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Simultaneous quantification of tenofovir, emtricitabine, rilpivirine, elvitegravir and dolutegravir in mouse biological matrices by LC-MS/MS and its application to a pharmacokinetic study

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Abstract

Combination antiretroviral (cARV) treatment is more common in human immunodeficiency virus (HIV) infection. In many instances, treatment regimen includes two or more combination of drugs from six different classes. Some of the antiretroviral combination medications are under study at preclinical and clinical stages. A precise method is required to quantify the drug concentration in biological matrices to study pharmacokinetic behavior and tissue distribution profile in animals and/or humans. We have developed and validated a sensitive and precise liquid chromatography-tandem mass spectrometry method for simultaneous quantification of selected antiretroviral drugs, tenofovir (TNF), emtricitabine (FTC), rilpivirine (RPV), dolutegravir (DTG) and elvitegravir (EVG) in mouse biological matrices. This method involves a solid phase extraction, simple isocratic chromatographic separation using Restek Pinnacle DB BiPh column (50mm $\times$ 2.1mm, 5 μ m) and mass spectrometric detection by an API 3200 Q Trap instrument. The total run time for each sample was 6 min. The method was validated in the concentration range of 5 to 2000 ng/mL for FTC, RPV, DTG, EVG and 10 to 4000 ng/mL for TNF respectively with correlation coefficients (r^2) higher than 0.9976. The results of intra and inter-run assay precision and accuracy were within acceptance limits for all the five analytes. This method was used to support the study of pharmacokinetics and tissue distribution profile of nanoformulated antiretroviral drugs in mice.

Keywords

Antiretroviral; LC-MS/MS; pharmacokinetic; tissue distribution; plasma

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1. Introduction

Antiretroviral therapy (ART) helps people with human immunodeficiency virus (HIV) live longer, healthier and reduces the risk of HIV transmission. An antiretroviral regimen generally consists of two nucleotide reverse transcriptase inhibitors (NRTIs) in combination with a third active antiretroviral drug from one of three drug classes: an integrase strand transfer inhibitor (INSTI), a non-nucleoside reverse transcriptase inhibitor (NNRTI), or a protease inhibitor (PI) with a pharmacokinetic enhancer [1].

Clinical trial data and retrospective evaluation of patient data in clinical care shows this treatment regimen has resulted in an increase of CD4 cells and decrease in plasma HIV RNA [2–4]. However adherence to the treatment is a major drawback due to pill burden, which can jeopardize the treatment progress [4]. Apart from the treatment adherence, rate of viral replication, reduced cellular drug uptake and maintenance of therapeutic concentrations within the intracellular compartment are limiting factors for HIV treatment. Long acting combination antiretroviral drugs (cARV) formulated in nanoparticles can counteract these deficiencies by offering patient convenience and sustained tissue levels [5–8]. The approved antiretroviral drugs, NRTIs: tenofovir (tenofovir alafenamide or tenofovir disoproxil fumarate), emtricitabine, NNRTI: rilpivirine, INSTIs: elvitegravir, dolutegravir were nanoformulated in different combinations using the United States Food and Drug Administration (USFDA) approved polymer and assessed for their pharmacokinetic and tissue distribution profile in mice.

Several simultaneous liquid chromatography-tandem mass spectrometry methods have been reported for simultaneous quantification of antiretroviral drugs for assay of NNRTIs and PIs [9–14], NRTIs, NNRTIs and PIs [15–18] in biological matrices. But for assay of NRTIs, NNRTIs and INSTIs combination in biological matrices only two methods have been reported [19–20]. Himes, et al. has developed a method for simultaneous quantification of multiple antiretrovirals in meconium; it has the disadvantage of long run time of 34 minutes [19]. Djerada, et al. also reported a method for simultaneous quantification of multiple antiretrovirals [20]; this method uses a gradient mobile phase and has poor retention for tenofovir. Also this method did not quantify emtricitabine, rilpivirine and dolutegravir. As the range of polarities are broad for the selected drugs of different classes, most of the methods reported involved gradient separation which resulted in long run times or gradient spikes in blank samples. To date, no analytical method has been reported with isocratic mobile phase composition for the simultaneous quantification of these selected class of drugs.

There was no method reported for the simultaneous quantification of all five drugs in mouse biological matrices such as plasma, liver, kidney, spleen, brain and SubQ injection site. The objective of this work was to develop and validate an accurate and precise method for the simultaneous quantification of tenofovir, emtricitabine, rilpivirine, elvitegravir, and dolutegravir in mouse biological matrices using LC-MS/MS.

2. Experimental

2.1. Chemicals and Reagents

Tenofovir (TNF) reference standard was purchased from United States Pharmacopeia, (Rockville, MD). Emtricitabine (FTC), elvitegravir (EVG) and rilpivirine (RPV) reference standards were purchased from Sequoia, Pangbourne, United Kingdom. Dolutegravir (DTG) standard was purchased from Biochempartner co., Ltd., China. Internal standards (tenofovir-d6, emtricitabine- ^{13}C , $^{15}\text{N}_2$, elvitegravir-d6, rilpivirine-d6 and dolutegravir-d4) were purchased from Toronto Research Chemicals Inc., Canada. Milli-Q water was obtained from in-house Milli-Q water purification system, Millipore, USA. LC-MS grade methanol, acetonitrile, formic acid and trifluoroacetic acid were purchased from Fisher Scientific, USA.

2.2. Instrumentation

An Agilent 1200 HPLC system (Agilent Technologies, CA, USA) coupled with AB Sciex API 3200 Q Trap with an electrospray ionization (ESI) source (Applied Biosystems, Foster City, CA, USA) was used. The LC-MS/MS system was controlled by Analyst 1.4.2 software.

2.3. Preparation of calibration curve standards and quality control samples

The stock solutions of FTC, EVG, RPV and internal standards (TNF-d6, FTC- ^{13}C , $^{15}\text{N}_2$, EVG-d6 and RPV-d6) were prepared in methanol. TNF stock solution was prepared in water:methanol (80:20, v/v). DTG and DTG-d4 stock solutions were prepared in dimethyl sulphoxide. All stock solutions were prepared at 1 mg/mL concentration. Two separate stocks of each analyte were prepared and used for preparation of calibration standards and quality controls. Working standard and internal standard (IS) spiking solutions were prepared from stock solutions by diluting with water:methanol (50:50, v/v). The working standard solutions prepared were used to prepare the calibration and quality control samples.

An eight-point calibration curve was prepared by spiking the previously screened blank human plasma with corresponding working standard solution not exceeding 5% v/v. The calibration curve was ranged from 5 to 2000 ng/mL for FTC, RPV, EVG and DTG and 10 to 4000 ng/mL for TNF. Similarly quality control samples were prepared at three concentrations 5/10 (LLOQ QC) 15/30 (low QC), 750/1500 (mid QC) and 1750/3500 ng/mL (high QC). Blank tissue matrices, liver, kidney, spleen and brain were collected from BALB/c mice. Tissues were homogenized with water (1:5, w/v) and spiked with appropriate working standard to prepare tissue quality control samples.

2.4. Sample Preparation

A 100 μL aliquot of plasma or tissue homogenate was mixed with 25 μL of IS spiking solution followed by 100 μL of 1 % trifluoroacetic acid and samples were vortexed for 30 sec. The discovery C18 SPE cartridges were equilibrated with 1mL of methanol followed by water and samples were loaded. Cartridges were washed with water followed by 5% methanol and eluted with 1 mL of methanol. Eluent was evaporated to dryness under the

stream of nitrogen at 40 °C, reconstituted with 100 µL of 50 % acetonitrile in water and 5 µL was injected into the LC-MS/MS instrument.

2.5. Liquid chromatography and mass spectrometric conditions

Chromatographic separation was carried out on Restek Pinnacle DB Biph (2.1mm × 50mm, 5µm) column with isocratic mobile phase consisting of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) (48:52 v/v) at a flow rate of 0.250 mL/min. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode. Electrospray ionization (ESI) source was operated in positive mode. The mass spectrometer parameters such as declustering potential (DP), collision energy (CE), cell exit potential (CXP) and entrance potential (EP) were optimized by infusing each analyte and internal standard using 500 ng/mL solution in 50% methanol. The source temperatures, ion spray voltage and gas pressures were optimized through flow injection analysis (FIA) by infusing mobile phase using LC. The source temperature, ion spray voltage and gas pressures (GS1 and GS2) were set at 400°C, 3000V, 45 and 55 psi respectively. The optimized MRM parameters such as DP, EP, CE and CXP and retention times for all the analytes and internal standards are presented in Table 1.

2.6. Method Validation

The validation of the developed method was carried out as per the USFDA guidelines [21] and validation parameters were chosen based on the application of analytical method [22].

2.6.1. Selectivity—Selectivity was tested by extracting plasma and tissues (liver, kidney, spleen, female reproductive tract, brain, lymph node, colon and small intestine) from three untreated mice. All the tissues were homogenized with water (1:5 w/v). The peak area counts at the retention of respective analytes in blank samples were compared against lower limit of quantification (LLOQ, 5 and 10 ng/mL of FTC, RPV, EVG DTG and TNF respectively) to calculate % interference.

2.6.2. Matrix effect and extraction efficiency—Matrix effect was evaluated by comparing peak areas from neat solution of analyte and blank matrix samples spiked with analyte post extraction. Matrix effect was evaluated at three concentrations (low, mid and high) for all the analytes. IS normalized matrix factor was calculated by dividing the matrix factor of analyte by the matrix factor of IS to determine ion suppression or enhancement.

Infusion matrix effect was also evaluated by injecting extracted blank sample while continuously infusing the neat solution (500 ng/mL of each analyte) post column. The ion suppression or enhancement at the retention times of analytes was determined by overlaying the chromatographic profile of extracted blank and calibration standard. Extraction efficiency was evaluated by comparing the peak areas of neat solution of analytes with peak areas from spiked plasma/tissue samples. Extraction efficiency was performed at three concentrations (low, mid and high QC).

2.6.3. Calibration Curves—Calibration curves were established with eight non-zero standards from 5 to 2000 and 10 to 4000 ng/mL for FTC, RPV, EVG, DTG and TNF

respectively in plasma. Peak area ratio (drug peak area/IS peak area) versus nominal concentration of analyte were plotted to generate calibration curve using linear regression. The weighting models $1/x$ and $1/x^2$ were compared for best fit.

2.6.4. Precision and Accuracy—Precision and accuracy of the method was evaluated by injecting three batches containing six replicates of quality controls at each of four concentrations (LLOQ QC, low, mid and high QC) in each batch. The inter-run and intra-run precision and accuracies were calculated as coefficient of variation (%CV) and bias (% of deviation between nominal and measured concentrations) respectively.

2.6.5. Stability Experiments—Stability of analytes has been tested to determine the effect of conditions that were expected to be encountered during sample handling and analysis. The stability experiments were carried out in plasma and selected tissue matrices. Bench top, freeze-thaw, processed sample stability and reinjection reproducibility were performed at low and high QC concentrations using six replicates at each level. The stability sample concentrations were back calculated from freshly spiked and processed calibration curve. The stock solution stability at refrigerated conditions ($2-8^{\circ}\text{C}$) was performed by comparing analyte peak areas of stability stock with freshly prepared stock.

2.7. Pharmacokinetic Study

The method has been successfully utilized for quantification of analytes in mice biological matrices, plasma, tissues such as, liver, kidney, brain, spleen, small intestine, female reproductive tract and lymph node and SubQ injection site. All protocols and procedures were approved by Institutional Animal Care and Use Committee (IACUC; Protocol #0989).

Briefly, the study in mice was conducted as follows: humanized female NSG mice were fasted overnight and given cARV nanoparticles through subcutaneous route. A combination nanoparticle containing TAF and EVG was administered at 100 mg/kg each drug. Blood from each mouse ($n=3$ at each time point) were collected at day 1, 4, 7, 10, 14, and 21 days after dosing. Mice were euthanized by CO_2 exsanguination and tissues harvested including liver, kidney, and SubQ injection site and snap frozen. The plasma was separated immediately after blood collection by centrifugation at 4000 rpm for 5 min. Tissues samples and plasma were stored at -80°C until LC-MS/MS analysis. The method is currently being used to study the pharmacokinetic/pharmacodynamic (PK/PD) relationship of cARV nanoparticles containing TAF, FTC and EVG.

3. Results and Discussion

3.1. Method Development

The goal of this work was to develop a sensitive and precise method for the simultaneous quantification of antiretroviral drugs in order to study pharmacokinetic and tissue distribution profile of long acting nanoformulated cARV drugs in mice. Due to the complexity of endogenous components in tissues and wide difference in partition coefficients of selected analytes, several challenges were encountered during method development. Considering the vast difference in partition coefficients of analytes (-4.13 ,

–0.90, 1.70, 4.67 and 5.47 for TNF, FTC, DTG, EVG and RPV respectively), initial method development was started with gradient mobile phase composition containing acetonitrile (A) and 2 mM ammonium acetate, pH 3.5 (B) at a flow rate of 300 μ L using C18 column (Accucore RP-MS, 2.1mm $\times$ 100mm, 1.8 μ m). Gradient program started at 30% of A, maintained for 3 min, increased up to 85% at 3.1 min, maintained this composition till 9 min and brought back to initial composition to re-equilibrate the column at 9.1 min for 2 min. This trial gave high retention time for elvitegravir (8.86 min) and resulted in bad peak shape. Another C18 column (Kinetex, 4.6mm $\times$ 75mm, 2.6 μ m) was tried. The retention time for elvitegravir was brought down to 6.68 min, but its peak shape had tailing and did not get enough sensitivity for tenofovir to quantify 10 ng/mL in biological sample.

Paying attention to the outstanding issues in the earlier trials, a specialized column (Restek, Ultra IBD, 2.1mm $\times$ 50mm, 5 μ m) was selected to test its suitability. A gradient run containing acetonitrile (A) and formic acid, 0.5% v/v (B) started at 95% B for 2 min, decreased to 20% at 2.5 min, maintained this composition till 6 min and brought back to initial composition to re-equilibrate the column at 6.1 min for 3 min. The retention times for tenofovir, emtricitabine, dolutegravir, rilpivirine and elvitegravir were found at 0.60, 0.78, 2.09, 5.16 and 6.18 min respectively. Peak shapes for all the analytes were good. But the peak retention times were not reproducible; this was fixed by increasing the column re-equilibration to initial condition. During further method development, carry over experiment was performed, which gave high carry over for elvitegravir. The detailed investigation on carry over resulted in the ruinous outcome (gradient spike), which was due to gradient switch over, it could not be resolved even after using long linear gradient. As this column also can be used under hydrophilic interaction chromatography (HILIC) conditions, an isocratic mobile phase containing acetonitrile and formic acid, 0.1% v/v at 50:50 ratio was tested. All the analytes were eluted within in a short run time of 4 min. But peak shape for tenofovir and rilpivirine were not well defined.

An alternative HILIC, biphenyl column (Restek, Pinnacle DB Biph, 2.1mm $\times$ 50mm, 5 μ m) was tested with an isocratic mobile phase containing acetonitrile and formic acid, 0.1% v/v (50:50) at a flow rate 400 μ L/min. The retention times were 0.47, 0.45, 0.63, 1.26 and 2.42 min for TNF, FTC, DTG, EVG and RPV respectively. Peak shapes for all the analytes were appropriate. With the exception of sensitivity for TNF, all the analytes had good sensitivity.

With the set chromatography and mass spectrometer parameters, extraction optimization was initiated with protein precipitation. Protein precipitation (PPT) with methanol and acetonitrile were investigated, but it had significant matrix effect (ion suppression) at the retention of tenofovir and emtricitabine. Pretreatment of the sample with buffers was also failed to minimize ion suppression. Liquid-liquid extraction (LLE) is more specific compared to PPT and has potential to reduce unspecific extraction of matrix components [23], hence LLE with different solvents were investigated. The polar analytes (TNF and FTC) were not adequately recovered to accomplish enough sensitivity. Solid phase extraction (SPE) is more specific than any other method, and was researched. Sample extraction was performed using discovery C18 cartridge (100 mg/mL), although the recoveries were not high, they were consistent at different concentration levels. Infusion matrix effect showed no significant ion suppression or enhancement at the retention time of

analytes. All the five analytes had good peak shape, reproducibility and 5/10 ng/mL limit of quantification was achieved with 100 μ L sample.

3.2. Method Validation

3.2.1. Selectivity—No significant interference in blank plasma and tissues was observed at the retention time of analyte(s) and internal standards due to endogenous compounds. The precision of peak areas for all the analytes at lower limit of quantification were found to be less than 7.8%. The representative chromatogram for plasma blank and LLOQ with internal standard are presented in Fig. 1.

3.2.2. Matrix effect and extraction efficiency—The mean IS normalized matrix factor for all the five analytes was between 0.97 and 1.16 with %CV 6.6 for all the five analytes. Infusion matrix effect experiment also revealed as shown in Fig. 2 there is no significant ion suppression or enhancement at the retention of analytes.

The mean extraction recoveries in plasma were found to be 22.1, 33.1, 41.6, 51.2 and 63.1% with %CV values of 6.8, 2.1, 2.6, 1.9 and 1.9% for TNF, FTC, DTG, EVG and RPV respectively as shown in Table 2.

3.2.3. Calibration curves—Calibration curves were linear in each assay over the range of 10 to 4000 ng/mL for TNF, 5 to 2000 ng/mL for FTC, DTG, EVG and RPV. A wide calibration range was tested for TNF based on the peak concentrations (3000 ng/mL) that were found in majority of tissues in earlier PK study of TAF [24]. The linear regression with 1/x² weighting has produced correlation coefficient greater than 0.9976 for all the five analytes over three different analytical runs. The observed mean back calculated concentrations with % accuracy, precision (%CV) and correlation coefficient (r^2) are presented in Table 3.

3.2.4. Precision and accuracy—The results of inter and intra-run assay precision and accuracy for all the five analytes were within the acceptable limits, %CV 15% and %RE, 100 $\pm$ 15% at each concentration level. The results of precision and accuracy are presented in Table 4. Intra run assay precision and accuracy in selected tissue matrix were studied and results were found to be within $\pm$ 15% of the nominal concentration.

3.2.5. Stability—Plasma samples stored on bench top, freeze-thaw (3 cycles) and processed samples stored at ambient temperature were stable for 22h. No significant difference in %RE was observed on reinjection of accepted analytical precision and accuracy run. Stock solutions were stable at 2–8°C for 9 days. All the stability sample results, % RE were within 85 to 115% and %CV 15%.

3.3. Pharmacokinetic study

There are tissue reservoirs that actively participate in the pathogenesis and persistence of HIV despite cART that eliminates virus from peripheral blood [25]. Authors are working on formulation of nanoparticles for combination antiretroviral drugs of different drug classes (NRTI, NNRTI and INSTI) to enhance tissue permeability and study their pharmacokinetics

and dynamics. For treatment of HIV, the recommended combination is two NRTIs with one drug from the other classes [1]. For prevention, it is a novel attempt to use two drugs from different classes. We have formulated nanoparticles using TAF (NRTI) and EVG (INSTI) to study their PK and tissue distribution profile. The study of tissue concentration and pharmacokinetic profile are necessary to determine dosage regimen necessary for protective/therapeutic levels of the combination antiretroviral drugs from nanoparticles. This method was successfully applied to study the pharmacokinetics and tissue distribution profile of TNF and EVG formulated into nanoparticles. This method is also currently being used to study the PK-PD relationship of nanoformulated combination antiretroviral drugs in HIV infected mice. The pharmacokinetic parameters of nanoformulated antiretroviral drugs are presented in Table 5, pharmacokinetic and tissue distribution profiles are presented in Fig. 3 and 4.

3.4. Significance over reported methods

Most of the reported methods for the simultaneous quantification of ARVs in biological matrices have either quantified NNRTI, PI [9–14] or NRTI, NNRTI and PI [15–18]. There are only two methods reported for simultaneous quantification of NRTI, NNRTI and INSTI [19–20]. No method was so far reported for the simultaneous quantification of these five analytes. Djerada, et al. has reported a novel method for simultaneous quantification of 15 ARVs with boceprevir which uses gradient mobile phase with high aqueous (95% v/v) and sub 2 μ column. As the back pressure is high with this combination of mobile phase and column, this method cannot be used with regular LC as front end. Whereas author has developed and validated a novel method with simple isocratic mobile phase. Also this method was validated for human plasma and mice tissue matrices.

Majority of the assays for quantification of tenofovir and emtricitabine involves use of anion exchange SPE for extraction due to low partition coefficient values and matrix effect associated with their poor retention on LC column. We have used C18 SPE cartridges for extraction, achieved desired sensitivity and had no matrix effect for all the analytes. The calibration range, precision and accuracy values for all the analytes were comparable to the reported methods [26–28].

4. Conclusion

A simultaneous assay for quantification of all five analytes in mouse biological matrices was developed, validated and successfully applied for studying pharmacokinetic and tissue distribution profile of nanoformulated antiretroviral drugs. The method described is precise, has short run time, excellent specificity and consistent recoveries. Clinical trials on these combination antiretroviral drugs are in progress [29]; this method can be extrapolated to high-throughput bioanalysis for clinical trials.

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Highlights

- A simultaneous method for quantification of five antiretroviral drugs having different partition coefficients was developed and validated.
- The method uses isocratic mobile phase unlike all the report methods and has good sensitivity and selectivity.
- Consistent recoveries with no significant matrix effect all the five analytes.
- The method was applied to pharmacokinetic and tissue distribution profiles of nanoformulated antiretroviral drugs.

Fig. 1. Representative chromatograms of TNF, FTC, DTG, EVG and RPV in blank plasma (left), extracted LLOQ (center) and corresponding internal standard (right).

Fig. 2.
Infusion matrix effect: overlaid chromatograms of extracted blank with post column infusion of aqueous sample at 500 ng/mL versus calibration standard.

Fig. 3. Mean (+ SEM) plasma concentration-time profile of tenofovir and elvitegravir following single subcutaneous injection (100mg/kg each) of drug nanoparticles to female humanized mice.

Fig. 4.
Tissue distribution profile of tenofovir and elvitegravir after single subcutaneous injection (100 mg/kg each) of drug nanoparticles to female humanized mice

Optimized MRM parameters and retention time (RT) of all analytes and internal standards (IS)

Table 1

Analyte/IS	MRM transition	DP (V)	EP (V)	CE (V)	CXP (V)	RT (min)
Tenofovir	288.0 → 176.2	65	5	35	3	0.80
Emtricitabine	248.1 → 130.1	40	5	20	4	0.81
Dolutegravir	420.1 → 277.2	65	5	35	3	1.19
Elvitegravir	448.2 → 344.1	50	5	40	4	2.35
Rilpivirine	367.2 → 195.1	75	10	50	3	4.02
Tenofovir-d6 (IS)	293.9 → 182.3	65	5	35	3	0.80
Emtricitabine- ¹³ C, ¹⁵ N ₂ (IS)	251.1 → 133.1	40	5	20	4	0.81
Dolutegravir-d4 (IS)	424.2 → 279.2	65	5	35	3	1.19
Elvitegravir-d6 (IS)	455.0 → 350.6	50	5	40	4	2.35
Rilpivirine-d6 (IS)	373.2 → 195.1	75	10	50	3	4.02

DP, declustering potential; EP, entrance potential; CE, collision energy; CXP, cell exit potential

Table 2

Mean extraction recoveries of all analytes in mouse biological matrices (n=18, six at each of low, mid and high QC concentration level)

Analyte	Plasma (Mean $\pm$ SD, %CV)	Liver (Mean $\pm$ SD, %CV)	Kidney (Mean $\pm$ SD, %CV)	Spleen (Mean $\pm$ SD, %CV)	Brain (Mean $\pm$ SD, %CV)
Tenofovir	22.1 $\pm$ 1.5, 6.8	23.1 $\pm$ 1.6, 6.7	22.6 $\pm$ 0.9, 3.8	24.2 $\pm$ 1.4, 5.9	21.3 $\pm$ 1.5, 6.8
Emtricitabine	33.1 $\pm$ 0.7, 2.1	34.8 $\pm$ 1.2, 3.3	33.0 $\pm$ 1.2, 3.5	31.6 $\pm$ 0.9, 2.7	34.7 $\pm$ 0.7, 1.9
Dolutegravir	41.6 $\pm$ 1.1, 2.6	40.7 $\pm$ 0.8, 1.9	41.3 $\pm$ 1.1, 2.6	41.3 $\pm$ 1.5, 3.6	41.9 $\pm$ 1.3, 3.0
Elvitegravir	51.2 $\pm$ 1.0, 1.9	51.3 $\pm$ 1.8, 3.4	51.0 $\pm$ 0.9, 1.7	50.7 $\pm$ 0.7, 1.3	48.0 $\pm$ 1.8, 3.8
Rilpivirine	63.1 $\pm$ 1.2, 1.9	62.2 $\pm$ 1.5, 2.4	59.9 $\pm$ 1, 1.6	61.9 $\pm$ 2.1, 3.3	53.4 $\pm$ 1.8, 3.4

%CV, percent coefficient of variation; SD, standard deviation

Table 3

Summary of calibration curve standards

Analyte	Nominal Conc (ng/mL)	Mean Calculated Conc (ng/mL) n=3	%CV	% Accuracy	r ²
Tenofovir	10	10.51	0.40	105.07	>0.9976
	20	18.18	1.45	90.91	
	100	98.22	2.15	98.22	
	500	527.21	3.87	105.44	
	1000	1006.11	1.70	100.61	
	2000	1980.62	2.41	99.03	
	3000	2997.99	1.28	99.93	
Emtricitabine	4000	4017.70	1.77	100.44	>0.9997
	5	4.96	0.20	99.18	
	10	10.22	0.21	102.18	
	50	49.16	1.75	98.31	
	250	248.42	0.68	99.37	
	500	507.28	0.36	101.46	
	1000	1015.74	2.10	101.57	
Dolutegravir	1500	1485.11	0.58	99.01	>0.9996
	2000	2013.21	0.93	100.66	
	5	5.00	0.53	99.94	
	10	9.97	1.56	99.73	
	50	50.20	2.02	100.41	
	250	255.86	0.82	102.34	
	500	512.29	1.73	102.46	
Elvitegravir	1000	973.88	1.28	97.39	>0.9988
	1500	1470.73	1.72	98.05	
	2000	2003.94	0.81	100.20	
	5	5.09	2.14	101.86	
	10	9.72	4.34	97.20	
	50	48.70	2.76	97.40	

Analyte	Nominal Conc (ng/mL)	Mean Calculated Conc (ng/mL) n=3	%CV	% Accuracy	r ²
	250	251.53	0.55	100.61	
	500	495.93	0.93	99.19	
	1000	978.86	2.45	97.89	
	1500	1578.63	0.23	105.24	
	2000	2033.91	1.03	101.70	
Rilpivirine	5	5.03	0.77	100.52	
	10	9.87	1.69	98.69	
	50	50.21	1.58	100.42	
	250	253.88	1.37	101.55	
	500	503.43	0.72	100.69	>0.9998
	1000	999.82	2.16	99.98	
	1500	1491.50	0.74	99.43	
	2000	1982.29	2.35	99.11	

%CV, percent coefficient of variation; %RE, percent relative error

Table 4

Summary of intra and inter-run precision and accuracy of all the analytes

Analyte	QC Level	Nominal (ng/mL)	Intra run (n=6) in Plasma			Inter run (n=18) in Plasma			Intra run (n=6) in Liver			Intra run (n=6) in Kidney		
			Mean observed conc. (ng/mL)	%RE	%CV	Mean observed conc. (ng/mL)	%RE	%CV	Mean observed conc. (ng/mL)	%RE	%CV	Mean observed conc. (ng/mL)	%RE	%CV
Tenofovir	LLOQ	10	10.56	5.60	5.42	10.32	3.20	4.33	10.75	7.50	3.48	10.63	6.30	4.73
	Low	30	28.73	-4.24	6.61	29.65	-1.17	3.21	31.25	4.17	2.93	31.24	4.13	3.41
	Mid	1500	1535.23	2.35	0.90	1524.04	1.60	1.15	1556.33	3.76	2.48	1505.69	0.38	3.01
	High	3500	3528.97	0.83	0.83	3498.87	-0.03	1.01	3601.04	2.89	1.98	3425.78	-2.12	2.46
Emtricitabine	LLOQ	5	5.22	4.40	3.63	5.1	2.00	2.24	5.25	5.00	3.75	5.13	2.60	3.65
	Low	15	15.38	2.51	3.54	15.04	0.27	3.15	16.21	8.07	2.57	15.26	1.73	3.34
	Mid	750	761.53	1.54	2.20	762.45	1.66	2.17	760.45	1.39	3.76	762.79	1.71	2.18
	High	1750	1723.61	-1.51	2.04	1735.78	-0.81	1.98	1814.87	3.71	1.1	1710.56	-2.25	1.63
Dolutegravir	LLOQ	5	5.01	0.20	2.24	4.97	-0.60	2.44	5.23	4.60	2.49	4.93	-1.40	4.58
	Low	15	16.64	10.94	4.80	16.14	7.60	3.26	16.57	10.47	2.38	16.44	9.60	3.67
	Mid	750	764.66	1.95	2.12	758.78	1.17	2.02	801.58	6.88	2.25	796.25	6.17	3.19
	High	1750	1758.69	0.50	6.14	1761.52	0.66	3.65	1796.69	2.67	1.19	1754.12	0.24	2.11
Elvitegravir	LLOQ	5	5.10	2.00	3.34	5.07	1.40	3.15	5.12	2.40	2.91	5.16	3.20	3.47
	Low	15	16.29	8.58	2.46	15.88	5.87	2.28	16.11	7.40	2.72	16.47	9.80	2.92
	Mid	750	816.21	8.83	1.87	791.13	5.48	1.29	820.34	9.38	1.15	801.48	6.86	2.41
	High	1750	1851.99	5.83	2.20	1799.08	2.80	1.99	1849.71	5.70	1.04	1820.54	4.03	1.64
Rilpivirine	LLOQ	5	5.05	1.00	3.45	5.14	2.80	3.33	5.03	0.60	3.72	4.89	-2.20	2.47
	Low	15	15.15	1.00	2.90	14.97	-0.20	2.78	15.77	5.13	3.04	15.21	1.40	2.05
	Mid	750	734.22	-2.10	4.08	745.45	-0.61	3.57	753.61	0.48	3.65	742.68	-0.98	2.04
	High	1750	1775.17	1.44	0.99	1766.28	0.93	1.02	1795.42	2.60	1.85	1711.22	-2.22	1.16

%CV, percent coefficient of variation; %RE, percent relative error

Table 5

Summary of pharmacokinetic parameters of nanoformulated tenofovir and elvitegravir following single subcutaneous injection (100mg/kg each) to female humanized mice.

Parameter	Tenofovir	Elvitegravir
C _{max} (ng/mL)	264.90 ± 142.57	174.33 ± 150.14
AUC _{all} (day*ng/mL)	1433.34 ± 428.88	627.04 ± 450.73
Cl (L/h)	1.20	6.26
Vz_F_obs (L)	2122.56	2711.15
K _{el} (1/day)	0.0136	0.0554
t _{1/2} (Day)	50.97	12.50
AUC _{all_Liver} (day*ng/mL)	5065.08 ± 777.97	515.05 ± 238.25
AUC _{all_Kidney} (day*ng/mL)	988.04 ± 184.07	1693.72 ± 276.28