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Real-Time Analysis of Tenofovir Release Kinetics Using Quantitative Phosphorus (^{31}P) Nuclear Magnetic Resonance Spectroscopy

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Abstract

The dialysis method is classically used for drug separation before analysis, but, does not provide direct and real-time drug quantification and has limitations affecting the dialysis rate. In this study, a phosphorus nuclear magnetic resonance (^{31}P -qNMR) method is developed for the real-time quantification of therapeutic molecules *in vitro*. The release kinetics of model drug tenofovir (TFV: anti-HIV microbicide) was analyzed in vaginal fluid simulant (VFS), semen fluid simulant (SFS), and human plasma (HP) from chitosan nanofibers (size ~100–200nm) using the NMR (direct) method and compared with dialysis/UV-Vis (indirect) method. The assay was linear in VFS/SFS (0.20–5.0mM), HP (0.30–5.0mM) and specific (no drug ^{31}P -qNMR chemical shift (~15ppm) interference with formulation/media components). LOD values were 0.075/0.10/0.20mM, whereas, LOQ values were 0.20/0.20/0.30mM in VFS/SFS/HP, respectively. The method was robust, precise (%RSE <2%), and accurate (%mean recovery: 90–110%). After 12h, ~75%/72%/70% w/w of TFV release was observed with ^{31}P -qNMR, compared to ~47%/52%/52% w/w by dialysis method in VFS/SFS/HP, respectively. Approximately 20% decrease in %drug release observed with dialysis method suggested an interference with drug transport process due to the dialysis membrane and the Gibbs-Donnan effect. Overall, ^{31}P -qNMR provides more accurate, real-time and direct drug quantification for effective *in vitro/in vivo* correlation.

Keywords

Controlled release; Encapsulation; HIV/AIDS; HIV vaginal microbicide; *In vitro/in vivo* correlations; Nanotechnology; NMR spectroscopy; UV/Vis spectroscopy

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INTRODUCTION

Accurately quantifying the amount of drug being delivered in the human body is a crucial requirement of any drug development process. Most quantitative techniques such as liquid chromatography (LC) and UV-Vis spectrometry require a dialysis membrane to separate the free drug released from their formulations and to determine it in biological fluids. In the dialysis method, drug formulations are enclosed in a dialysis bag with an appropriate molecular weight cutoff (MWCO) membrane in the donor compartment (Figure 1A). A sink condition has to be maintained to separate the drug in the donor compartment with respect to the receiving compartment. The sink condition is achieved using a volume of medium at least three times higher than that of a saturated solution of the drug contained in the formulation being tested¹. The quantity of drug detected in the receiving compartment is generally considered as the amount of drug released from formulations. However, experimental results of release quantification methods utilizing dialysis membrane can be misleading since donor compartment does not satisfy the sink condition in majority of cases²⁻⁴. In addition, dialysis method has some potential limitations and several considerations should be taken into account in drug quantitation as summarized below.

(a) The volume of drug release medium inside the dialysis membrane should be at least 6-to-10-fold lower to maintain the sink conditions and provide a driving force for drug transport out of the membrane. (b) The outer media must be agitated to prevent the accumulation of polymer degradation products, especially for protein formulations. Agitation can affect the drug release kinetics because the concentration equilibrium time is prolonged if the bulk media is not stirred due to the formation of the unstirred water layer. (c) At least two media changes are needed over the time-period of 12-24 h. (d) An appropriate membrane surface molecular weight cutoff and surface area must be selected. (e) Dialysis is a two-step process: diffusion of drug from the carrier to the interior of the dialysis membrane and then the external reservoir. The drug release is governed by the partition between two phases (donor and receiving compartments) separated by the dialysis membrane²⁻⁴ (Figure 1B). (f) Dialysis bag with a higher drug concentration requires a longer-time for the drug concentration equilibrium between compartments. (g) Several process variables to be optimized and affecting the dialysis rate include the pH, temperature, time, concentration, volume, stirring speed, sampling time. (h) Concentration gradient between inside and outside of the dialysis bag may affect the observed release rate. (i) Achievement of drug concentration equilibrium in the outer media is slow (if the membrane surface area is small) or too quick (if the surface area is very large). (j) Due to two diffusion barriers (drug carrier matrix and dialysis membrane), the drug release kinetics is reduced to various degrees depending on drug/carrier systems potentially leading to significant errors. (k) This approach cannot be used if the drug binds to the polymer or dialysis membrane. (l) The dialysis method is not capable of direct and real-time quantification of drug release kinetics.

These limitations make the dialysis method a less than ideal choice for drug release profiling. Hence, there is an urgent and critical need for developing a direct, real-time, and specific method for the quantification of drug release kinetics. Therefore, in this study, a realtime solution-state quantitative phosphorus nuclear magnetic resonance (³¹P-qNMR)

method was developed and validated for the analysis of phosphorus containing therapeutic molecules in biological fluids. It is noteworthy that phosphorus is among the top 10 elements commonly found in the pharmaceutical drugs and one of the most frequently represented heteroatoms in addition to sulfur and fluorine⁵. The developed NMR method was applied for real-time quantification of model drug (tenofovir) release kinetics from its nanoformulations.

Quantitative NMR spectroscopy

NMR spectroscopy is one of the most powerful and widely used forms of spectroscopy in pharmaceutical sciences since its discovery in early 1950s⁶⁻⁸. Quantitative NMR (qNMR) is one of the techniques in drug analysis that offer several advantages over other analytical techniques including the use of a universal reference standard for quantifications^{7, 9, 10}. The widespread acceptance of NMR for quantitative measurements can be attributed to specific advantages such as, (i) possibility to determine structures at a molecular level, (ii) no need for intensity calibrations in case of determination of ratios since signal area is directly proportional to the number of nuclei, (iii) relatively short measuring times, (iv) NMR nondestructive character, (v) no need for prior isolation of analyte in a mixture, (vi) possibility of a simultaneous determination of more than one analyte in a mixture, and (vii) easy sample preparation and handling⁶⁻⁸.

NMR spectroscopy in drug release analysis from nanoformulations

Recently, Zhang et al., have published a proton (¹H) NMR method¹⁰ to quantify an anti-HIV topical vaginal microbicide drug (tenofovir: TFV) in vaginal fluid simulant (VFS) and in a mixture of vaginal and seminal fluid simulant (VSFS) buffers. Authors have compared NMR method with dialysis membrane method to analyze drug release pattern using deuterated solvents in ¹H-NMR spectroscopy, making this method less economic and not routinely applicable in biological fluids. Carbon NMR (¹³C-NMR) spectroscopy is another commonly used NMR approach for versatile identification of carbon atom electronic environments. However, most drugs contain more than one carbon and some of these carbons are in very similar electronic environments. As a result, the overlap of the ¹³C signal is inevitable, especially in relatively large compound. Furthermore, the abundance of ¹³C active nuclei is relatively low (~1.1%) requiring higher sample amount/concentration in ¹³C-NMR assay. Implementation of ¹³C-qNMR spectroscopy is thus less economical and sensitive considering γ the gyromagnetic ratio and abundance factors in routine, especially for *in vivo* assays¹¹.

³¹P-qNMR is an excellent technique for studying phosphorus containing compounds and typically gives rise to a single sharp peak that corresponds to single phosphorous atom being a part of the drug molecule. ³¹P-qNMR has been proven to measure small quantities of phosphorus containing molecules *in vivo*¹². There are essentially five main motivations for ³¹P-qNMR spectroscopy investigation for real-time quantitation of such compounds. First, ³¹P nucleus has a relatively higher gyromagnetic ratio γ (17.235 MHzT⁻¹) in comparison to that of ¹³C (10.705 MHz T⁻¹) nucleus^{13, 14}. Second, ³¹P nucleus has a 100% natural isotopic abundance which makes ³¹P-qNMR more economical and sensitive (~379-times more considering both γ and abundance factors) than ¹³C-NMR. Third, like ¹H

nucleus, ^{31}P nucleus has a nuclear spin of $\frac{1}{2}$, which makes NMR spectra relatively easy to interpret. Fourth, there is no need of expensive deuterated solvents in ^{31}P -qNMR compared to ^1H -NMR, which simplifies the sample preparation process. Fifth, ^{31}P -NMR exhibits wider ordinary range of chemical functionalities and shifts from about $\delta 250$ to $-\delta 250$ (not primarily due to the diamagnetic shielding magnitude, but rather induced by paramagnetic shielding tensors).

Considering the drawbacks related to ^{13}C -NMR and ^1H -NMR assays, in this study, a specific, and direct ^{31}P -qNMR method was developed and validated for the real-time quantification of therapeutic molecules in biological fluids such as vaginal fluid simulant (VFS), seminal fluid simulant (SFS), and human plasma (HP). An anti-HIV topical microbicide tenofovir (TFV) was selected as model phosphorus containing drug (Figure 2A). TFV is a nucleotide reverse-transcriptase inhibitor under the category of antiretroviral drugs, a weakly acidic, water-soluble, stable, and low molecular weight (MW: 287.213 Da) drug^{15, 16}. TFV is a Food and Drug Administration (FDA) approved vaginal microbicide (in its pro-drug form)¹⁷. The developed NMR method was applied for real-time quantification of TFV release kinetics from its nanoformulations in the above biological fluids.

A chitosan based nanofibers (NFs) formulations of TFV was fabricated using electrospinning method. Chitosan is a linear amino-polysaccharide, composed of 2-amino-2-deoxy- β -D-glucan combined with glycosidic linkages and its solubility is pH dependent. Chitosan exhibits many advantages in formulation development, including biocompatibility, biodegradability, and a low immunogenicity^{18, 19}. Electro-spinning offers great flexibility in selecting biomaterials in drug delivery applications. Employing different techniques, such as blending, coaxial process, and emulsification methods, incorporating drugs into NFs via electro-spinning techniques is very versatile. Therefore, electro-spun NFs have been extensively studied in drug delivery applications^{20–23} and offer potential advantages such as high drug loading, flexibility in the drug formulation shapes, high surface-to-volume ratio and, high porosity^{21, 24}. Furthermore, the encapsulation of instable molecules in NFs can potentially enhance their stability against harsh biological/physiological environments.

MATERIALS and METHODS

Chemicals

Tenofovir (TFV) was purchased from Beijing Zhongshuo Pharmaceutical Technology Development Co. Ltd. (Beijing, China). Chitosan (MW 50-190 kDa), thioglycolic acid, poly (ethylene oxide) (PEO: MW 900 kDa), were purchased from Sigma Aldrich (St. Louis, MO, USA). Poly (DL-lactide) (PLA) was obtained from Purac Biomaterials (Lincoln, IL, USA). Deionized water for all experiments was obtained through a Millipore Milli-Q water purification system (Millipore Corp., Danvers, MA, USA). All other chemicals were of analytical grades and used as obtained from suppliers. The pH of all solutions was adjusted using either hydrochloric acid (HCl) or sodium hydroxide (NaOH) solutions prepared accordingly and measured using a SevenEasy pH meter (Mettler Toledo, Schwerzenbach, Switzerland) under ambient temperature conditions (23-24°C).

³¹P-qNMR spectroscopy analysis of TFV

The experimental procedure for ³¹P-qNMR spectroscopy was carried out on a Varian 400 MHz spectrometer (Palo Alto, CA, USA) with a Varian two channel probe. Vnmrj software (version 2.2) (Palo Alto, CA, USA) was used to process the data. Preliminary analyses were performed to get the optimized NMR conditions such as number of scans, temperature, pulse width, and relaxation delay. Typically, spectra were acquired without proton decoupling with a 45° pulse width of 5.05 μs. Therefore, no nuclear Overhauser effect (transfer of *nuclear* spin polarization from one spin bath to another spin bath via cross-relaxation) would be present. The relaxation delay and number of scans were set at 25 sec and 256, respectively. Relaxation delay is one of the most important parameters in qNMR assays. In order to make most nuclei fully relaxed, 3-5 times of T_1 (Spin-lattice or longitudinal relaxation time) is required. The T_1 of the phosphorous atom of TFV was 5 sec which typically requires up to 25 sec ($5 \times T_1$) as relaxation delay.

The line-width of the NMR signal directly depends on T_2 (spin-spin or transverse relaxation time) which varies for different molecules ($\text{Line-width} = (\pi T_2)^{-1}$). T_2 is the time required for the transverse magnetization to fall to approximately 37% (1/e) of its initial value. T_2 is usually very small for large molecules such as polymers or proteins, which gives rise to very broad peaks. Therefore, if the drug is free in solutions, phosphorous signal can be easily detected due to sharp peaks. However, if drug is encapsulated inside formulations, the drug peak generated could be very broad because of the slow tumbling.

Shimming (adjusting the resolution of NMR signals by optimizing homogeneity of the magnetic field) was applied to every sample in order to obtain a similar line width in each spectrum. Baseline correction, phase adjustment, and integral calculation were carried out manually using MestReNova LITE 5.2.5-4731 software (Escondido, CA, USA). NMR assay was performed at 37°C with a spinning frequency of 20 Hz. Kinetics experiments were applied where intensities of ³¹P peak of TFV was utilized to quantify the amount of TFV released from nanoformulations. The total time for one release experiment was 12 h. The integration of ³¹P peak of TFV was used to quantify the amount of released drug.

TFV sample preparation for ³¹P-qNMR spectroscopy analysis

TFV samples were prepared at concentrations of 0.10 - 5.0 mM in VFS (pH: ~4.2), SFS (pH: ~7.8), and HP (pH: ~7.4). These VFS and SFS buffers were prepared according to previous reports^{25, 26}. However, commercially available pooled HP (Innovative Research, Inc., Novi, MI, USA) was used. A stock solution of TFV (1 M) in respective biological fluids was diluted with the same fluid to yield solutions in the concentration range of 0.10 - 5.0 mM. NMR analysis was performed by taking 500 μl of each sample in a 5-mm outer diameter NMR tubes (Wilma-LabGlass, Vineland, NJ, USA) and analyzed using ³¹P-qNMR method. Orthophosphoric acid (OPA: 85% v/v) was used as the external reference compound. The integrated peak area of ³¹P atom in TFV was used to estimate its amount in each sample.

Validation of ^{31}P -qNMR method

^{31}P -qNMR method was validated according to the International Conference on Harmonization (ICH) guidelines Q2:R1²⁷ for several parameters as explained below.

Limit of quantification (LOQ) and limit of detection (LOD)—LOQ and LOD values represent the lowest quantity of a substance that can be distinguished from the absence of that substance, and concentration at which quantitative results can be reported with a high degree of confidence within a stated confidence limit, respectively²⁷. In this study, LOQ and LOD values were determined using signal-to-noise (S/N) ratios of 10:1 and 3:1, respectively.

Linearity and calibration curve—Linearity is the ability of a method to elicit test results that are directly proportional to analyte concentration within a given concentration range. The drug calibration plot related the peak areas to the drug concentration in the range of 0.20 - 5.0 mM in VFS, SFS, and 0.30 - 5.0 mM in HP. The intercept, slope, and correlation coefficient (*R*) values were determined by linear regression analysis. Blank drug release medium was used as the baseline reference.

Precision and accuracy—Precision and accuracy represent the level of measurement that yields consistent results (no random errors) and true results (no systematic errors) when repeated, respectively. Accuracy is the percent of the therapeutic molecule recovered by assay. Accuracy of ^{31}P -qNMR method was calculated for three quality controls (QC) samples (0.20 mM, 0.75 mM, and 5.0 mM in VFS, and SFS, and 0.30 mM, 0.75 mM, and 5.0 mM in HP). Accuracy results were reported as percent mean recovery. Precision was assessed using the same three QC samples and reported as percent relative standard error (%RSE). Intra-day (within-day) and inter-day precision and mean recovery (over a period of one week) were determined. Repeatability expresses the precision of the method under the same operating conditions over a short time interval. Repeatability was analyzed by using three QC samples of TFV (as stated above), in triplicate ($n = 3$), each a day in VFS, SFS and HP.

Robustness—The robustness of an analytical assay is its ability to remain unaffected by small variations in assay parameters. Following changes in ^{31}P -qNMR method parameters were examined: temperature (in °C), relaxation delay (in seconds), and 45° pulse width (in μs). The variations in ^{31}P -qNMR peak area of TFV at the concentration of 5.0 mM were calculated and an acceptance criterion of %RSE < 2% was considered for each parameter.

Specificity—Specificity of a method was determined by analyzing the potential interference peaks generated by formulation/biological media components to the analyte peak. ^{31}P -qNMR spectrum of TFV in VFS/SFS/HP was compared to that of drug-free as well as TFV loaded nanoformulations suspended in these biological fluids to ensure the specificity.

Application of the developed ^{31}P -qNMR method

The developed ^{31}P -qNMR method was applied to determine the release profile of TFV in its nanoformulations as explained below.

Fabrication of chitosan based TFV loaded electro-spinning NFs—TFV

encapsulated chitosan NFs were prepared by using a coaxial electro-spinning method²¹. Initially, chitosan-thioglycolic acid conjugate (TCS) was synthesized by introducing thioglycolic acid (TGA) to chitosan via amide bond formation mediated by a carbodiimide (EDC/NHS) coupling reaction¹⁸. In order to confirm the effective conjugation between chitosan and TGA, ¹H-NMR spectroscopy analysis was performed. Samples were dissolved in deuterium oxide (D₂O) and spectra were observed using a Varian (Palo Alto, CA, USA) 400 MHz spectrometer with a Varian two channel probe. V_{nmrj} 2.2 (Palo Alto, CA, USA) software was used to process the experimental data. Based on the results of NMR spectroscopy, the conjugation between chitosan and TGA was confirmed (amide bond formation: data not shown)²¹.

Chitosan is a cationic polysaccharide and the amino groups (pKa ~6.5) on the polymer backbone are positively charged below the pKa value. During the electro-spinning process, the repulsive force between ionized amino groups within the polymer backbone limit its electro-spinnability and inhibits the formation of continuous fiber under the high electric field²⁸. Therefore, the electro-spinning of pure TCS typically forms beads and droplets instead of ultrafine fibers²⁹. To overcome this drawback and assure continuous NF formation, PLA and PEO were used. PLA is biodegradable aliphatic polyester, and has been approved by the FDA for human clinical applications. PLA is a good fiber-forming polymer^{30, 31}. Moreover, PLA helps crosslink and solidify the shell by forming hydrogen bound with chitosan³². Some works have been devoted to the manufacture PLA/chitosan composites^{33, 34}. In our preliminary study, it was observed that NFs became more uniform when equal amounts of TCS and PLA were used. Thus, to formulate the chitosan NFs, the TCS conjugate was mixed with PLA in a ratio of 1:1 (% w/w) and used as the shell material.

PEO, a well-known fiber-forming polymer^{35, 36} was used as a core material to enhance the fiber formation capability in the electro-spinning process. PEO as a fiber-forming polymer has attracted a great attention due to its water-soluble, biodegradable, and biocompatible properties³⁷. Thus, a blend of TFV and PEO was used as core material in the electro-spinning process. The PEO core solution acts as a carrier that forms a stable Taylor cone and draws out the shell chitosan solution by forming a continuous jet ejection³⁸.

A coaxial spinneret was used to allow for the injection of core solution into the shell solution at the tip. The core composite solution was typically prepared by dissolving PEO into 50% v/v formic acid aqueous solution at a concentration of 20 mg/ml and stirring over night to yield a homogeneous solution. TFV was dissolved in the same solution at a concentration of 50 mg/ml. To prepare the shell composite solution, TCS-PLA blend (1:1 w/w) was dissolved in pure formic acid at the concentration of 3% (w/v) and tripolyphosphate (TPP) was added in the solution at the ratio of TCS: TPP is 8:1 (w/w). The core and shell composite solutions were placed in two glass syringes (Becton, Dickinson and Company, NJ, USA), and pushed by two syringe pumps (Cole-Parmer Instrument Company, IL, USA) with the feeding rate of 0.05 ml/h for both solutions. A high voltage power supply (Gamma High Voltage Research, Inc., FL, USA) was used to provide a voltage of 15 kV to the nozzle. Chitosan NFs were collected onto an aluminum collector connected to the ground electrode. NFs mat was

peeled from the collector, vacuum dried at room temperature and stored in a vacuum desiccator until further uses.

Surface morphology, size distribution, percentage yield and drug loading of chitosan NFs—Chitosan NFs were characterized for their surface morphology, and size distribution. The surface morphology was analyzed by scanning electron microscopy (SEM). Briefly, a small amount of NFs was put onto a grid. The membrane was mounted on a 1/200 SEM stubs with double-sticky carbon tape. NFs samples were then sputter coated with 20 nm thickness of gold and visualized under a Philips SEM 515 microscope (Eindhoven, The Netherlands) and observations were performed at an accelerating voltage of 5 kV. The diameter of individual NFs was analyzed using Image Pro® Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA). The following measurements were made: average fiber diameter, average bead diameter, and the approximate ratio of bead area to total bead and fiber area. The diameter of NFs in the SEM images was estimated based on a count of at least 100 fibers.

The percent drug loading (%DL) was determined indirectly from the supernatant after ultra-centrifugation and appropriate dilution using UV-Vis spectroscopy at a wavelength of 259 nm³⁹. The process yield of NFs recovery was determined using mass balance calculation. To determine the drug loading, approximately 1 mg of TFV loaded NFs were submerged in 50 ml of water for 48 h. In this scheme, the blend of hydrophilic TFV and the PEO core of the NFs was completely dissolved and extracted in water leaving an empty shell as proven by transmission electron microscopy (TEM) in a recent publication²¹. After ultra-centrifugation, the TFV concentration in water was detected using UV-Vis spectroscopy at the wavelength of 259nm. The percent drug loading was calculated by using Equation 1:

$$\text{Drug ; loadins (\%)} = \frac{\text{Total ; amount ; of ; TFV}}{\text{Total ; amount ; of ; NFs}} \times 100\% \quad (1)$$

In vitro TFV release analysis from chitosan NFs using dialysis (indirect) method—Chitosan NFs (1 mg/ml) in VFS, SFS and HP were added in the dialysis bag (Spectra/Por Float-A-Lyzer G2, MWCO 3.5-5 KD, Spectrum Laboratories Inc. Rancho Dominguez, CA, USA) were placed into a dialysis tube containing 20 ml of release media. The whole system was kept at 37°C water bath with a shaking speed of 60 rpm. At predetermined time intervals up to 12 h, 1 ml of the sample was taken out and replaced by fresh release media to maintain the sink conditions. The concentration of TFV in the dialysis solution was determined by UV-Vis spectroscopy at 259 nm. Each experiment was run in triplicate (n = 3) together with a blank control. The percent cumulative drug release profile of TFV was measured using a calibration curve of TFV in the concentration range of 1 - 10 µg/ml³⁹.

Real-time in vitro drug release analysis of chitosan NFs using ³¹P-qNMR (direct) method—In order to determine the real-time, *in vitro* drug release kinetics, chitosan NFs loaded with TFV at the concentration of 1 mg/ml were dispersed in VFS, SFS and HP. The samples were prepared in NMR glass tubes and analyzed by taking 1 ml of

each sample using this ^{31}P -qNMR method at 37°C with a spinning frequency of 20 kHz. Data were collected at different time points in real-time measurements for 12 h (0.5, 1, 2, 3, 4, 6, 8, 10 and 12 h), using 256 scans. The percent drug release was measured using a calibration curve of TFV in the concentration range of 0.20 - 5.0 mM in VFS and SFS, and 0.30 - 5.0 mM in HP.

In vitro drug release kinetics of TFV in chitosan NFs—Drug release data of TFV loaded chitosan NFs were analyzed by using various *in vitro* kinetic models^{15, 40} Microsoft Excel add-in DDSolver program⁴¹ was used for data modeling. Kinetic models used were zero-order, first-order, Higuchi, Korsmeyer-Peppas, and Weibull models. The criteria for selecting the most appropriate model were based on the coefficient of determination (R^2) and the Akaike Information Criterion (AIC)⁴¹. AIC is used to measure goodness of fit based on maximum likelihood. The selection of an appropriate model was based on both higher R^2 values and lower AIC values.

Statistical data analysis

Experimental values were generally presented as mean $\pm$ standard deviation (SD) of triplicate determinations ($n = 3$) unless otherwise mentioned. The histogram and size distribution curves were generated by IBM® SPSS® Statistics software Version 23 (IBM Corp., Armonk, NY, USA) for at least 100 NFs per sample. Statistical analysis was evaluated using Students *t* test. The p value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

^{31}P -qNMR method development and validation

^{31}P -qNMR method of TFV was developed and validated according to the ICH guidelines²⁷. The method was linear over the tested concentration range of TFV in VFS (0.20 - 5.0 mM) (Figure 2B), SFS (0.20 - 5.0 mM) (Figure 2C), and in HP (0.30 - 5.0 mM) (Figure 2D) with R^2 value > 0.999 . The LOD values were found to be 0.075 mM (VFS), 0.10 mM (SFS), and 0.20 mM (HP), whereas, the LOQ values were 0.20 mM for VFS and SFS, and 0.30 mM for HP, respectively. NMR spectrum of free TFV was observed to give a specific single resonance line corresponding to its single phosphorous atom (Figure 2A). ^{31}P -qNMR peak of TFV was observed at ~ 15.7 ppm (Figure 3A), ~ 15.1 ppm (Figure 3C), ~ 13.7 ppm (Figure 3E), and ~ 15.7 ppm (Figure 3G) in VFS, SFS, HP and water, respectively. Results of repeatability analysis showed that NMR method was reproducible and showed consistent results in terms of the chemical shift value and area of TFV (data not shown). NMR method was accurate with a percent mean recovery value between 90% to 110% (Table 1) and precise as the %RSE values for intra-day, and inter-day precision were less than 2% for all three QC samples tested in VFS, SFS, and HP (Table 2). The % RSE for each of the robustness parameters tested at TFV concentration of 5.0 mM in each of three biological fluids was $< 2\%$ (Table 3). Results confirmed that the developed ^{31}P -qNMR method was robust, accurate and precise.

Specificity analysis demonstrated the lack of interference peaks between the signal of interest (^{31}P -NMR peak of TFV) and any other peak from components of the VFS (Figure

3B), SFS (Figure 3D), HP (Figure 3F), and OPA (Figure 3G), respectively. Chemical shifts in the TFV ^{31}P -NMR peak were slightly different in each biological fluid which might be due to the difference in their chemical environment and functional group associated with ^{31}P in each media. The water and VFS did not have any phosphorus atom containing components²⁶ therefore, no ^{31}P chemical shift was observed in water (Figure 3G) and blank VFS (Figure 3B) spectrum. However, the presence of sodium phosphates (mono and dibasic) in SFS^{25, 26} showed a peak at ~ 1.7 ppm (Figure 3D) in blank SFS.

Because the external standard (OPA) does not take into account the bulk properties of the sample, it is well documented in this field that chemical shifts in ^{31}P NMR usually depend on factors such as the sample concentration, type of solvent, and the co-existence of other compounds. Therefore, the reported chemical shifts for the same compound could vary by 1 ppm or more, especially for phosphate groups ($\text{P}=\text{O}$) as it is the case in this study (Figure 4C). It is noteworthy that ^{31}P -NMR chemical shifts are independent of formal oxidation numbers but mainly depend on the actual electron density on phosphorus, bond angles, and π -bonding. Above factors affected the chemical shift value of TFV (~ 15.1 ppm) in SFS compared to that observed in water and VFS (at ~ 15.7 ppm in both the fluids). The presence of inorganic hydrogen and dihydrogen phosphates in HP⁴² exhibit chemical shift ~ 2.5 ppm and in between 0-(-2) ppm (Figure 3F). These peaks have been slightly shifted in the presence of TFV and also had significant effects on the chemical shift value of TFV. Therefore, TFV peak was observed at ~ 13.7 ppm (Figure 3G). There were chemical shifts between different solvents, but there was still specificity (i.e. no overlap between analyte and solvent peaks). This was partly due to pH effects. These variations in chemical shifts did not change the observed specificity, so long as the T2 did not change drastically and a proper calibration curve was used (i.e. calibration in the appropriate solvent). It is noteworthy that T_2 values are actually fundamental to this study: the essence of the argument is as follows – TFV molecules that are bound up in a larger particle will have very short T_{2s} effectively rendering them invisible in the NMR spectrum (because of their very broad linewidths). TFV molecules that are free in solution will have relatively long T_2 s giving nice narrow lines that are easily integrated to yield concentration. This concept is the central idea that makes this form of analysis possible.

Overall, results clearly showed that TFV peak was well separated from the biological fluid components (Figure 3). Two limitations of this study are the limited sensitivity for TFV and the lack of validated HPLC/MS. The LOQ values in the NMR assay for the tested drug in this study are relatively higher (in microgram/ml) compared to HPLC or Mass Spec analysis. However; these values are comparable to data from other techniques such as in-line UV detection methods. The lack of validated HPLC/MS method (to be considered in future studies). The regulatory requirements for UV-Vis spectrophotometers are defined in the United States Pharmacopeia (USP) XIII, Section 851 on “Spectrophotometry and Light Scattering. Although, the UV and visible spectra of substances generally do not have a high degree of specificity, they remain highly suitable for quantitative assays. Moreover, they are useful supplementary identification methods for many substances.

Application of the developed ^{31}P -qNMR method in real-time *in vitro* TFV release analysis from chitosan NFs

TFV NFs formulation development—The developed ^{31}P -qNMR method was applied for the real-time quantification and release profile of TFV from its chitosan based NFs formulation fabricated using electro-spinning method (Figure 4A). SEM images revealed that NFs were successfully formulated with a smooth surface morphology (Figure 4B). The mean diameter of NFs was 158.51 ± 59.20 nm ($n = 100$) (Figure 4C). The % DL, and % yield of NFs were calculated as 14.20 ± 2.12 % w/w, and 63.18 ± 1.16 % w/w, respectively, ($n = 3$).

In vitro TFV release kinetics analysis—Drug release data modeling showed that ~75%, ~72%, and ~70% w/w TFV release was observed in case of NMR method in VFS (Figure 5A), SFS (Figure 5B), and HP (Figure 5C), respectively. To evaluate the magnitude of potential interference of dialysis membrane with quantification of drug released from nanoformulations, the concentration of TFV release from chitosan NFs in the receiving compartment was measured using UV-Vis method. Results showed that only ~47%, ~52%, and ~52% w/w of TFV release was observed using dialysis method in VFS (Figure 5A), SFS (Figure 5B), and HP (Figure 5C), respectively.

Approximately 20% decrease in the percent drug release observed with the dialysis method suggested the interference of dialysis membrane with drug transport process and the Donnan membrane effect due to presence of cationic polymer (chitosan) (as explained in Figures, 1A and 1B). The Gibbs-Donnan effect describes the behavior of charged particles near a semi-permeable membrane, often failing to distribute evenly across the two sides of the membrane. In this study, TFV is a negatively charged due to the presence of phosphate groups. Chitosan (from at NFs surface or resulting from degradation process) is a cationic polymer due to the presence protonated amino groups in the acidic environment. Due to the presence of dialysis membrane with its low molecular weight cut off (<5kD), the entrapped or free chitosan polymer (MW 50-190kDa) is unlikely to cross dialysis membrane pores leading to charge-charge interaction between the drug and polymer in donor compartment and thus uneven distribution of drug molecules on each side of the dialysis membrane (between donor and receiving compartments). It was reasonably speculated from results that TFV did not achieve equilibrium during the release period analyzed by the dialysis method. Therefore, data obtained for a drug release using the dialysis method will not be accurate in term of release kinetics and mechanism (e.g. burst release effect and the amount of drug measured in the receiving compartment could be significantly underestimated).

In vitro TFV release kinetic modeling to elucidate drug release mechanism—In HP and SFS, the *in vitro* kinetics of TFV release from NFs was best explained by Korsmeyer-Peppas model for both direct (no dialysis/NMR) and indirect (dialysis/UV Spec) assays (Table 4). The Korsmeyer-Peppas model also best explained release in VFS by dialysis method/UV spec analysis. However, in VFS analyzed by NMR, the first order was the best model (Table 4). First order model describes the drug dissolved in pharmaceutical dosage forms for example water soluble drugs in a porous material. Korsmeyer-Peppas model represents the drug release from a polymeric system and the value of exponent n

characterizes the mechanism of drug release from the formulation. The exponent n value enables to classify the drug release mechanism as Fickian diffusion ($n = 0.5$), anomalous transport (diffusion and erosion controlled release) ($0.45 < n < 1$), and super case-II transport (erosion and degradation of polymer) ($n = 1$). However, in case of a cylinder (NFs are considered as a cylindrical system), an n value of 0.45 instead of 0.50, and 0.89 instead of 1.0 should be applied^{40, 43}.

In the case of NMR measurements, TFV release mechanism was mostly anomalous ($0.45 < n < 1$) in VFS, SFS and HP. However, in the case of dialysis (UV-Vis) measurements, TFV release mechanism was only anomalous in VFS but occurred through Fickian diffusion ($n < 0.45$) in both SFS and HP (Table 4). This clearly indicated that NFs erosion process was not captured in most media by the release model when one relies only on the indirect method. Perhaps, the absence of phosphate based compounds in VFS media overshadows this release problem. Therefore, it might be misleading to refer to the dialysis method, which further demonstrated the importance of developing a real-time quantification method such as ³¹P-qNMR assay as described in this study. Overall, drug release mechanism dramatically differed using Korsmeyer-Peppas model for cylinders [e.g. diffusion only ($n < 0.45$) in SFS, HP by indirect method and both erosion and diffusion ($0.45 < n < 0.89$) by direct method].

CONCLUSION

In this study, a ³¹P-qNMR method was demonstrated to be a simple, and precise way to quantify phosphorus containing drug molecules from their nanoformulations in different biological fluids such as VFS, SFS, and HP. The ability for real-time and direct quantification of therapeutic molecules have the potential of providing more accurate information about drug release kinetics for better *in vitro* and *in vivo* correlation studies in drug development. NMR method has demonstrated a significant difference in the release rate and mechanism of TFV from NFs in biological fluids compared to the indirect (dialysis) assay.

Comparing the direct (no dialysis/NMR) and the indirect (dialysis/UV-Vis) method of the drug release quantitation; NMR method appeared to have at least four distinct advantages:

1. It is a direct and real-time quantification method, where dialysis membrane was not required. If the *in vitro* profile was determined using a dialysis method, the concentration of drug in release media might be underestimated due to interactions between drug molecules and dialysis membrane and the two-step process (diffusion of drug from the carrier to interior of the dialysis membrane and then to the outer reservoir)^{2-4, 44} (Figure 1B). When extrapolated to the *in vivo study*, the concentration of drug based on indirect method would be lower than the actual concentration, which could potentially lead drug overdose and toxicity.
2. NMR method allowed for more data points to be collected in the real-time scenario which provided a more accurate and dynamic nanoformulations drug release profile measurement.

3. UV-Vis spectroscopy measurements and related chromatographic assays are based on the Beer-Lambert Law. But, the absorbance can only be related to drug concentration if the molar absorptivity is known for a drug molecule having a chromophore group^{45, 46}. However, the NMR method does not depend on the drug molecule's molar absorptivity and chromophore groups.
4. No specific sample preparation method was required as it was a direct measurement, thus saving valuable research time and resources.

Conversely, it is well known that phosphorus atom plays an important role in biological and physiological processes since it is part of the genetic materials (deoxyribonucleic acid: DNA) as well as of adenosine triphosphate (ATP). Thus, ³¹P-qNMR method for phosphorus quantitation developed in this study can also be widely applied in the analysis of many other biological agents. It is anticipated that the developed ³¹P-qNMR method will be applicable to a large number of phosphorous containing drugs and their release kinetics/mechanism analysis *in vitro* and *in vivo* (if they can successfully pass the 'specificity' test).

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References

1. Stamatopoulos K, Batchelor HK, Alberini F, Ramsay J, Simmons MJ. Understanding the impact of media viscosity on dissolution of a highly water soluble drug within a USP 2 mini vessel dissolution apparatus using an optical planar induced fluorescence (PLIF) method. *Int J Pharm.* 2015; 495(1): 362–373. [PubMed: 26363111]
2. Modi S, Anderson BD. Determination of drug release kinetics from nanoparticles: overcoming pitfalls of the dynamic dialysis method. *Mol Pharm.* 2013; 10(8):3076–3089. [PubMed: 23758289]
3. Zambito Y, Pedreschi E, Di Colo G. Is dialysis a reliable method for studying drug release from nanoparticulate systems?-A case study. *Int J Pharm.* 2012; 434(1–2):28–34. [PubMed: 22617795]
4. Xie L, Beyer S, Vogel V, Wacker MG, Mantele W. Assessing the drug release from nanoparticles: Overcoming the shortcomings of dialysis by using novel optical techniques and a mathematical model. *Int J Pharm.* 2015; 488(1–2):108–119. [PubMed: 25847513]
5. Smith BR, Eastman CM, Njardarson JT. Beyond C, H, O, and N! Analysis of the elemental composition of U.S. FDA approved drug architectures. *J Med Chem.* 2014; 57(23):9764–9773. [PubMed: 25255063]
6. Malet-Martino M, Holzgrabe U. NMR techniques in biomedical and pharmaceutical analysis. *J Pharmaceut Biomed Anal.* 2011; 55(1):1–15.
7. Holzgrabe U, Deubner R, Schollmayer C, Waibel B. Quantitative NMR spectroscopy - Applications in drug analysis. *J Pharmaceut Biomed Anal.* 2005; 38(5):806–812.
8. Holzgrabe U. Quantitative NMR spectroscopy in pharmaceutical applications. *Prog Nucl Mag Res Sp.* 2010; 57(2):229–240.
9. Malz F, Jancke H. Validation of quantitative NMR. *J Pharmaceut Biomed Anal.* 2005; 38(5):813–823.
10. Zhang C, Zhang T, Oyler NA, Youan BB. Direct and real-time quantification of tenofovir release from pH-sensitive microparticles into simulated biological fluids using ¹H nuclear magnetic resonance. *J Pharm Sci.* 2014; 103(4):1170–1177. [PubMed: 24765652]

11. Clendinen CS, Stupp GS, Ajredini R, Lee-McMullen B, Beecher C, Edison AS. An overview of methods using (13)C for improved compound identification in metabolomics and natural products. *Front Plant Sci.* 2015; 6:611. [PubMed: 26379677]
12. Zatina MA, Berkowitz HD, Gross GM, Maris JM, Chance B. 31P nuclear magnetic resonance spectroscopy: noninvasive biochemical analysis of the ischemic extremity. *J Vasc Surg.* 1986; 3(3): 411–420. [PubMed: 2936903]
13. Maltsev S, Hudson SM, Sahu ID, Liu L, Lorigan GA. Solid-state NMR (31)P paramagnetic relaxation enhancement membrane protein immersion depth measurements. *J Physic Chem B.* 2014; 118(16):4370–4377.
14. Lumata LL, Merritt ME, Malloy CR, Sherry AD, van Tol J, Song L, Kovacs Z. Dissolution DNP-NMR spectroscopy using galvinoxyl as a polarizing agent. *J Magn Reson.* 2013; 227:14–19. [PubMed: 23246650]
15. Agrahari V, Zhang C, Zhang T, Li W, Gounev TK, Oyler NA, Youan BB. Hyaluronidase-Sensitive Nanoparticle Templates for Triggered Release of HIV/AIDS Microbicide In Vitro. *The AAPS J.* 2014; 16(2):181–193. [PubMed: 24343770]
16. Agrahari V, Putty S, Mathes C, Murowchick JB, Youan BB. Evaluation of degradation kinetics and physicochemical stability of tenofovir. *Drug Test Anal.* 2015; 7(3):207–213. [PubMed: 24817173]
17. Abdool Karim Q, Abdool Karim SS, Frohlich JA, Grobler AC, Baxter C, Mansoor LE, Kharsany AB, Sibeko S, Mlisana KP, Omar Z, Gengiah TN, Maarschalk S, Arulappan N, Mlotshwa M, Morris L, Taylor D, Group CT. Effectiveness and safety of tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women. *Science.* 2010; 329(5996):1168–1174. [PubMed: 20643915]
18. Meng J, Zhang T, Agrahari V, Ezoulin MJ, Youan BB. Comparative biophysical properties of tenofovir-loaded, thiolated and nonthiolated chitosan nanoparticles intended for HIV prevention. *Nanomedicine (Lond).* 2014; 9(11):1595–1612. [PubMed: 24405490]
19. Meng J, Sturgis TF, Youan BB. Engineering tenofovir loaded chitosan nanoparticles to maximize microbicide mucoadhesion. *Eur J Pharm Sci.* 2011; 44(1–2):57–67. [PubMed: 21704704]
20. Agrahari V, Meng J, Ezoulin MJ, Youm I, Dim DC, Molteni A, Hung WT, Christenson LK, Youan BC. Stimuli-sensitive thiolated hyaluronic acid based nanofibers: synthesis, preclinical safety and in vitro anti-HIV activity. *Nanomedicine (Lond).* 2016; 11(22):2935–2958. [PubMed: 27785967]
21. Meng J, Agrahari V, Ezoulin MJ, Zhang C, Purohit SS, Molteni A, Dim D, Oyler NA, Youan BC. Tenofovir Containing Thiolated Chitosan Core/Shell Nanofibers: In Vitro and in Vivo Evaluations. *Mol Pharm.* 2016; 13(12):4129–4140. [PubMed: 27700124]
22. Nista SV, Bettini J, Mei LH. Coaxial nanofibers of chitosan-alginate-PEO polycomplex obtained by electrospinning. *Carbohydr Polym.* 2015; 127:222–228. [PubMed: 25965478]
23. Sill TJ, von Recum HA. Electrospinning: applications in drug delivery and tissue engineering. *Biomaterials.* 2008; 29(13):1989–2006. [PubMed: 18281090]
24. Tseng YY, Liu SJ. Nanofibers used for the delivery of analgesics. *Nanomedicine (Lond).* 2015; 10(11):1785–1800. [PubMed: 26080700]
25. Owen DH, Katz DF. A review of the physical and chemical properties of human semen and the formulation of a semen simulant. *J Androl.* 2005; 26(4):459–469. [PubMed: 15955884]
26. Owen DH, Katz DF. A vaginal fluid simulant. *Contraception.* 1999; 59(2):91–95. [PubMed: 10361623]
27. ICH. Q2 (R1) Harmonized tripartite guideline: Validation of analytical procedures: text and methodology; International Conference on Harmonization ICH; Geneva, Switzerland. 2005.
28. Mckee MG, Wilkes GL, Colby RH, Long TE. Correlations of Solution Rheology with Electrospun Fiber Formation of Linear and Branched Polyesters. *Macromol.* 2004; 37(5):7.
29. Mina B-M, Leeb SW, Limb JN, Youb Y, Leeb TS, Kangc PH, Park WH. Chitin and chitosan nanofibers: electrospinning of chitin and deacetylation of chitin nanofibers. *Polymer.* 2004; 45:6.
30. Soti PL, Weiser D, Vigh T, Nagy ZK, Poppe L, Marosi G. Electrospun polylactic acid and polyvinyl alcohol fibers as efficient and stable nanomaterials for immobilization of lipases. *Bioprocess Biosyst Eng.* 2016; 39(3):449–459. [PubMed: 26724947]

31. Chiu JB, Liu C, Hsiao BS, Chu B, Hadjiargyrou M. Functionalization of poly(L-lactide) nanofibrous scaffolds with bioactive collagen molecules. *J Biomed Mater Res A*. 2007; 83(4): 1117–1127. [PubMed: 17593546]
32. Jiang S, Lv J, Ding M, Li Y, Wang H, Jiang S. Release behavior of tetracycline hydrochloride loaded chitosan/poly(lactic acid) antimicrobial nanofibrous membranes. *Mater Sci Eng C Mater Biol Appl*. 2016; 59:86–91. [PubMed: 26652352]
33. Li Y, Chen F, Nie J, Yang D. Electrospun poly(lactic acid)/chitosan core-shell structure nanofibers from homogeneous solution. *Carbohydr Polym*. 2012; 90(4):1445–1451. [PubMed: 22944401]
34. Ignatova M, Manolova N, Markova N, Rashkov I. Electrospun non-woven nanofibrous hybrid mats based on chitosan and PLA for wound-dressing applications. *Macromol Biosci*. 2009; 9(1):102–111. [PubMed: 18855947]
35. Ji Y, Ghosh K, Shu XZ, Li B, Sokolov JC, Prestwich GD, Clark RA, Rafailovich MH. Electrospun three-dimensional hyaluronic acid nanofibrous scaffolds. *Biomaterials*. 2006; 27(20):3782–3792. [PubMed: 16556462]
36. Ji Y, Ghosh K, Li B, Sokolov JC, Clark RA, Rafailovich MH. Dual-syringe reactive electrospinning of cross-linked hyaluronic acid hydrogel nanofibers for tissue engineering applications. *Macromol Biosci*. 2006; 6(10):811–817. [PubMed: 17022092]
37. Bhattarai N, Edmondson D, Veiseh O, Matsen FA, Zhang M. Electrospun chitosanbased nanofibers and their cellular compatibility. *Biomaterials*. 2005; 26(31):6176–6184. [PubMed: 15885770]
38. Pakravan M, Heuzey MC, Ajji A. Core-shell structured PEO-chitosan nanofibers by coaxial electrospinning. *Biomacromol*. 2012; 13(2):412–421.
39. Agrahari V, Youan BB. Sensitive and rapid HPLC quantification of tenofovir from hyaluronic acid-based nanomedicine. *AAPS PharmSciTech*. 2012; 13(1):202–210. [PubMed: 22215290]
40. Costa P, Sousa Lobo JM. Modeling and comparison of dissolution profiles. *Eur J Pharm Sci*. 2001; 13(2):123–133. [PubMed: 11297896]
41. Zhang Y, Huo M, Zhou J, Zou A, Li W, Yao C, Xie S. DDSolver: an add-in program for modeling and comparison of drug dissolution profiles. *The AAPS J*. 2010; 12(3):263–271. [PubMed: 20373062]
42. Sullentrop F, Moka D, Neubauer S, Haupt G, Engelmann U, Hahn J, Schicha H. 31P NMR spectroscopy of blood plasma: determination and quantification of phospholipid classes in patients with renal cell carcinoma. *NMR Biomed*. 2002; 15(1):60–68. [PubMed: 11840554]
43. Dash S, Murthy PN, Nath L, Chowdhury P. Kinetic modeling on drug release from controlled drug delivery systems. *Acta Pol Pharm*. 2010; 67(3):217–223. [PubMed: 20524422]
44. Shetab Boushehri MA, Lamprecht A. Nanoparticles as drug carriers: current issues with in vitro testing. *Nanomedicine (Lond)*. 2015; 10(21):3213–3230. [PubMed: 26548350]
45. Agrahari V, Meng J, Zhang T, Youan BB. Application of Design of Experiment and Simulation Methods to Liquid Chromatography Analysis of Topical HIV Microbicides Stampidine and HI443. *J Anal Bioanal Tech*. 2014; 5(1):1–10.
46. Pelillo M, Cuvelier ME, Biguzzi B, Gallina Toschi T, Berset C, Lercker G. Calculation of the molar absorptivity of polyphenols by using liquid chromatography with diode array detection: the case of carnosic acid. *J Chromatogr A*. 2004; 1023(2):225–229. [PubMed: 14753688]

Figure 1.

(A) Dialysis membrane method of quantitative *in vitro* drug release analysis from drug formulations in biological media. (a) Initial state. (b) Equilibrium state. (B) Membrane partitioning in dialysis method of quantitative *in vitro* drug release analysis

Figure 2.

(A) Chemical structure of tenofovir (TFV). Linearity of ^{31}P -qNMR method in: (B) Vaginal fluid simulant (VFS). (C) Seminal fluid simulant (SFS). (D) Human plasma (HP)

Figure 3.

Specificity analysis and representative ^{31}P -qNMR peaks of tenofovir (TFV): (A) TFV in vaginal fluid simulant (VFS). (B) Blank VFS. (C) TFV in seminal fluid simulant (SFS). (D) Blank SFS. (E) TFV in human plasma (HP). (F) Blank HP. (G) TFV with orthophosphoric acid (OPA) in water

Figure 4.

(A) Coaxial electro-spinning fabrication setup of chitosan nanofibers (NFs). (B) Surface morphology of NFs; scale bar, 5 μm . (C) Size distribution histogram of NFs averaged using at least 100 measurements per group

Figure 5. Percent cumulative drug release (% w/w) profile of tenofovir (TFV) loaded chitosan nanofibers (NFs) analyzed by ^{31}P -qNMR method and dialysis bag method in vaginal fluid simulant (VFS), seminal fluid simulant (SFS), and human plasma (HP)

Table 1

Accuracy (%mean recovery) data for tenofovir (TFV) ^{31}P -qNMR method in vaginal fluid simulant (VFS), seminal fluid simulant (SFS), and human plasma (HP).

Biological media	Concentration (mM)	Mean recovery $\pm$ RSE (%) (n = 3)		
		Intra-day	Inter-day	
		Within-a day	Day-2	Day-3
VFS	0.20	102.8 $\pm$ 2.7	102.1 $\pm$ 1.7	101.2 $\pm$ 1.7
	0.75	96.0 $\pm$ 2.3	99.6 $\pm$ 0.9	97.6 $\pm$ 1.9
	5.00	103.5 $\pm$ 0.2	103.8 $\pm$ 0.3	103.6 $\pm$ 0.3
SFS	0.20	94.7 $\pm$ 1.6	98.9 $\pm$ 1.3	96.1 $\pm$ 1.4
	0.75	95.6 $\pm$ 1.2	101.6 $\pm$ 1.5	93.8 $\pm$ 1.6
	5.00	106.5 $\pm$ 0.3	107.9 $\pm$ 0.2	105.9 $\pm$ 0.2
Human plasma	0.30	105.2 $\pm$ 2.9	104.6 $\pm$ 2.1	103.5 $\pm$ 2.4
	0.75	96.7 $\pm$ 1.9	96.7 $\pm$ 1.9	94.8 $\pm$ 1.5
	5.00	99.9 $\pm$ 1.8	99.9 $\pm$ 1.8	99.3 $\pm$ 1.9

Table 2

Precision (%RSD) data for tenofovir (TFV) ^{31}P -qNMR method in vaginal fluid simulant (VFS), seminal fluid simulant (SFS), and human plasma (HP).

Biological media	Concentration (mM)	Precision as %RSE (n = 3)		
		Intra-day	Inter-day	
		Within-a day	Day-2	Day-3
VFS	0.20	1.65	1.65	1.04
	0.75	0.44	0.44	1.14
	5.00	0.11	0.11	0.16
SFS	0.20	1.70	1.70	1.49
	0.75	1.25	1.25	1.63
	5.00	0.31	0.31	0.19
Human plasma	0.30	1.64	1.22	1.37
	0.75	1.11	1.11	0.81
	5.00	1.05	1.05	1.09

Robustness analysis data for tenofovir (TFV) ^3P -NMR method in vaginal fluid simulant (VFS), seminal fluid simulant (SFS), and human plasma (HP).

Table 3

Biological Media (TFV Conc. 5mM in each)	Robustness Parameters Temperature ($^{\circ}\text{C}$)			Relaxation Delay (sec)			45 degree Pulse Width (μs)		
	% RSE (n = 3)			% RSE (n = 3)			% RSE (n = 3)		
	35	37	39	4.8	5.0	5.2	4.85	5.05	5.25
VFS	0.30	0.43	0.34	0.22	0.43	0.15	0.12	0.43	0.28
SFS	0.17	0.21	0.22	0.24	0.21	0.24	0.15	0.21	0.27
Human plasma	0.91	0.98	0.72	0.70	0.98	0.79	0.81	0.98	0.64

in vitro drug release kinetics models parameters of tenofovir (TFV) loaded chitosan nanofibers (NFs).

Table 4

Biological fluid	Kinetic model	Direct (³¹ P-qNMR) assay			Indirect (dialysis: UV-Vis) assay		
		Parameters	R ² *	AIC**	Parameters	R ² *	AIC**
VFS	Zero-order	k ₀ =7.227	0.876	52.726	k ₀ =2.366	0.826	21.893
	First-order	k ₁ =0.121	0.976	39.656	k ₁ =0.027	0.860	20.899
	Korsmeyer-Peppas	k _{KP} =13.608	0.954	46.739	k _{KP} =5.323	0.924	19.876
		n=0.711			n=0.634		
	Higuchi	k _H =20.810	0.892	51.677	k _H =7.010	0.901	19.179
SFS	Weibull	α=9.077	0.977	41.204	α=18.888	0.920	20.105
		β=1.050			β=0.692		
	Zero-order	k ₀ =7.029	0.653	65.275	k ₀ =4.958	0.305	33.391
	First-order	k ₁ =0.120	0.908	53.342	k ₁ =0.071	0.553	31.199
	Korsmeyer-Peppas	k _{KP} =20.106	0.998	22.448	k _{KP} =18.767	0.970	19.694
Human plasma		n=0.511			n=0.393		
	Higuchi	k _H =20.537	0.997	21.489	k _H =15.149	0.931	21.828
	Weibull	α=4.789	0.994	31.337	α=4.824	0.955	21.708
		β=0.697			β=0.473		
	Zero-order	k ₀ =6.801	0.716	63.399	k ₀ =5.040	0.440	33.065
Human plasma	First-order	k ₁ =0.112	0.931	50.7189	k ₁ =0.073	0.678	30.301
	Korsmeyer-Peppas	k _{KP} =18.414	0.999	13.916	k _{KP} =17.921	0.980	18.293
		n=0.537			n=0.423		
	Higuchi	k _H =19.793	0.996	25.003	k _H =15.346	0.963	19.487
	Weibull	α=5.335	0.996	26.744	α=5.147	0.972	20.143
		β=0.725			β=0.517		

* Coefficient of determination.

** Akaike Information Criterion.