kimwipe was used to remove excess formulation from the outside of the pipette tip. Using a volumetric pipette, 9.9 mL of Diluent was added into a 20 mL glass scintillation vial. A 100 µL aliquot of the suspension was accurately transferred into the glass scintillation vial, and the pipette was used to pipette up and down with the diluent to clear out any formulation stuck within the pipette tip. This resulted in a 1:100 dilution. The mixture was briefly vortexed to mix, and the samples were sonicated for 10 minutes. After sonication, 1 mL of the extraction mixture was transferred into a 1.5 mL tube to centrifuge and pellet the lipids for 10 minutes at ambient temperature and 14,500 rpm. As much of the supernatant as possible (about 800 µL) was collected into an HPLC vial for HPLC-UV quantification.

For the dialysate, 40 mL of dialysate for each sample was freeze-dried. The freeze-dried material was resuspended with 4 mL of ethanol and shaken for 1 hour to fully dissolve the solutes. The mixture was then centrifuged at 3,000 rpm. A 3.2 mL aliquot of the supernatant was taken and nitrogen dried. The dried material was then extracted with 250 µL of ethanol, centrifuged again at 14,000 rpm, and the supernatant was taken for HPLC-UV analysis. The HPLC-UV system was set according to **table 5**.

Table 4. Preparation of TLC-ART 301 Standards. The concentration of 3TC, DTG, and TFV in the standards are the same and shown in column 4.

Standard ID	Volume of 100% Stock Standard added (µL)	Volume of Diluent added (µL)	Concentration of the

			drugs(mg/mL)
100% Std	1000	0	0.1
50% Std	500	500	0.05
25% Std	250	750	0.025
10% Std	100	900	0.01
5% Std	50	950	0.005
1% Std	100 (from 10% standard)	900	0.001
0.5% Std	100 (from 5% standard)	900	0.0005
0.1% Std	200 (from 0.5% standard)	800	0.0001
0.05% Std	50 (from 1% standard)	950	0.00005

Table 5. HPLC-UV Running Conditions for TLC-ART301 Assay.

Parameter	Setting	
Channel A	1.25 mM tetrabutylammonium perchlorate in 20% acetonitrile, 20 mM ammonium phosphate buffer	
Channel B	85% acetonitrile, 15% methanol	
Flow Rate	0.5 mL/min	
Column	Machery-Nagel Nucleodur Polartec 100 x 3 mm, 3 μm particle size (C18)	
Gradient	Time (min)	%B
	0.00	0
	0.01	20
	1.00	20
	10.00	75
	13.00	75
	15.00	20
	16.00	20
Run Time (min)	16.00	
Column Temperature	Ambient	
Autosampler Temperature	Ambient	
Injection Volume	10 μL	
Detector Wavelength	254 nm	

2.4 Lipid quantification of the TLC-ART by HPLC-CAD

2.4.1 Chemicals

Methanol (MeOH), HPLC grade, Fisher Cat. # A452-4 or equivalent.

Ammonium Acetate, ACS grade, JT Baker Cat. # 0599-08 or equivalent.

Milli-Q or HPLC Grade water, J.T. Baker Cat. # 4218-03 or equivalent

DSPC Lipid, Corden, Cat# LP-04-076

mPEG-$_{2000}$ DSPE Lipid, Corden, Cat# LP-04-039

2.4.2 Equipment

Alliance HPLC System, which consists of the following sub-systems

Waters e2695 Separations Module, part # 186269506 or equivalent

Waters Column Heater, part # 186269506 or equivalent

CAD, Corona Veo RS or equivalent

Peak Scientific Corona Nitrogen 3010 – N_2 Generator or equivalent

Agilent ChemStation software, version C.01.05, or equivalent

Kinetex 5 µm C18 100 Å, LC Column 50 ×2.1 mm, Part No: 00B-4601-AN

High performance ZORBAX guard fittings kit, Cat# 820999-901

C8 Guard Cartridge, 4 ×2.0 mm, 5 µm particle size, Phenomenex, Part No: AJ0-4289

Waters brand 12x32 mm, screw-top vial with cap and bonded, pre-slit PTFE/Silicone Septa P/N 186000989

Insert 0.25 mL conical bottom for WM 12 × 32 vial

Axygen 1.5 mL microtubes, clear, homo-polymer, boil-proof

General laboratory glassware

Vortexer, analytical balance with readability of 0.0001 g, sonicator

pH Meter, Fisher Scientific Accumet XL15 or equivalent

Sample Concentrator with regulated nitrogen supply, Techne DRI-BLOCK DB-3A heat block and Sample Concentrator Cat. #1100500 or equivalent

2.4.3 Methods

Mobile phase A was made by preparing 4 mM ammonium acetate, and mobile phase B was made by preparing 4 mM ammonium acetate in methanol. Fresh DSPC and mPEG-$_{2000}$ DSPE standards were made daily. Using a sterile spatula, 10 mg of DSPC was added into a 20 mL glass scintillation containing 10 mL methanol to produce a 1.0 mg/mL stock. Steps were repeated for mPEG-$_{2000}$ DSPE. Both DSPC and mPEG-$_{2000}$ DSPE stocks were vortexed and then sonicated for 10 minutes. Both stocks were submerged into a water bath set to 50–55 °C to facilitate the dissolution of the lipid powders. Dissolution was considered complete when both stock solutions were visually homogeneous, colorless, and transparent. The lipid stock solutions were vortexed to mix well. With the 1 mg/mL lipid standards, 2.5 mL of DSPC and 1.25 mL of mPEG-$_{2000}$ DSPE were measured into a new glass scintillation vial. A total of 1.25 mL of Methanol was pipetted into the glass scintillation vial, and the solution was vortexed. The stock solution was prepared to contain 0.5 mg/mL DSPC and 0.25 mg/mL mPEG-$_{2000}$ DSPE. This solution served as the working standard used for the standard curve dilutions. The standard curve was prepared according to **table 6**.

Table 6. Preparation of TLC-ART lipid Standards.

Label	Amount of Standard (0.5 mg/ml DSPC, 0.25mg/mL mPEG-$_{2000}$ DSPE) added (μL)	Methanol (μL)	Conc. of TLC-ART301 Lipids (mg/mL)	
			DSPC	mPEG-$_{2000}$ DSPE
Standard 0	100	900	0.05	0.025
Standard 1	200	800	0.1	0.05
Standard 2	400	600	0.2	0.1
Standard 3	600	400	0.3	0.15
Standard 4	800	200	0.4	0.2

A positive displacement pipet was used for pipetting viscous samples such as formulated TLC-ART 101 and 301. A Kimwipe was used to wipe the outside of the pipet tip before the sample was added to the diluent. Methanol (4.95 mL) was dispensed into two separate 20mL glass scintillation vials (replicates of 2). A 50 μL aliquot of TLC-ART suspension was dispensed into the 20 mL glass scintillation vial. This process was repeated 1 time to prepare a total of 2 glass vials containing TLC-ART 301 suspension at a 1:100 dilution. A 100 μL aliquot of the diluted suspension from each vial was dispensed into a microcentrifuge tube containing 900 μL of methanol for a total of 1000-fold dilution. The mixture was briefly vortexed to mix well. The microcentrifuge tubes were then centrifuged at approximately 14,000 rpm for 10 minutes at 25 °C to pellet any undissolved lipids. For each preparation, about 800 μL of the supernatant was transferred to an HPLC autosampler vial for HPLC-CAD analysis.

Table 7. HPLC-CAD Running Conditions for TLC-ART Assay.

Parameter	Setting	
Channel A	4mM Ammonium Acetate Buffer	
Channel B	4mM Ammonium Acetate in Methanol	
Flow Rate	0.33 mL/min	
Guard Column	C8 Guard Cartridge, 4 x 2.0mm, 5 µm particle size	
Column	Kinetex 5 µm C18 100 Å, LC Column 50 x 2.1 mm	
Gradient	Time	%B
	0.00	80
	10.00	100
	20.00	100
	25.00	80
Run Time	25 min	
Column Temperature	55°C	
Autosampler Temperature	40ºC	
Injection Volume	10 µL	
CAD settings	Evaporation Temp 35ºC Filter: 5.0 sec Power Function: 1.0 Data Collection Rate: 2.0 Hz	

As for the dialysate, 40 mL of dialysate for each sample was freeze-dried. The freeze-dried material was resuspended with 4 mL of ethanol, then vortexed and shaken for 1 hour to fully dissolve the solutes. The mixture was centrifuged at 3,000 rpm. A 3.2 mL aliquot of the supernatant was taken and nitrogen dried. The dried material was then extracted with 250 µL of ethanol, centrifuged again at 14,000 rpm, and the supernatant was taken for HPLC-CAD analysis. The HPLC-CAD system was set according to **table 7**.

2.5 Calculation of recovery and AE of TLC-ART

Two ways were used to calculate the *in vitro* AE from the dialysis system. First, we assumed that drugs associated with the lipid nanoparticle could not perfuse from the dialysis tubing, while the free drugs could perfuse freely from the semipermeable membrane. When the system came to equivalent, since the volume of dialysate was 200 times larger than the retentate, we could assume that all free drugs perfused into the dialysate, and all the drugs we measured from the retentate were bonded to DcNP. In that case, nominal AE could be calculated from the following equation:

$$\text{AE}=\frac{Drug\ amount\ in\ DcNP\ after\ dialysis}{Drug\ amount\ in\ DcNP\ before\ dialysis}\times 100\% \tag{1}$$

Since equation 1 was based on the drug concentration retained within the semi-permeable membrane before and after the dialysis, we referred to it as a nominal AE, based on retentate of the dialysis method. It was assumed that the materials released and permeated across the dialysis membrane could be accounted in the dialysate. Given they were diluted into a much larger volume, it was more challenging to measure drug levels with precision.

In a mass balanced approach, the percentage of AE could be described with the following equation.

$$\text{AE}= \frac{Drug_{in}}{Drug_{in} + Drug_{out}} \times 100\% \qquad (2)$$

In equation 2, $Drug_{in}$ meant the drug amount in the retentate and $Drug_{out}$ meant the drug amount in the dialysate. We could calculate AE from both the data measured from the dialysate and retentate by equation 2. This AE was the mass-balance AE because it did not assume the mass balance before and after the dialysis.

To check the mass balance of the dialysis system, we used the percent recovery of a certain API or lipid.

$$Recovery\% = \frac{Amount\ in\ the\ post{-}dialysis\ formulation + Amount\ in\ dialysate}{Amount\ in\ the\ pre{-}dialysis\ formulation} \times 100\% \qquad (3)$$

Recovery% could show the ratio of the amount of an API or excipient after dialysis versus the amount of it before dialysis, so it could show the mass balance of the system. A 100% recovery meant that all we put into the dialysis system are found after dialysis in the system.

3 Results

3.1 The chromatography and standard curves for the TLC-ART dialysis system

Figures 4, 5 and 6 show the chromatogram of the APIs and excipients in TLC-ART 101 and 301, and **figure 7** shows the standard curves. It can be seen that the drugs and lipids were separated successfully in the HPLC-UV and HPLC-CAD. Also, the standard curves were good with an R^2 of at least 0.997.

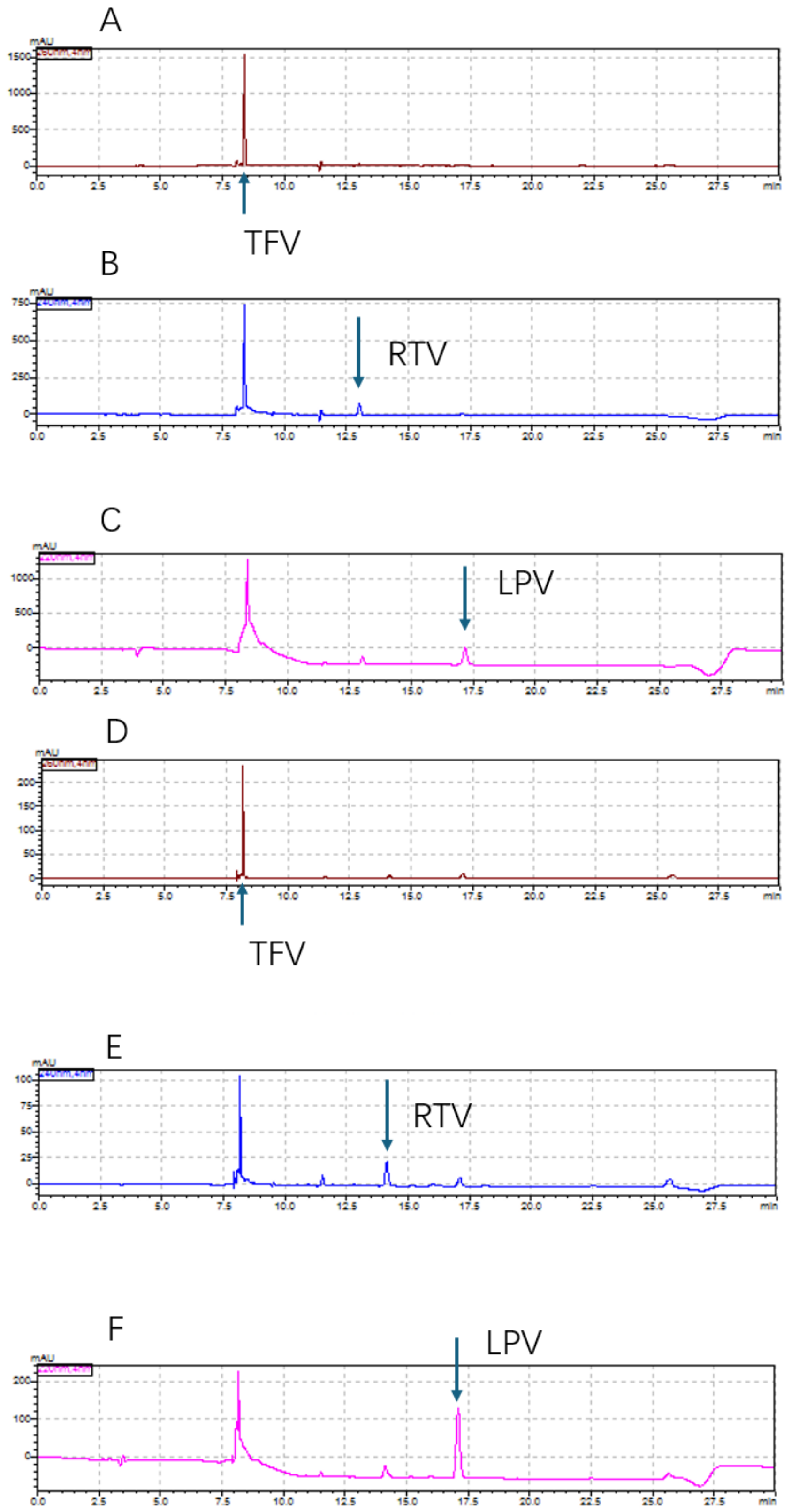


Figure 4. The chromatogram of the drugs in TLC-ART 101. A, TFV standard detected at 260 nm; B, RTV standard detected at 240 nm; C, LPV standard detected at 220 nm; D, TFV sample detected at 260 nm; E, RTV sample detected at 240 nm; F, LPV sample detected at 220 nm.

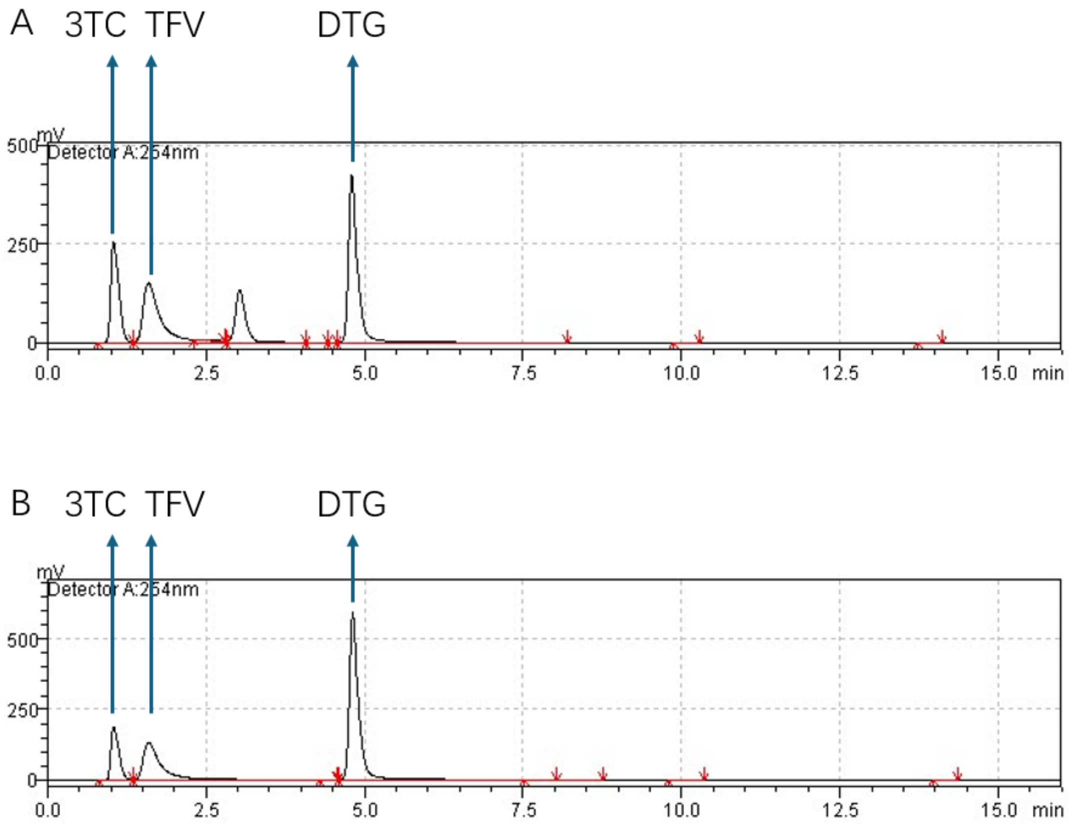


Figure 5. The chromatogram of the drugs in TLC-ART 301. A, 3TC, TFV and DTG standard detected at 254 nm; B, 3TC, TFV and DTG sample detected at 254 nm.

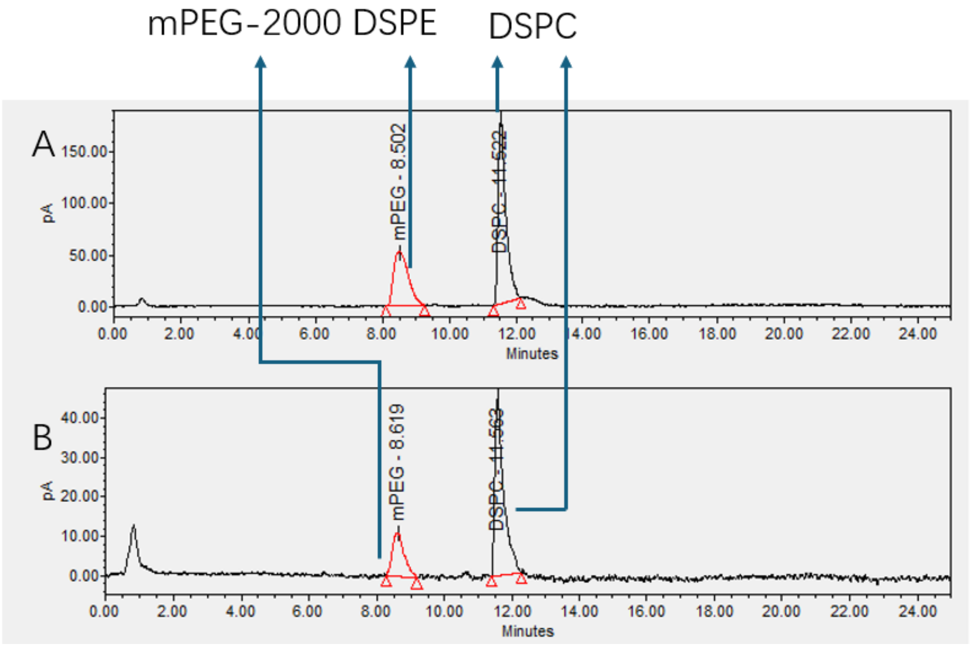


Figure 6. The chromatogram of the lipids in TLC-ART 101 and 301. A, standard detected at 254 nm; B, 3TC, TFV and DTG sample detected at 254 nm; B, sample detected at 254 nm; B, 3TC, TFV and DTG sample detected at 254 nm.

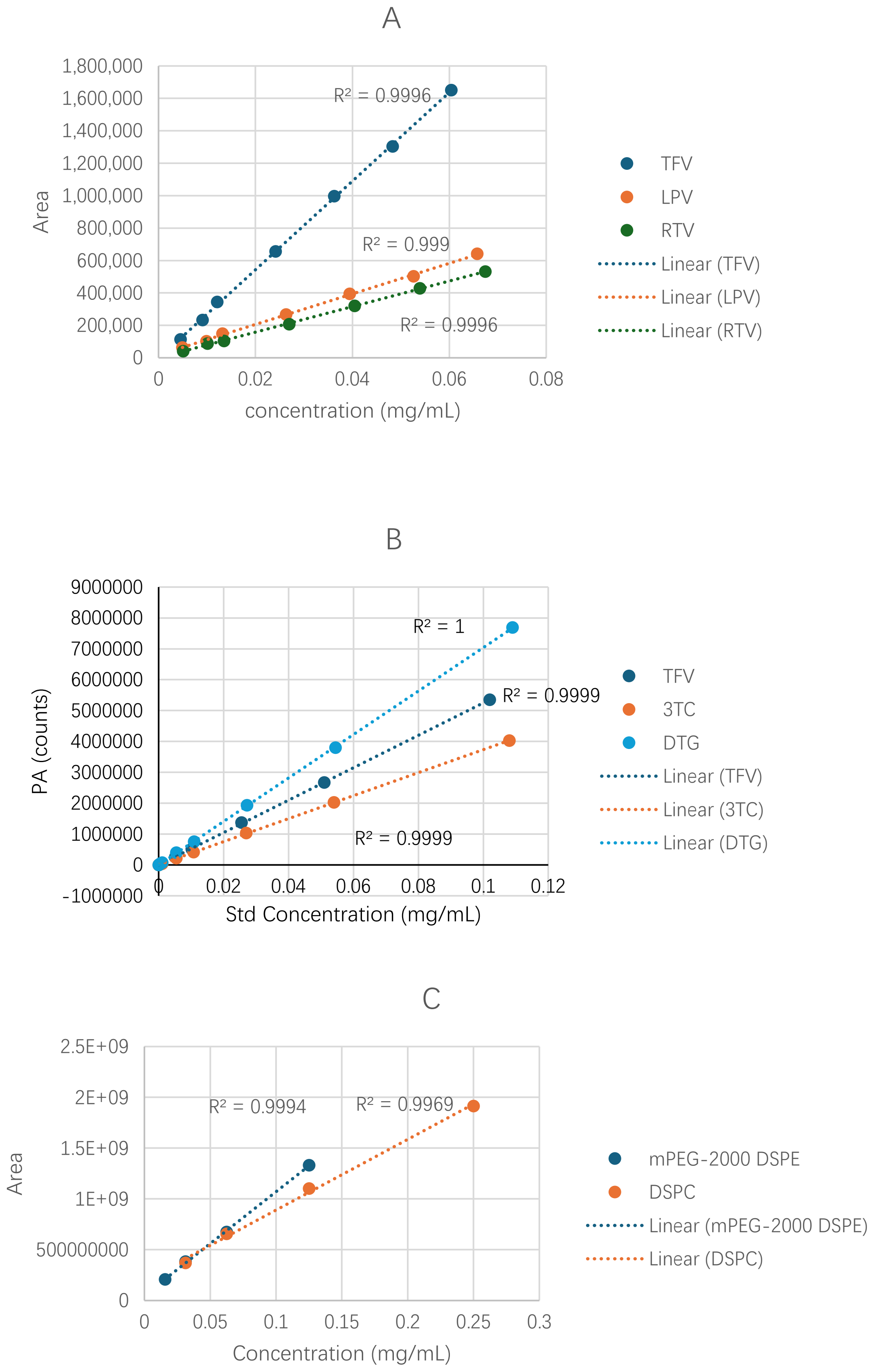


Figure 7. Standard curves for the TLC-ART 101 and 301 APIs and excipients. A, standard curve for drugs in TLC-ART 101 measured by HPLC-UV; B, standard curve for drugs in TLC-ART 301 measured

by HPLC-UV; C, standard curve for drugs in TLC-ART 101 and 301 measured by HPLC-CAD.

3.2 TLC-ART dialysis system quantification

In the dialysate of TLC-ART 101, all APIs and excipients except DSPC appeared, while all APIs and excipients appeared in the dialysate of TLC-ART 301. The result of Recovery% is shown in **figure 8**. Except the TFV in TLC-ART 101, all APIs and excipients had a recovery of 100% in the range of CV.

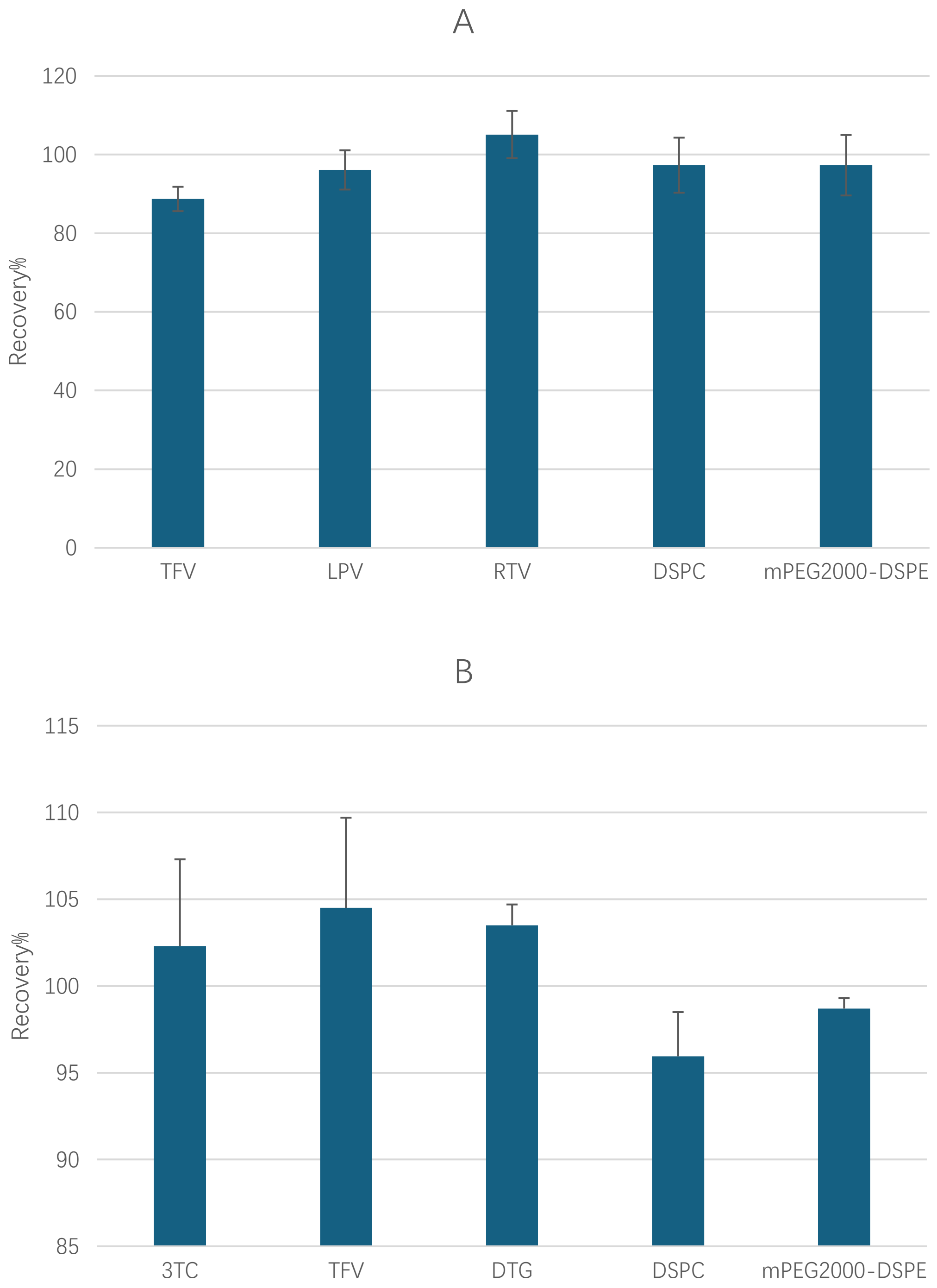
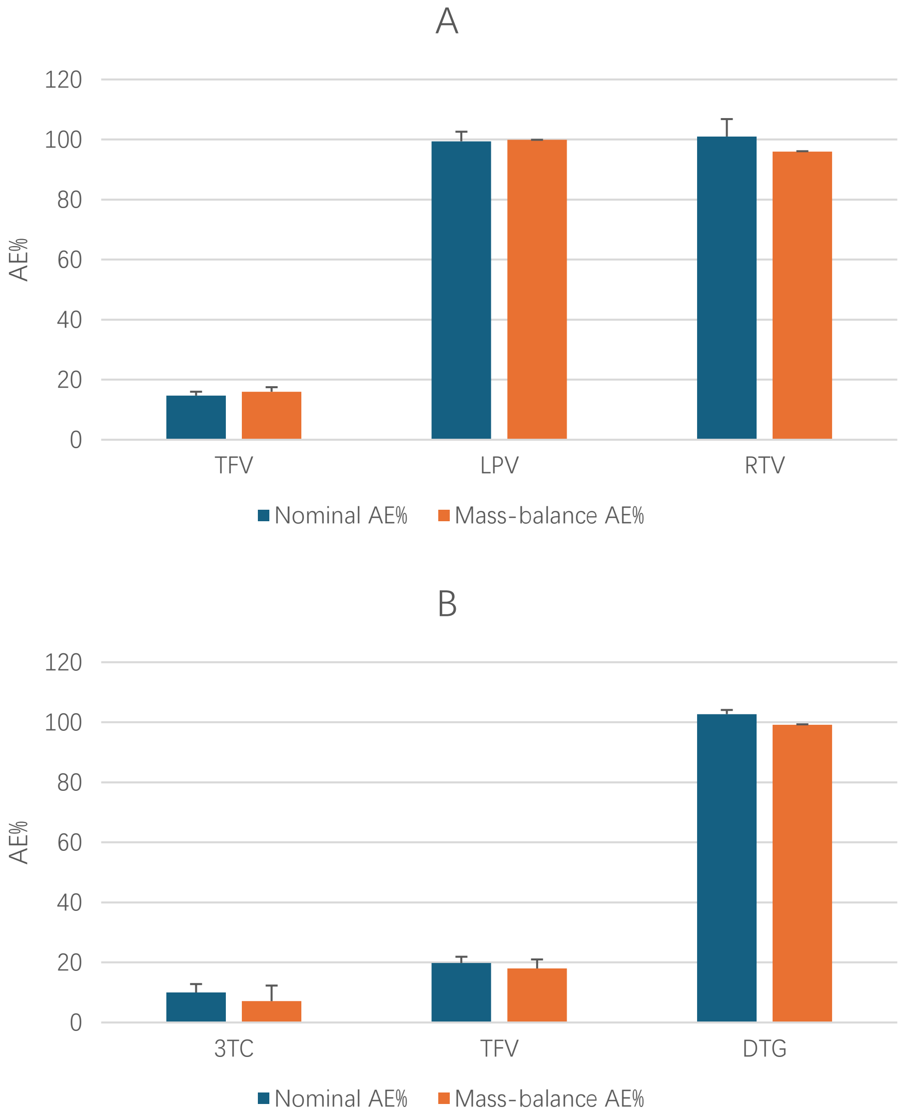


Figure 8. Recovery% of APIs and excipients in TLC-ART 101 in the dialysis experiments. A: TLC-ART 101; B: TLC-ART 301.

Also, the AE of different APIs were calculated using equations (1) and (2), the results are shown in **Figure 9**. **Figure 9A and B** compare the results got from equation (1) and (2). LPV, RTV and DTG are hydrophobic, so they are bonded tightly to the TLC-ART nanoparticles and have a high AE. TFV and 3TC are hydrophilic drugs, so they are not highly associated with the TLC-ART nanoparticles. The AE results were consistent with our previously published results. **Figure 9C and D** check the correlation of the nominal AE and mass-balance AE, and the result shows a high correlation with an R^2 of 0.99 and a slope of 0.98.



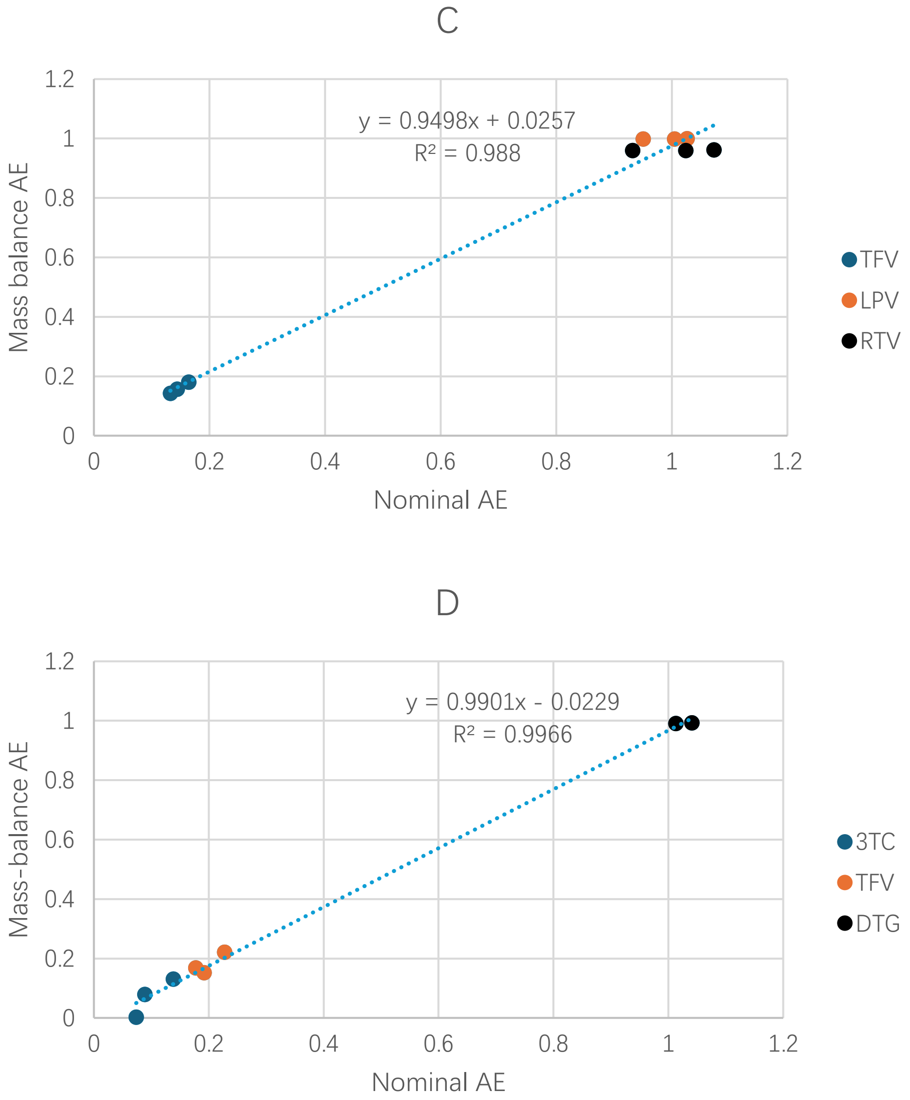


Figure 9. The AE of TLC-ART 101 and 301 APIs were determined from dialysis experiment. Nominal AE was calculated from equation (1) and mass-balance AE was calculated from equation (2). A: TLC-ART 101; B: TLC-ART 301; C: The plot of the correlation of nominal AE versus mass-balance AE of APIs in TLC-ART 101 determined from dialysis experiment, with a slope of 0.95 and R^2 of 0.99; D, The plot of the correlation of nominal AE versus mass-balance AE of APIs in TLC-ART 301 determined from dialysis experiment, with a slope of 0.99 and R^2 of 1.00.

Figure 10 shows the proportion of the lipid in the dialysate versus the total lipid amount. It is noteworthy that in the dialysate of TLC-ART 101, only mPEG-$_{2000}$ DSPE is found and no DSPC is found. It can be seen that although lipids did exist in the dialysate, the proportion of it is relatively small compared to the total lipid amount. Also, the proportion of lipid in the dialysate varies between different TLC-ART formulations. In TLC-ART 101, the molecular proportion of TFV to mPEG$_{2000}$-DSPE was 14975.5. In TLC-ART 301, the molecular proportion of TFV to DSPC was 59.5 and TFV to mPEG$_{2000}$-DSPE was 619.5.

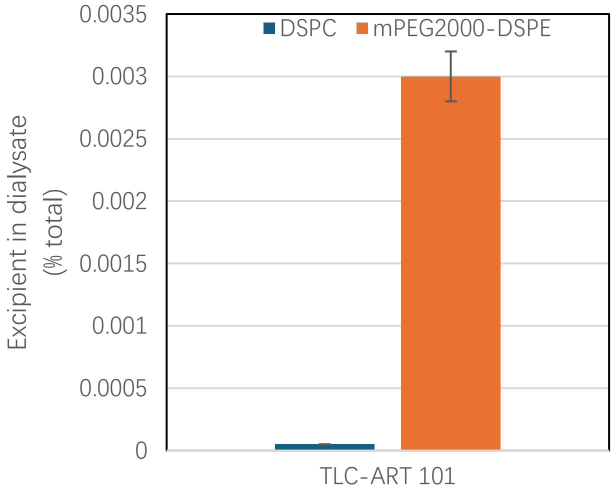


Figure 10. Proportion of lipids in the dialysate versus the lipids in the dialysate plus the lipid in the post-dialysis formulation

4 Discussion

4.1 The mass balance of the dialysis system of TLC-ART 101 and 301

By using percent recovery, we show that the dialysis system is mass balanced for all the APIs and excipients except for TFV in TLC-ART 101. This may result from the underestimate of TFV in the dialysate. According to our hypothesis, TFV can combine with the mPEG-$_{2000}$ DSPE to form micelles. The TFV in the micelles may have a different retention time in the HPLC-UV, which would result in an underestimation of it in the dialysate. In fact, there are several peaks around the peak for TFV in the result of the HPLC-UV. Among them, one of the peaks may be the TFV-mPGE$_{2000}$ DSPE micelles we are looking for.

Proof of the mass balance of the dialysis system can provide a reliable foundation for the analysis of AE of the APIs in TLC-ART. We calculated AE of two different methods and compared them in **figure 9**. We can see that the two methods are consistent with a slope of 0.94 and R^2 of 0.99 for TLC-ART 101 and slope of 0.99 and R^2 of 1.00 for TLC-ART 301. So, we proved that the contradiction between the *in vitro* and the *in vivo* AE of TFV was not from the mass balance problem of the dialysis system.

4.2 The AE of the dialysis system of TLC-ART 101 and 301

If our hypothesis is correct, the conflict between *in vitro* and *in vivo* AE resulted from the TFV combining with mPEG-$_{2000}$ DSPE, which would result in an overestimation of *in vivo* AE. Other reasons can also lead to an overestimation of *in vivo* AE. For example, the microenvironment of the DcNP could increase the AE of TLC-ART 101 *in vivo*. Since we were performing the experiment in sink condition, at room temperature and in normal saline buffer, it is very different from the *in vivo* conditions. There is no protein in our dialysis system, which may also bind to TFV or may facilitate the association of TFV with lipids in the body.

We could not find literature comparing the AE of TFV *in vitro* and *in vivo*. For the *in vitro* AE, the literatures did not take mass balance into account. They either measured the free drug amount or the drug amount in the nanoparticle and compared it to the theoretical total drug amount.[29–31]

We found that in the dialysate, there existed lipids perfused from the dialysis tubing, but their amounts were so small that it is challenging to tell whether they perfused with TFV, forming micelles or they were simply not associated with the DcNP. Also, it was shown in **figure 10**, that significantly more lipids were found in the dialysate of TLC-ART 301 than that in TLC-ART 101. It is possible that the dialysate of TLC-ART 301 might have been contaminated by the opening of the dialysis tubing, or that some formulation leaked into the dialysis buffer when clipping the opening of the dialysis tubing. Lastly, it is interesting that only mPEG-$_{2000}$ DSPE was found in the dialysate of TLC-ART 101. The dialysate of TLC-ART 101 was concentrated 133-fold in an attempt to detect DSPC, but still no DSPC was detected (data not shown). So, we can conclude that the amount of DSPC in the dialysate of TLC-ART 101 is too low to detect. Since the MWCO of the dialysis tube we used in the experiment is 6-8 kDa, and mPEG-$_{2000}$ DSPE has a molecular weight of about 2860, it is possible to perfuse from the dialysis tubing if it is not associated with the DcNP. However, because only mPEG-$_{2000}$ DSPE perfused from the dialysis tubing, while DSPC of molecular weight of 790.2 did not, it seems to support our hypothesis.

In the literature, we found another method to check the AE *in vivo,* where fluorescence microscopy was used to observe the dynamic changes of the lipid nanoparticles *in vivo*.[32] In the future, we can try this method and compare the outcome with our current results.

5 Conclusion

In this thesis, we determined the mass balance and the AE of Class II products TLC-ART 101 and 301. We found the mass balance of the dialysis system of TLC-ART 101 and 301 was met except for the TFV in TLC-ART 101. We also calculated the AE of each drug in those formulations using two different methods considering or not the mass balance of the system. Both methods were consistent with each other. It requires further research to discover why TFV has such a high AE *in vivo* but has a low AE *in vitro*.

6 Acknowledgements

First, I want to thank my mentor Dr. Rodney Ho and Dr. Qingxin Mu for their guidance. I also want to thank laboratory members at the Ho lab, Masa Eguchi Zachary Stephen, Xiaolin Xu, Rachele Fratte, Jason Lo, Arial Scott and Kenneth Garbutt for their help with this research. I also want to thank the funding and resource support received through the National Institutes of Health (NIH) for the UM1, R61/33 grant funded UW Targeted, Long-acting Drug Combination or TLC-ART program. The support of the WHO's Global Funder Unitaid through GLAD project is acknowledged for making TLC-ART 301 product available for my thesis work. Editorial input of Aaron Haley is greatly appreciated. Most importantly, I want to thank my family for supporting my graduate study.

7 Table of abbreviations

1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC)
Acetonitrile (ACN)
Active pharmaceutical ingredient (API)
Association efficiency (AE)
AUC (area under the curve)
Charged Aerosol Detector (CAD)
Dolutegravir (DTG)
Drug-combination nanoparticle (DcNP)
Human immunodeficiency virus (HIV)
Lamivudine (3TC)
Long-acting (LA)
Lopinavir (LPV)
Molecular weight (MW)
Molecular weight cut off (MWCO)
Nucleoside reverse transcriptase inhibitor (NRTI)
People living with HIV (PLWH)
Pharmacokinetics (PK)
Resolution per minute (rpm)
Reversed-phase high-performance liquid chromatography (RP-HPLC)
Ritonavir (RTV)
Sodium; [(2R)-2,3-di(octadecanoyloxy)propyl] 2-(2-methoxyethoxycarbonylamino) ethyl phosphate (mPEG-$_{2000}$ DSPE)
Targeted LA combination antiretroviral therapy (TLC-ART)
Tenofovir (TFV)
Trifluoroacetic acid (TFA)

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