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Plasma Protein Binding Determination for Unstable Ester Prodrugs: Remdesivir and Tenofovir Alafenamide

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A B S T R A C T

Remdesivir (RDV) and tenofovir alafenamide (TAF) are prodrugs designed to be converted to their respective active metabolites. Plasma protein binding (PPB) determination of these prodrugs is important for patients with possible alteration of free fraction of the drugs due to plasma protein changes in renal impairment, hepatic impairment, or pregnancy. However, the prodrugs' instability in human plasma presents a challenge for accurate PPB determination. In this research work, two approaches were used in the method development and qualification for PPB assessment of RDV and TAF. For RDV, dichlorvos was used to inhibit esterase activity to stabilize the prodrug in plasma during equilibrium dialysis (ED). The impact of dichlorvos on protein binding was evaluated and determined to be insignificant by comparing the unbound fraction (f_u) determined by the ED method with dichlorvos present and the f_u determined by an ultrafiltration method without dichlorvos. In contrast to RDV, TAF degradation in plasma is ~3-fold slower, and TAF stability cannot be improved by dichlorvos. Fit-for-purpose acceptance criteria for the TAF PPB method were chosen, and an ED method was developed based on these criteria. These two methods were then qualified and applied for PPB determinations in clinical studies.

Introduction

While a free drug can transverse across cell membranes and interacts with intracellular targets,[1] a protein-bound drug is unable to cross membranes due to its high molecular mass. Plasma protein binding (PPB) is used at various stages of drug development, including therapeutic index estimation, *in vitro* to *in vivo* extrapolation, first-in-human dosage determination, drug-drug interaction prediction, and in pharmacokinetic modeling.[2,3] PPB assays determine the drug free fraction, f_u, used for the calculation of the unbound drug concentrations in plasma. The impact of PPB on drug half-life depends on route of administration, extraction ratio, and volume of distribution of the drug.[4,5] For most drugs, reduced PPB does not affect drug exposure (i.e., area-under-the-curve) because the increased free plasma concentration can be counterbalanced by greater elimination through metabolism or excretion. However, PPB may be relevant in populations with altered protein binding due to changes in protein synthesis, turnover, and excretion.[2,5−7] A dose adjustment may therefore be required for populations with reduced levels of systemic proteins, such as renal- and hepatic-impaired patients, pregnant individuals, and infants.[5] These populations not only have altered PPB, but also have other physiological changes that can affect drug exposure. To ensure safety in these populations, a pharmacokinetic (PK) study with PPB data can assist in determining whether a dose adjustment is needed.

Nucleoside prodrugs (ProTides), characterized by a nucleoside analog attached to a phosphate masked by an amino acid ester and an aromatic group,[8,9] are often used in antiviral therapy. Remdesivir (RDV), an RNA polymerase inhibitor for COVID-19 treatment,[10,11] and tenofovir alafenamide (TAF), a nucleotide analog reverse

Abbreviations: ACN, acetonitrile; ADME, absorption, distribution, metabolism, and excretion; DMSO, dimethyl sulfoxide; ED, equilibrium dialysis; f_u, unbound fraction; HM, healthy-matched; LC, liquid chromatography; MS, mass spectrometry; NSB, nonspecific binding; PPB, plasma protein binding; PK, pharmacokinetic(s); QC, quality control; TTE, time-to-equilibrium; UF, ultrafiltration.

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transcriptase inhibitor for the treatment of HBV and HIV, are part of the ProTide drug class and are designed as substrates for various esterases. The unbound fractions of RDV and TAF can be hydrolyzed in plasma prior to permeation into cells, where they are metabolized into corresponding active phosphorylated forms that inhibit viral RNA replication (Fig. 1a-b). While the ProTides themselves are inactive, the administered parent molecule for the active metabolites can ultimately affect efficacy, and thus PK characterization of these ProTides, including PPB, is essential in drug development.

Both RDV and TAF are considered to be highly protein-bound by regulatory agencies. EMA guidelines consider >90 % PPB as "highly bound",[12] while FDA considers >80 % (renal)[13] and >90 % (hepatic)[14] as "highly bound." Both agencies recommend the usage of unbound concentrations in addition to the total drug concentrations for the calculations of PK parameters and suggest follow-up PPB studies to demonstrate PPB concentration independence if PPB is high. Preliminary data showed RDV to be 88−93.6 % bound to plasma proteins, while its two major metabolites, GS-704277 and GS-441524, are only 1−2 % bound.[15,16] TAF was 80-95 % bound to proteins,[17,18] based on an ultrafiltration method using spiked [^{14}C]-TAF with scintillation counting detection, done at a time when the impact of plasma container tubes/bags was not taken into consideration.[19] Because of the high degree of binding, development of PPB methods for RDV and TAF (redevelopment for improvement) were needed as their PPB studies are required by regulatory agencies. Moreover, more robust and accurate methods were needed to distinguish the PPB differences among patient cohorts with different degrees of impairments or with plasma protein concentration variations.

While accurate and reproducible determination of f_u for a compound remains a general challenge for the bioanalytical laboratory,[20]

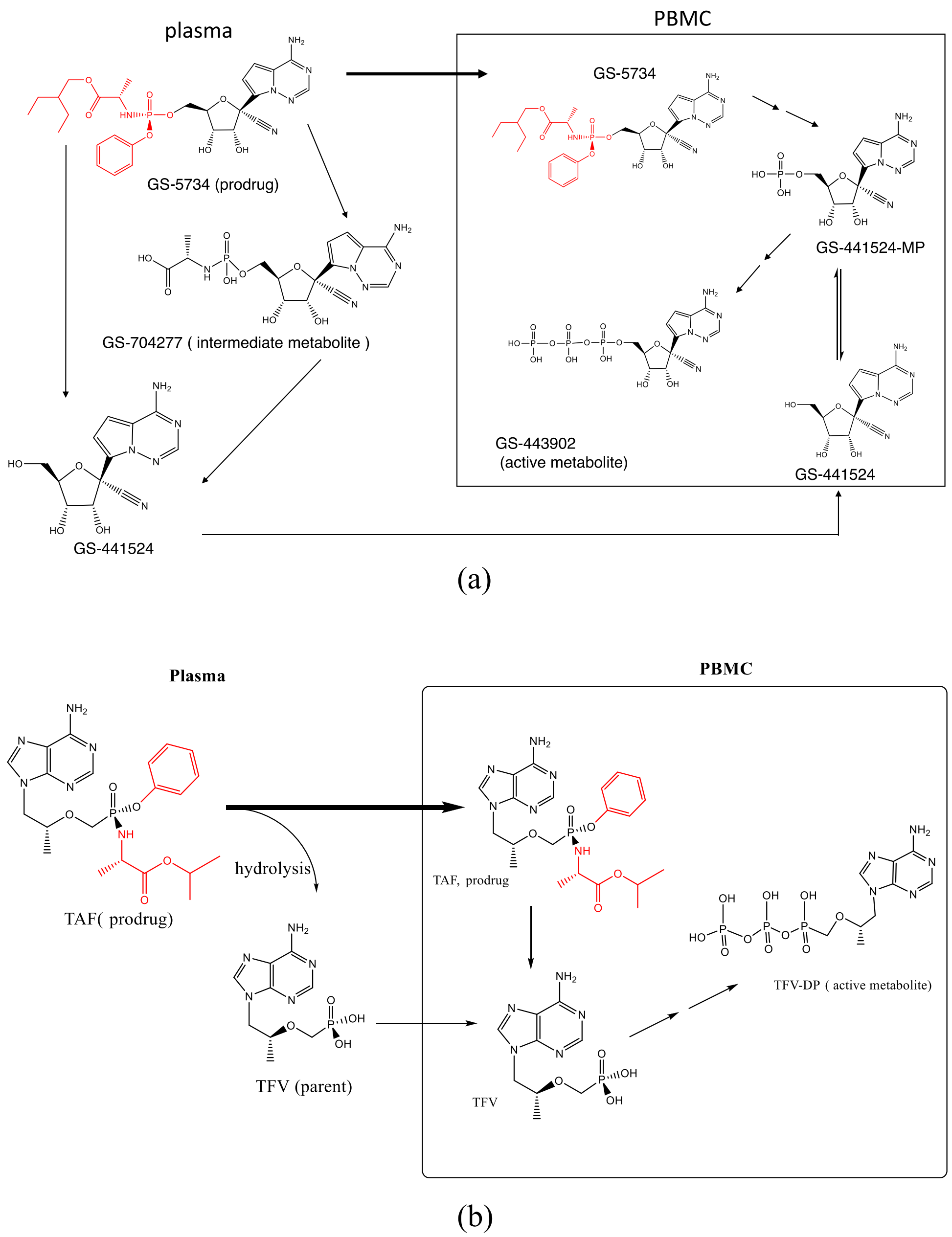


Figure 1. a. The conversion scheme of RDV (GS-5734) to intermediate metabolites GS-704277 and GS-441524 in plasma, and their permeation into PBMC and conversion to the active metabolite GS-443902 in PBMC. b. The conversion scheme of TAF to TFV in plasma, their permeation into PBMC and their conversion to TFV-DP in PBMC. The promoiety is shown in red.

it is more so for unstable prodrugs designed to be cleaved by esterases. Of several commercial protein binding methods available, the most common methods for PPB binding assays are equilibrium dialysis (ED),[21] ultrafiltration (UF)[22,23] and ultracentrifugation.[24] While ultracentrifugation lacks a membrane and thus often has low absorption to the device, it requires long centrifugation times (>6 h) at up to 300,000 g, which is problematic for unstable compounds. In addition, the technical requirement of precise pipetting below the top layer of lipoproteins makes this method low-throughput and error-prone. For UF, its short experimental time (<1hr) makes this method an attractive and viable option for unstable drugs. However, its nonspecific binding (NSB),[21] the volume ratio of ultrafiltrate to starting volume, and the impact of centrifugal force can impact the unbound fraction.[25,26] Considered as the gold standard high-throughput assay, ED is not impacted by nonspecific binding if equilibrium is achieved. However, ED requires relatively long equilibrium times, typically of several hours, making this method unfit for unstable compounds.

However, a possible solution for drug instability during ED is to stabilize the drug in plasma during the ED incubation. Among stabilization methods for unstable compounds, such as the usage of low temperatures (ie 4 °C), addition of weak acid,[27,28] enzymatic inactivation, enzymatic inhibition, and sample dilution,[2] only the latter two are applicable for PPB as they don't alter the physiological conditions of 37 °C and pH 7.4. Because sample dilution requires sensitive detection assays for compounds with high PPB, we targeted the use of enzyme inhibitors for stabilization. Besides reducing esterase activity, an ideal inhibitor should not alter the pH of plasma, coagulate plasma, destabilize the drug, or interfere with protein binding or analyte detection. Esterase inhibitors that have been used include diisopropylfluorophosphate, paraoxon, phenylmethane-sulfonylfluoride, bis(4-nitrophenyl)-phosphate, sodium fluoride, acetylcholine, eserine, thenoyltrifluoro acetone, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, H-D-Phe-Phe-Arg-chloromethyl ketone and EDTA[29] for the inhibition of plasma esterases, such as butyrylcholinesterase, paraoxonase, and albumin.[30,31] Recently, butyrylcholinesterase was shown to hydrolyze RDV into metabolite GS-704277 in plasma[32] and was irreversibly inhibited by the dichlorvos, an organophosphate also known as 2,2-dichlorovinyl dimethyl phosphate (DDVP).[33] This inhibitor had been previously used to stabilize the ester prodrug oseltamivir in plasma.[34]

In this study, we developed a plasma ED protein binding method for RDV using dichlorvos as a stabilizer and studied its impact on PPB and sample recovery. Being a more stable prodrug than RDV, TAF resists early enzymatic cleavage following oral administration and remains largely intact until it is taken up by target cells.[35−39] Since TAF was not stabilized by dichlorvos, we developed a plasma ED protein binding method without using a stabilizer and used fit-for-purpose acceptance criteria. These PPB methods were qualified and were applied to plasma samples to determine f_u.

Experimental

Chemical and Materials

Potassium Phosphate Buffer

A 0.133 M potassium phosphate buffer was prepared by combining 190 mL of 0.133 M monobasic potassium phosphate with 810 mL of 0.133 M dibasic potassium phosphate. Sodium chloride (9.0 g) was dissolved in 1.0 L of the potassium phosphate buffer. The pH was adjusted to 7.4 (pH = 7.4 ± 0.1), and the buffer was refrigerated until use.

Blank Human Plasma

Blank human plasma containing K_2EDTA as anticoagulant was pooled from at least three males and three females and was used for the method development and qualification. The blank plasma was obtained from BioIVT (Westbury, NY) and was stored at -70 °C. It was completely thawed at room temperature and centrifuged at approximately 500 g for 5 min to remove any precipitate before initiating an experiment. This blank human plasma was used in the preparation of plasma quality control (QC) samples and in mixed matrix preparation for buffer sample collection at the end of ED.

Preparation of Stock Solutions, Secondary Spiking Stock Solutions and Internal Standards

The primary stock solution of RDV (1000 μg/mL) was prepared in acetonitrile (ACN):dimethyl sulfoxide (DMSO) (1:1, v/v), and secondary RDV spiking stock solutions (100, 200, and 400 μg/mL) were prepared by diluting with the same solvent. The primary stock solution of TAF (1000 μg/mL) was prepared in DMSO. The secondary TAF spiking stock solutions (20, 100, and 200 μg/mL) were prepared by diluting the primary stock solution with DMSO.

All stock solutions and working solutions were stored at approximately -20 °C until use. The internal standard (ISTD) solutions were 50 ng/mL ISTD GS-829143 in methanol:water:formic acid (50:50:0.1, v/v/v), 40 ng/mL GS-652864 in methanol, and 50 ng/mL ritonavir in ACN.

Preparation of Spiked Predose Samples and Plasma Quality Control (QC) Samples

All spiked predose and quality control samples were prepared fresh on the day of the experiment. For the RDV experiments, dichlorvos prepared in DMSO was spiked into predose or blank human plasma to obtain a nominal concentration of 0.5 mM, and after a brief vortex, an appropriate volume of RDV stock solution was spiked into each aliquoted sample to obtain a nominal concentration of 800 ng/mL. After the addition of RDV and dichlorvos, the final volume of ACN and DMSO in spiked RDV plasma was 0.25 % and 0.75 %, respectively. The total volume of organic solvent was 1 %. For the TAF experiments, an appropriate volume of TAF stock solution was spiked into blank plasma samples to obtain a nominal concentration of 500 ng/mL for QC samples. The final volume of DMSO in TAF spiked QC plasma was 0.5 %. Warfarin plasma QC samples were also prepared fresh in polypropylene tubes by adding 10 μL of 200 μg/mL warfarin stock solution into 2 mL blank human plasma to obtain a nominal concentration of 1000 ng/mL. QC samples had <1 % organic solvent and were used as positive controls that were tested at the same time as clinical plasma samples during protein binding determinations.

Protein Binding Method Development

ED Method with Dichlorvos in Comparison to the UF Method Without Dichlorvos for RDV PPB Determination

To determine if dichlorvos impacted RDV plasma protein binding, a UF method without dichlorvos was developed to compare the ED method with dichlorvos. In the UF method, 1000 ng/mL RDV in plasma without dichlorvos was spun for 20 min at 1200 *g* in CentriFree 30K tubes (MilliporeSigma, Burlington, MA). The filtrate representing the free fraction was collected and analyzed for f_u. To determine the recovery, the ultrafiltrate was placed into a new Centrifree device and centrifuged at 1200 *g* for 20 min before collecting the second round of ultrafiltrate. Since RDV is highly bound and relatively lipophilic (log P = 1.249), NSB correction would be necessary to prevent underestimation of f_u. Though Tween solutions can correct for NSB, they may interfere with protein binding and were not used.[25,40]

For ED using the Harvard Dialysis device (Harvard Apparatus, Holliston, MA), RDV at 600, 1800, and 6000 ng/mL was dialyzed with 1 mM dichlorvos for 3 h. Having larger surface area for dialysis, the

Harvard Dialysis device was used instead of the Rapid Equilibrium Dialysis (RED) device (Thermo Scientific, Rockford, IL) to achieve equilibrium quickly. The unbound fractions determined by the ED and UF methods were then compared.

The Optimization of Stabilizer Concentration

The stabilities of RDV in human plasma and phosphate buffer were determined in triplicate per timepoint over a period of 24 h. RDV (1600 ng/mL) was added into phosphate buffer and blank plasma spiked with dichlorvos at concentrations of 0, 0.5, 1, and 2 mM. Each of the solutions was apportioned into aliquots for incubation at 37 °C. At times 0, 1, 6, and 24 h, three tubes of each solution were removed from incubation. To create a 50:50 plasma:buffer matrix for LC-MS analysis, plasma samples were diluted with buffer, while buffer samples were diluted with blank plasma spiked with 0.5 mM dichlorvos. A similar design was implemented using 0 or 0.5 mM dichlorvos and 500 ng/mL TAF tested over 0, 2, 4, 6, and 8 h. Ratios of concentrations or peak areas were used to determine f_u. Samples were analyzed on the same day of the experiment or stored at approximately -70 °C until analysis. The established frozen stabilities of RDV and TAF in plasma are 392 days and 520 days, respectively, which were considered to be a relevant surrogate of the 50:50 buffer plasma mixture.

Determination of Time-to-Equilibrium (TTE) and Concentration Dependence

Equilibrium dialysis was conducted using a single-use plate-based RED device containing dialysis membranes with a molecular weight cut-off of ~8000 Da. The TTE experiment was conducted in triplicate using human plasma containing 0.5 mM dichlorvos at two nominal RDV concentrations based on clinical relevance,[41] of 400 ng/mL and 1600 ng/mL RDV, which corresponded to approximately 20 % and 80 % average C_{max} of a renally-impaired cohort with dose adjustment. Time zero samples were mixed with blank buffer containing potassium phosphate buffer to create the 50:50 plasma: buffer matrix and were stored at -70 °C until analysis. Spiked human plasma (200 μL) and blank phosphate buffer (400 μL) were placed into respective donor and receiver chambers of the RED device. The dialysis plate was shaken at 250 rpm (VWR, Standard Orbital Shaker 3500) and incubated at 37 °C. At 2, 4, 6, and 8 h, three plasma aliquots of each RDV concentration were removed from incubation and diluted with blank buffer. Three buffer aliquots of each RDV concentrations were removed from incubation and diluted with blank plasma containing 0.5 mM dichlorvos. Equilibrium was considered reached when either the buffer/plasma concentration ratio (f_u) or the buffer concentration difference between two consecutive time points was ≤15 %.

For TAF, a similar design was implemented using 100 ng/mL and 1000 ng/mL TAF, of which the range captured both the average C_{max} and the maximum C_{max} seen in subjects after a standard 25 mg dose.[17,42,43] Concentrations were tested over 2, 3, 4, and 6 h but did not include dichlorvos as a stabilizer. Samples were analyzed on the same day of the experiment or stored at approximately −70 °C until analysis. Equilibrium was considered reached when the buffer/plasma concentration ratio (f_u) or buffer concentration difference between two consecutive time points is ≤30 %. The widened acceptance criterion was due to the instability of TAF at the experimental pH at 37 °C.

Interday Precision and Recovery

Protein binding was conducted on two separate days to evaluate day-to-day precision. On the day of the experiment, spiked plasma (200 μL) and blank phosphate buffer (400 μL) were placed into appropriate RED dialysis chambers. For pre-dialysis samples, three 100 μL aliquots of spiked plasma were transferred into tubes containing 100 μL of blank phosphate buffer. Triplicates of human plasma spiked at 0.5 mM dichlorvos and 800 ng/mL RDV or at 100, 500, 1000 ng/mL TAF were put on an orbital shaker at ~250 rpm in a humid incubator at 37 ± 1 °C with 5 % carbon dioxide. RDV was incubated for 6 h while TAF was incubated for 4 h. Known for its high protein binding, warfarin (1000 ng/mL) in human plasma was used as a positive control, in separate wells of the device on both days of the experiment. At the end of dialysis, a plasma:buffer (1:1, v:v) matrix was made for each chamber either by combining a 100 μL post-dialysis plasma with 100 μL blank buffer, or by combining a 100 μL post-dialysis buffer with 100 μL of blank plasma. The plasma:buffer samples were then stored at -70 °C until analysis.

Sample Preparation and Analysis

RDV

Stability, time-to-equilibrium, and inter-day protein binding samples were analyzed for RDV using a qualified LC-MS/MS method with calibrators prepared in a mixed matrix of human plasma containing 0.5 mM dichlorvos:phosphate buffer (1:1, v/v). For the extraction of standards, QC samples, and study samples, protein precipitation of sample aliquots (50 μL) was initiated by adding 50 μL methanol: water:formic acid (50:50:0.1, v/v/v) containing 50 ng/mL of ISTD GS-829143 and 500 μL of acetonitrile:formic acid (100:1.0, v/v). After vortexing 5 min, samples were centrifuged at ~1900 *g* for 15 min. A TomTec Quadra4 transferred 450 μL of the resulting supernatant from each tube into a clean 96-well plate. Each supernatant was evaporated in a 40 °C bath under nitrogen stream and then reconstituted with 150 μL of methanol:water:formic acid (50:50:0.1, v/v/v). The reconstituted samples were vortexed for 4 min at low-medium speed and centrifuged at ~1900 *g* for 2 min before analysis.

RDV extracts were injected on a Shimadzu Nexera UPLC system connected to an API 4000 Triple Quadrupole mass spectrometer (Sciex, Framingham, MA). RDV was eluted on a 2.1 × 50 mm, 3.5 μm XBridge Phenyl column (Waters Corporation, Milford, MA) at 40 °C. Mobile phases consisting of 5mM ammonium formate in water:formic acid (100:0.1, v/v) (MPA) and ACN:water:formic acid (95:5:0.1, v/v/v) (MPB) were run at 500 μL/min for the following gradient: 0-0.2 min at 30 % B, 0.2-2.0 min from 30 % to 100 % B, 2.0-2.6 min held at 100 % B, 2.6-2.8 min to starting conditions and 2.8-3.4 min for re-equilibration. RDV and its ISTD GS-829143 were detected by positive mode electrospray ionization using multiple-reaction monitoring for RDV (603.3 > 402.2 m/z) and ISTD GS-829143 (606.4 > 402.2 m/z). The calibration range for RDV analysis ranged from 1 to 500 ng/mL.

TAF

For the extraction of standards, QC samples, and study samples, protein precipitation of sample aliquots (50 μL) was initiated by adding 50 μL methanol containing 40 ng/mL of GS-652864 as internal standard and 500 μL of acetonitrile:formic acid (100:1, v/v). After vortexing for 5 min, the samples were centrifuged at ~1900 *g* for 15 min. After a TomTec Quadra4 transferred 450 μL of the resulting supernatant from each tube into a clean 96-well plate, each supernatant was evaporated to dryness in a 40 °C bath under nitrogen stream, reconstituted with 150 μL of acetonitrile:water:formic acid (30:70:0.1, v/v/v), vortex mixed for 4 min at low-medium speed, and centrifuged at ~1900 *g* for 2 min before analysis.

An LC-MS/MS assay for the determination of TAF in human plasma:ultrafiltrate (1:1,v/v) mixed matrix was qualified for a method using UPLC-MS/MS. A 4 μL aliquot of each sample extract was injected to a Waters Acquity UPLC equipped with a 2.1 × 50 mm, 1.7 μm BEH C18 column (Waters Corporation) at 40 °C. Mobile phases consisting of 5mM ammonium formate in water:formic acid

(100:0.1, v/v) (MPA) and ACN:H_2O:formic acid (95:5:0.1, v/v/v) (MPB) were run at 400 μL/min to create the following gradient: 0−0.3 min at 20 % B, 0.3−2.0 min from 20 % to 100 % B, 2.0-2.8 min maintained at 100 % B, 2.8-3.0 min to starting conditions and 3.0−3.5 for re-equilibration. Using API-4000 (Sciex), TAF and its ISTD GS-652864 were detected by positive mode electrospray ionization using multiple-reaction monitoring for TAF (477.2 > 176.1 m/z) and ISTD GS-652864 (484.1 > 176.2 m/z). The calibration range for TAF analysis ranged from 1 to 200 ng/mL.

Warfarin

Warfarin in mixed matrix of human plasma: phosphate buffer (1:1, v/v) was determined by an LC-MS/MS method using peak area ratio. For the extraction of controls and study samples, protein precipitation of sample aliquots (25 μL) was initiated by adding 200 μL ACN containing 50 ng/mL of ritonavir as internal standard. Samples were vortexed at high speeds for 5 min and centrifuged at ~1900 *g* for 15 min. A TomTec Quadra4 transferred 100 μL of the resulting supernatant from each tube into a clean 96-well plate containing 100 μL of phosphate buffer. The samples were then vortexed for 4 min at low-medium speed and centrifuged at ~1900 *g* for 2 min before analysis.

A 2 μL aliquot of each sample extracts was injected into the LC-MS/MS system consisting of a Shimadzu Nexera UPLC system in tandem with an API 4000 Triple Quadrupole mass spectrometer (Sciex). Warfarin was eluted using a 2.1 mm × 50 mm, 1.8 μm Acquity UPLC HSS T3 column (Waters Corporation). Mobile phases consisting of H_2O:ACN (70:30, v/v) (MPA) and water:methanol:isopropanol:ACN: DMSO:formic acid (20:20:20:20:20:0.05, v/v/v/v/v/v) (MPB) were flowed at 600 μL /min for the following gradient: 0-0.1 min at 20 % B, 0.1-1.5 min from 20 % to 95 %B, 1.5-1.9 min maintained at 95 % B, 1.9-2.0 min to starting conditions and 2.0-2.8 min for re-equilibration. Transitions for warfarin (309.4 > 163.0 m/z) and ritonavir (721.3 > 296.1 m/z) were monitored.

Equations and Formulas

Percent Recovery, NSB, and Adjusted f_u Determination for UF

Percent recovery, percent nonspecific binding (NSB) and adjusted percent unbound were calculated using the following equations:

$$\%\,\text{Recovery} = \text{post filtration of the ultrafiltrate} / \text{pre filtration of the ultrafiltrate} * 100$$

$$\%\,\text{NSB} = 1 - \text{Recovery}$$

$$\text{Adjusted}\ \%\text{unbound} = \text{postfiltration}/\text{prefiltration} * (1 + \%\,\text{NSB}/100)$$

Percent Unbound and Percent Recovery Determination for ED

The percent unbound and percent recovery were calculated using the following equations:

$$\%\text{unbound} = C_b / C_p * 100$$

$$\%\text{Recovery} = (C_b * V_b + C_p * V_p)/(C_{pi} * V_p) * 100$$

Where C_b and C_p are post-dialysis buffer and post-dialysis plasma concentrations, respectively, while V_b and V_p are loaded buffer volume (400 μL) and plasma volume (200 μL), respectively. C_{pi} is the initial mean concentration of spiked plasma at time zero.

Precision and Recovery for Interday Analysis

Precision and recovery were evaluated with the following formula:

$$\%\text{CV} = \text{standard deviation}/\text{mean} * 100$$

$$\%\,\text{Recovery} = (C_{\text{post-dialysis plasma}} * V_{\text{post dialysis plasma}} + C_{\text{post-dialysis buffer}} * V_{\text{post dialysis buffer}}) / (C_{\text{pre-dialysis plasma}} * V_{\text{initial plasma}})$$

Results

The Impact of Adding Dichlorvos on PPB

Due to the short spin time of 20 min used in the UF method, a stabilizer was not needed to be added to samples. Using RDV at 1 ug/mL, the UF method without dichlorvos resulted 6.04 ± 0.41 % unbound corrected for NSB. At concentrations of 0.6, 1.8, and 6 ug/mL, the ED method with dichlorvos resulted 6.52 ± 0.23 %, 6.44 ± 0.79 %, and 7.40 ± 0.76 % for the percent unbound. The comparison between UF without dichlorvos and ED with dichlorvos shows that the stabilizer has no noticeable impact on the protein binding of RDV (Table 1).

Optimized Dichlorvos Concentration in Stabilizing RDV

The stability of RDV at 37 °C was tested in phosphate buffer without dichlorvos and in human plasma with and without dichlorvos at various concentrations in human plasma (Fig. 2**a**). In phosphate buffer without dichlorvos, RDV did not show dramatic degradation (<15 % in 6 h and <20 % in 24 h). In plasma without dichlorvos, RDV degraded to 29 % of the initial concentration by 6 h and completely degraded by 24 h. The addition of dichlorvos stabilized RDV at 37 °C for at least 24 h, at which concentrations were still within 15 % of the initial concentration. Mean unbound fractions in samples with dichlorvos concentrations of 0.5, 1, and 2 mM were not different at 24 h (p =0.4908). Dichlorvos at 0.5 mM was chosen for follow-up studies.

TAF Not Stabilized by Dichlorvos

TAF was not more stable in phosphate buffer than in plasma; the degradation rates were similar in both matrices (Fig. 2**b**). In plasma, TAF had degraded to 77 % of the initial concentration by 6 h either with or without 0.5 mM dichlorvos, suggesting that TAF was not stabilized by dichlorvos. Based on assumed linear degradation rates in plasma without dichlorvos, TAF was ~3x more stable than RDV in human plasma. TAF was deemed stable until 4 h and retains ≤ 15 % of

Table 1
Comparison between ultrafiltration without dichlorvos and equilibrium dialysis with dichlorvos.

Method	RDV conc	Dichlorvos Conc.	%Unbound	%Unbound Adjusted*
Ultrafiltration	1 μg/mL	0	4.72 ± 0.32	6.04 ± 0.41
Equilibrium Dialysis**	0.6 μg/mL	1 mM	6.52 ± 0.23	6.52 ± 0.23
Equilibrium Dialysis**	1.8 μg/mL	1 mM	6.44 ± 0.79	6.44 ± 0.79
Equilibrium Dialysis**	6.0 μg/mL	1 mM	7.40 ± 0.76	7.40 ± 0.76

* The %unbound using ultrafiltration method was adjusted using non-specific binding (NSB) data.
** Equilibrium dialysis (Harvard Dialysis Device) was conducted at 37 °C with 1 mM dichlorvos for 3 h dialysis time.

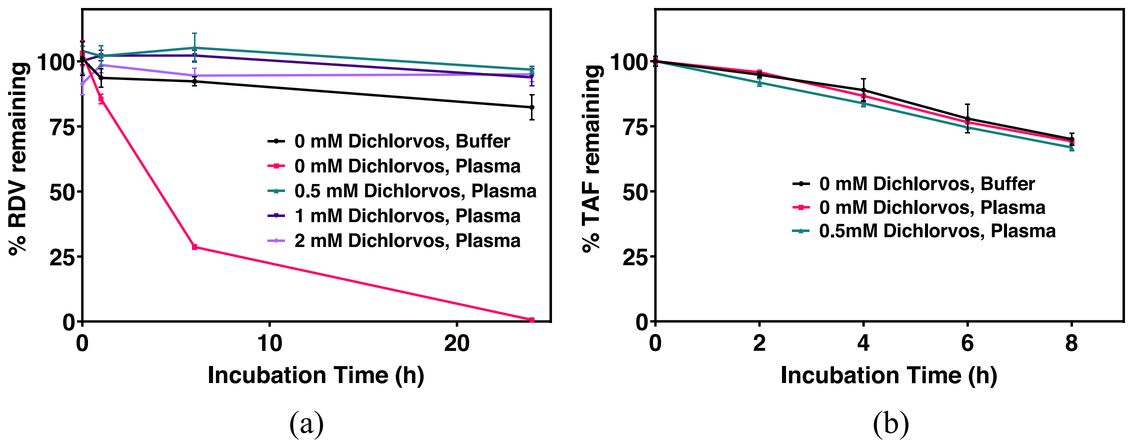


Figure 2. a. Stability of RDV at 37 °C in phosphate buffer without dichlorvos and in human plasma and with and without dichlorvos at various concentrations in human plasma. b. Stability of TAF at 37 °C in phosphate buffer without dichlorvos and in human plasma with and without 0.5 mM dichlorvos. Mean and SD are depicted using $n = 3$.

the initial concentration in human plasma and phosphate buffer during that time.

Time-to-Equilibrium and Concentration Dependence

Time-to-equilibrium (TTE) determinations for RDV with 0.5mM dichlorvos and TAF without dichlorvos at two analyte concentration levels are shown in Fig. 3a-b.

At clinically relevant RDV concentrations of 400 ng/mL and 1600 ng/mL, the f_u difference in human plasma was ≤15 % between 6 and 8 h, indicating that equilibrium was established after 6 h of dialysis based on our preset acceptance criteria. From the stability and TTE results, a 6 h dialysis time was selected for the protein binding determination using ED.

Due to the inability to stabilize TAF in human plasma, the acceptance criteria for TTE determination were expanded from ≤15 %. to ≤30 % between successive timepoints. For TAF, the buffer concentration percent difference between 4 and 6 h was 25 % at 100 ng/mL and 14 % at 1000 ng/mL. Because TAF was acceptably stable in plasma at 37 °C within 4 h and the percent buffer concentration difference between 4 and 6 h was within 30 %, the 4 h time point was selected for TAF dialysis time.

The unbound fractions and recoveries for RDV and TAF were concentration independent (Fig. 4a-b). Protein binding did not show concentration dependence at an RDV concentration range from 400 to 1600 ng/mL and a TAF concentration range of 100 to 1000 ng/mL. The changes in f_u and percent recovery for RDV and for TAFs were <15 % over the stated concentration ranges.

Intraday and Interday Precision and Recovery

RDV demonstrated a protein binding value of ~95 %. Compared to the overall mean percent unbound value (4.25 ± 0.374 %), the percent difference of interday percent unbound was within 10 %. Unbound values for TAF concentrations of 100, 500, and 1000 ng/mL in human plasma were 6.06 ± 0.22 %, 6.30 ± 0.41 %, and 6.34 ± 0.13 %, respectively, on day 1 and were 5.54 ± 0.04 %, 6.11 ± 0.20 %, and 6.42 ± 0.29 %, respectively, on day 2.

The percent unbound values between day 1 and day 2 were comparable at all tested concentrations; overall percent unbound values were approximately 6 %. Recoveries were 96-101 % for RDV and 90-94 % for TAF. Warfarin mean percent unbound values in human plasma were 0.89 ± 0.08 % for day 1 and 0.86 ± 0.05 % for day 2. The warfarin protein binding data in human plasma were consistent with literature (~ 99 % binding).

Application of the Methods

Accurate and precise determination of PPB is important for characterizing and interpreting any PK changes for highly protein-bound

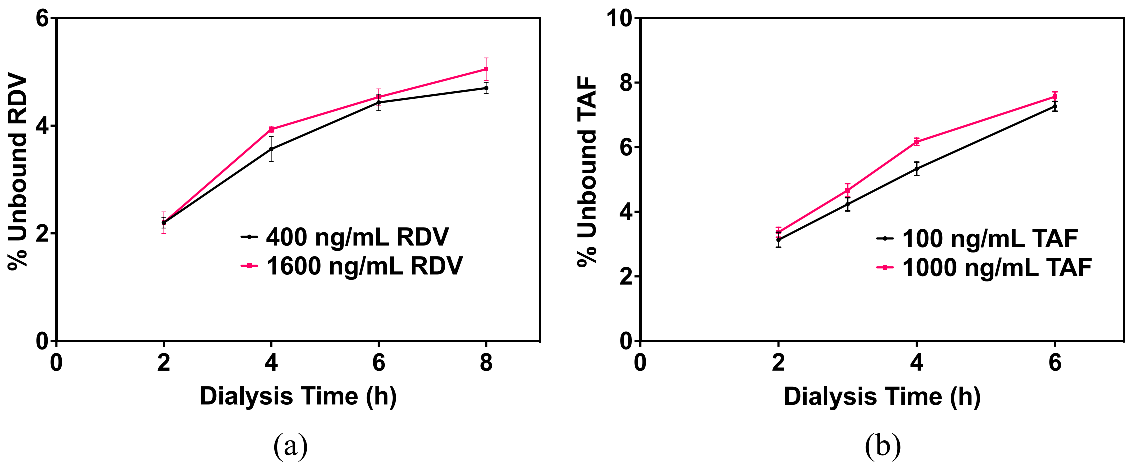
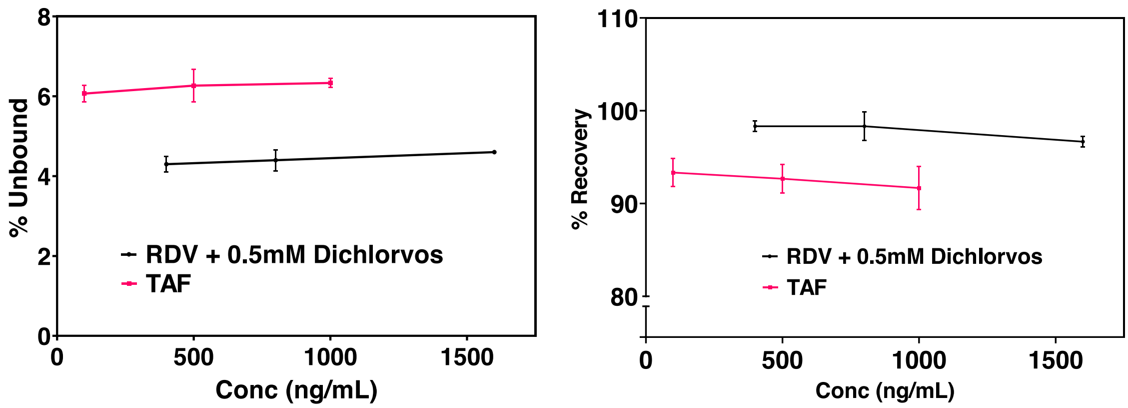


Figure 3. a. Time-to-Equilibrium determination for RDV with 0.5mM dichlorvos at two concentration levels. b. Time-to-Equilibrium determination for TAF at two concentration levels. Mean and SD are depicted using $n = 3$.



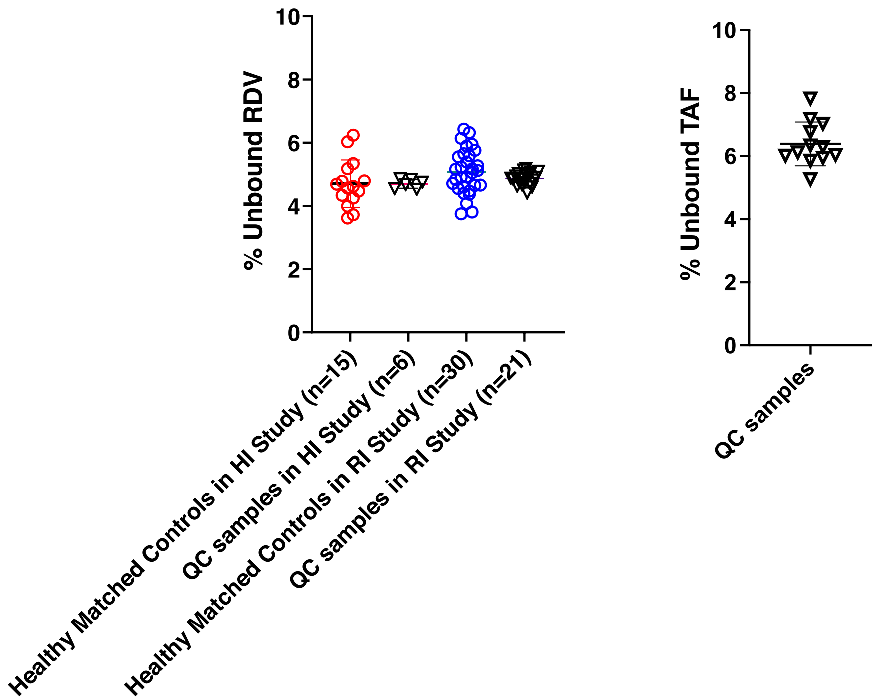
(a) (b)

Figure 4. a. Concentration dependence of %unbound at 400, 800, 1600 ng/mL of RDV with 0.5 mM dichlorvos and at 100, 500, and 1000 ng/mL of TAF without dichlorvos. b. Concentration dependence of %recovery at 400, 800, 1600 ng/mL RDV with 0.5 mM dichlorvos and at 100, 500, and 1000 ng/mL TAF without dichlorvos. Mean and SD are depicted using $n = 3$.

drugs, particularly in populations with altered protein binding. PPB methods for RDV and TAF were developed, qualified, and used to assess PPB in clinical studies. The RDV PPB method was applied to clinical samples from studies evaluating the PK of RDV in hepatically- and renally impaired participants and their healthy-matched (HM) control participants. For the hepatic study, the mean f_u values for the HM controls and spiked assay QCs were 4.4 ± 0.7 % and 4.7 ± 0.1 % respectively. Similarly, the mean f_u values for the HM controls and spiked assay QCs in the renal study were 5.0 ± 0.7 % and 4.8 ± 0.2 %, respectively (Fig. 5a). For TAF, spiked assay QC samples analyzed with samples from a clinical study had a mean f_u of 6.5 ±0.7 % (Fig. 5b).

Discussion

RDV and TAF are highly bound prodrugs that are unstable in plasma. For these two prodrugs we have developed precise equilibrium dialysis PPB methods that use two different approaches to resolve the instability issues of ester compounds. For RDV, the esterase inhibitor dichlorvos stabilized the drug in plasma for at least 24 h. For TAF, a second approach of setting wider, fit-for-purpose acceptance criteria for PPB parameters in method development and qualification were implemented. The degradation rates of TAF in plasma and buffer were similar and suggest inherent chemical instability rather than esterase activity as the reason for TAF's instability at 37 °



(a) (b)

Figure 5. a. % Unbound fractions of RDV in healthy-matched controls and QC samples in hepatic impairment (HI) and renal impairment (RI) studies. b. Unbound fractions of TAF in quality control samples.

C and pH 7.4. Although TAF was not stabilized by dichlorvos, its relatively slower degradation rate in plasma compared to RDV (degradation to 77 % vs 29 % of the initial concentration by 6 h) allowed implementation of a widened set of criteria during method development; specifically, f_u between two consecutive timepoints for TTE were expanded from 15 % to 30 %, and % recovery was reduced from ≥80 % to ≥50 %, while the criteria for interday precision (%CV) remained at 15 %.

Published data show RDV to be 88-93.6 % bound to plasma,[15,16] and the literature data are compatible with our interday results showing protein binding of 95 %. Prior data suggest TAF to be 80-95 % bound to proteins (unpublished data, not shown). Additional advances in PPB methods developed after the earlier PPB assessments were conducted have identified and addressed various concerns and weaknesses in methodology, including limitations of ultrafiltration methods, radiopurity of spiked [^{14}C]-TAF, external effects of plasma collected in container bags, and the impact of TAF stability on PPB assessment. Our interday results show that TAF is 94 % bound, which is at the upper range of previously reported data.

In conclusion, quantitative PPB assessment is applied in multiple stages throughout drug development and plays an important role in PK characterization of highly protein-bound drugs. In particular, results from quantitative PPB assessment are used to further inform PK characterization of special populations with altered plasma protein concentrations. Here we describe two ED approaches that were developed to determine PPB of prodrugs RDV and TAF and implemented for determinations in clinical studies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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