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UPLC-MS/MS quantification of nanoformulated ritonavir, indinavir, atazanavir, and efavirenz in mouse serum and tissues

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Abstract

Animal pharmacokinetic and tissue distribution assays of antiretroviral therapeutic drugs require accurate drug quantification in biological fluids and tissues. Here we report a simple, rapid, and sensitive ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method for quantification of commonly used antiretroviral drugs ritonavir (RTV), indinavir (IDV), atazanavir (ATV), and efavirenz (EFV) in mouse serum and tissues (liver, kidney, lung, and spleen). These antiretroviral drugs are currently the cornerstones of common therapeutic regimens for human immunodeficiency virus (HIV) infection. Chromatographic separation was achieved using a gradient mobile phase (5% acetonitrile in methanol and 7.5 mM ammonium acetate (pH 4.0)) on an ACQUITY UPLC[®] BEH Shield RP 18 column. All compounds eluted within a seven min run time. Lopinavir was used as an internal standard. Detection was achieved by dual positive and negative ionization modes on a quadrupole linear ion trap hybrid mass spectrometer with an electrospray ionization (ESI) source. The dynamic range was 0.2–1000 ng/mL for RTV, IDV, and ATV, and 0.5–1000 for EFV. The method was validated and showed high and consistent intra-day and inter-day accuracy and precision for all analytes. This method is used to support the preclinical development studies of targeted- and sustained-release combination ART (nanoART). The current data demonstrate a 1.5–4 fold increase in serum and tissue AUC of nanoformulated ATV, RTV, and EFV administered to mice when compared to native drug. In addition, the tested formulation enhanced exposure of the same anti-HIV drugs in mouse tissues.

Keywords

Antiretroviral drugs; UPLC-MS/MS; Pharmacokinetics; Tissue distribution

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1. Introduction

Combination antiretroviral therapy (cART) has dramatically reduced HIV-associated mortality and morbidity and as such remains the mainstay for treatment of human immunodeficiency virus (HIV) infection [1,2]. Currently, five antiretroviral drug classes are available for clinical use: nucleoside and non-nucleoside reverse transcriptase inhibitors (NRTIs and NNRTIs), protease inhibitors (PIs), entry inhibitors, and integrase inhibitors [3].

Despite such advances, a major limitation in treatment rests in the need for lifelong daily regimens associated with adverse drug reactions [4,5]. As such, reduced adherence to therapy leads to suboptimal viral suppression, risk of treatment failure, comorbid conditions and poor clinical outcomes. Complicating matters include secondary virus-associated tissue injuries elicited as a direct consequence of ongoing viral replication including the behavior, cognitive, and motor impairments linked to HIV-associated neurocognitive disorders (HAND) [6]. The development of nanoformulated ART (nanoART) that can release drug slowly into blood over periods of days to weeks and serve to target cell and tissue viral reservoirs may reduce systemic toxicities while improving adherence. Thus, nanoART could sustain drug exposure to viral reservoirs to ART and as such positively impact these concerns [7–9]. If the potential of nanoART can be realized, such drug delivery systems could revolutionize treatments for HIV disease and as such improve treatment outcomes through improvements in patient compliance of complex drug regimens. Based on these, recent efforts have focused on developing nanoART using homogenization of crystalline coated surfactants [10–12] or by wet milling [9]. Ritonavir (RTV), indinavir (IDV), atazanavir (ATV), and efavirenz (EFV) were chosen due to their hydrophobicity and common inclusions into cART regimens. These drugs, in general, are well tolerated and exhibit potent viral suppression [13–15]. The physicochemical properties and cellular uptake, drug release and antiviral activities of the nanoART were previously described [9].

In vitro models are used to evaluate the performance of the developed formulations by screening for their cell uptake, release, and antiretroviral activities in human monocyte-derived macrophages. Best performing formulations in laboratory assays [9,10] were next assessed for their pharmacokinetic (PK), tissue distribution, efficacy, and toxicity profiles in animal models including rodent. Therefore, a valid bio-analytical method for the simultaneous quantification of cART in *in vivo* fluids and tissues is required to support the PK, tissue distribution, and other preclinical studies involved in the development of these formulations. Inevitably these same evaluations will be utilized as part of the clinical testing of the nanoART formulations in infected humans.

Several high-performance liquid chromatography (HPLC) methods with UV detection have been used for the assay of PIs and NNRTIs [16–19]. More recently, ultra-performance liquid chromatography (UPLC) methods with diode array detection were used in the analysis of anti-HIV drugs to achieve faster and higher-resolution separation [20]. However, the low selectivity of UV detection requires more elaborate sample preparation and chromatographic conditions to achieve complete baseline separation of all peaks and to avoid interferences from the endogenous components of the biological matrices. For these reasons, LC methods with mass spectrometry (MS) detection were developed to quantify individual PIs and NNRTIs. A major drawback associated with single stage MS detectors is their relatively low sensitivity and selectivity [21–23]. Therefore, due to its superior selectivity and sensitivity, a number of liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods have recently been developed for the quantification of PIs and NNRTIs in plasma [3,24–27], dried blood spots [28,29], peripheral blood mononuclear cells [30–33], and hair [34] in humans. To date, no bio-analytical assays have been validated for the simultaneous quantification of all four nanoformulated anti-HIV drugs of interest in mouse serum and

tissues such as liver, kidney, lung, and spleen. More elaborate extraction procedures are usually required for the analysis of tissue compared to plasma/serum samples, due to the complexity of the endogenous components of tissues. Our present study described the development and validation of a UPLC-MS/MS method for the determination of three PIs (RTV, IDV, and ATV) and one NNRTI (EFV) in mouse serum and tissues. This method was validated according to FDA guidelines to ensure it provides the accuracy, precision, selectivity, and sensitivity required to support all preclinical studies associated with the development of nanoART. Furthermore, this method was applied to monitor mouse serum and tissue drug concentrations after a single *i.v.* bolus dose of a candidate nanoART formulation composed of a combination of RTV, ATV, and EFV.

2. Experimental

2.1. Chemicals and reagents

RTV and IDV-sulfate were purchased from Shengda Pharmaceutical Co., Taizhou, Zhejiang and Longchem Co., Shanghai, China, respectively. ATV-sulfate was purchased from Gymp Laboratories of America Inc. (Westbury, NY, USA). EFV was obtained from Hetero Labs Ltd. (Hyderabad, India). Lopinavir (LPV) was purchased from Toronto Research Chemicals Inc. (North York, Ontario, Canada). HPLC-grade methanol, acetonitrile, ammonium acetate, ammonium formate, ammonium hydroxide, formic acid, and acetic acid were obtained from Fisher Scientific (Fair Lawn, NJ, USA).

2.2. Instrumentation

A Waters ACQUITY UPLC system (Waters, Milford, MA) coupled to an Applied Biosystem 4000 Q TRAP[®] quadrupole linear ion trap hybrid mass spectrometer with an electrospray ionization (ESI) source (Applied Biosystems/MDS Sciex, Foster City, CA) was used. The UPLC-MS/MS system is controlled by Empower Pro 6.0 and Analyst 1.4.2 software, respectively. All chromatographic separations were performed with an ACQUITY UPLC[®] BEH Shield RP 18 column (1.7 μ m, 100 $\times$ 2.1 mm) equipped with an ACQUITY UPLC C₁₈ guard column (Waters, Milford, MA).

2.3. Liquid chromatographic and mass spectrometric conditions

The mobile phase consisted of 5% acetonitrile (ACN) in methanol (MeOH) (mobile phase A) and 7.5 mM ammonium acetate, adjusted to pH 4.0 using 10 M acetic acid (mobile phase B), at a total flow rate of 0.3 mL/min. The gradient profile was held at 70% mobile phase A for 5.0 min, increased linearly to 90% mobile phase A in 0.1 min, held at 90% for 0.8 min, and brought back to 70% mobile phase A in 0.1 min followed by 1-min re-equilibration. The injection volume of all samples was 10 μ L.

The mass spectrometer parameters, such as temperature, voltage, gas pressure, etc., were optimized by infusing each analyte and the internal standard (IS) using a 1.0 μ g/mL solution in 50% MeOH via a Harvard '22' standard infusion syringe pump (Harvard Apparatus, South Natick, MA, USA). RTV, IDV, and ATV were detected in the positive ionization mode, whereas EFV was quantified in the negative ionization mode. Source temperature, curtain gas, gas-1, gas-2, collision gas pressure, Q1/Q3 resolution, and interface heater were set at 600 $^{\circ}$ C, 20 AU, 40 AU, 40 AU, medium, unit, and on, respectively, whereas ion spray voltages were set at 4500 V and -4500V for positive and negative ionization modes, respectively. The multiple reaction monitoring (MRM) transitions for each analyte and IS, as well as their respective optimum MS parameters, such as declustering potential, entrance potential, collision energy, and cell exit potential, are shown in Table 1.

2.4. Preparation of standard solutions

The stock solutions of RTV, IDV, ATV, EFV and IS were prepared in MeOH at a concentration of 10 mg/mL. From these stock solutions, working standard solutions containing IS were prepared by dilution with 50% MeOH in H₂O. Blank tissues of interest were homogenized in deionized H₂O (1:2 w/v), and 100 µL aliquots of blank matrices were spiked with 10 µL of appropriate spiking standards, before extraction, to prepare calibration curves in the range of 0.2–1000 ng/mL for PIs and 0.5–1000 ng/mL for EFV.

2.5. Sample preparation

Tissue matrices of interest were homogenized in deionized H₂O (1:2 w/v). 1 mL of ice-cold ACN was added to a 100 µL serum or tissue homogenate sample spiked with 10 µL IS (2.0 µg/mL lopinavir). Samples were vortexed for 3min, shaken continuously for 15 min, and centrifuged at 16,000 g for 10 min. The supernatant was aspirated, evaporated under vacuum at room temperature, reconstituted in 100 µL of 50% MeOH in H₂O, and sonicated for 5min. After centrifugation at 16,000 g for 10 min, 10 µL of each sample was used for LC-MS/MS analysis. For all samples, the final concentration of IS was 200 ng/mL.

2.6. Analytical method validation

Serum, liver, kidney, lung, and spleen tissues were collected from 6 untreated mice. All tissues were homogenized in deionized water (1:2 w/v). 100 µL aliquots of resulting homogenates were spiked with 10 µL of the appropriate standard solution containing 2.0 µg/mL IS to construct a calibration curve with the range of 0.2–1000 ng/mL for RTV, IDV, and ATV, whereas the dynamic range for EFV was 0.5–1000 ng/mL. Calibration standards were analyzed in three independent validation runs. Calibration curves (area ratio of analyte:IS vs. nominal concentration) were fitted by least-squares linear regression using 1/x² weighting scheme.

The method was validated using 5 QC points for each calibration curve and the concentrations of the QC points were 0.2, 0.5, 50, 800, and 1000 ng/mL for RTV, IDV, and ATV, whereas the concentrations of the QC points were 0.5, 1.0, 50, 800, and 1000 ng/mL for EFV. Accuracy and precision of the method were determined by assaying five replicates of each QC point using freshly prepared calibration curves in three separate runs. Intra-day accuracy and precision were calculated from the % bias [% (Measured – Theoretical)/Measured concentration] and relative standard deviation [%RSD = %Standard Deviation/Mean], respectively, for the 5 replicates of each QC point. Inter-day precision was calculated similarly using the 15 replicates of each QC point from the three validation runs. Accuracy and precision were considered to be acceptable when it was found to be less than 15%, except for LLOQ where 20% deviation was allowed. The limit of detection (LOD) was defined as the concentration that produced a signal three times above the noise level.

2.7 Extraction recovery and matrix effect

Absolute extraction recoveries were determined for each QC point in each matrix from the ratio of the analyte peak area in samples spiked before extraction compared to the corresponding peak areas in untreated samples prepared in neat solution. The degree of matrix effect of each matrix was examined for each QC point in each matrix from the ratio of the analyte peak area in samples spiked post-extraction compared to the corresponding peak areas in untreated samples prepared in neat solution.

To ensure that analytes contained in the nanoformulations have the same extraction as the non-formulated analytes, the analyte peak areas from blank matrix samples spiked with equi-molar amounts of nanoART and non-formulated analytes were compared. In addition, the excipient suppression/enhancement effect on the MS signal of analytes was examined

using matrix samples pre-spiked with blank nanoART formulation that do not contain any analytes.

2.8. Preparation of nanoART

Nanoformulated RTV, IDV, ATV and EFV were prepared by wet-milling as described by Nowacek et al. [9]. The nanoformulations consisted of crystalline drug surrounded by a thin layer of surfactant. The nanoformulation excipients were IDV and ATV coated with poloxamer 188 (P188) and sodium dodecyl sulfate; RTV and EFV coated with P188 and 1,2-distearoyl-phosphatidylethanol-95 amine-methyl-polyethyleneglycol conjugate-2000. The nanoART suspensions were lyophilized and resuspended in phosphate buffered saline (PBS) to yield the appropriate concentration of drug for administration of a 200 μ L *i.v.* bolus dose. Drug concentrations of the original nanoART suspension were determined by HPLC with UV detection [9].

2.9. Animal studies

Six-week-old male BALB/cJ mice were purchased from Jackson Laboratory (Bar Harbor, ME). Food (7012 Teklad LM-485 Mouse/Rat Sterilizable Diet, Harlan, Madison, WI) and water were provided *ad libitum*. Mice were housed in the laboratory animal facility according to the American Animal Association Laboratory Animal Care guidance. All protocols and procedures were approved by the Institutional Animal Care and Use Committee at the University of Nebraska Medical Center. NanoART were suspended in PBS and administered to each mouse as a single *i.v.* bolus dose via the tail vein. A combination nanoART was administered as a single dose containing 0.31 mg RTV, 0.9 mg ATV and 1.25 mg EFV in a total volume of 200 μ L. After dosing, mice were returned to their home cage and cage-side observation was performed on the day of dosing and at least daily for the remainder of the study. At 0.25 hr, 1 hr, 6 hr, 1 day, 7 day, and 14 day post-dose, 3 mice per treatment group were anesthetized by isoflurane anesthesia and approximately 500 μ L of blood was collected from each mouse by cardiac puncture into BD microtainer[®] serum separator tubes (BD Biosciences, Franklin Lakes, NJ). Mouse liver, kidney, lung and spleen were harvested from the same animals. Blood samples were centrifuged at 1,000 g for 10 min to obtain serum. Serum and tissue samples were stored at -80° until LC-MS/MS analysis.

The pharmacokinetic parameters, such as total clearance (CL), half-life ($t_{1/2}$), area under the curve (AUC), and steady-state volume of distribution (V_{ss}) were determined using the bolus intravenous input noncompartmental analysis module of WinNonlin (version 1.5, Pharsight, Mountain View, CA).

3. Results and discussion

3.1. Method development

Sensitive and specific methods are required for antiretroviral drug detection in order to support PK, tissue distribution, and toxicology studies required for translation of preclinical to clinical investigations. Inevitably such evaluations would be included as part of the clinical testing of the nanoART formulations. Although several high-performance liquid chromatography (HPLC) methods with UV detection are used commonly for PIs and NNRTIs assays [16–19], they lack precision for complex detections in body fluids and tissues. To meet this change UPLC methods with diode array detection were developed and included in the analysis of ART in order to achieve faster and higher-resolution separations [20]. However, the low selectivity of UV detection requires more elaborate sample preparation and chromatographic conditions to achieve complete baseline separation of all peaks and to avoid interferences from the endogenous components of the biological

matrices. For these reasons, LC methods with MS detection were developed to quantify the current drug and drug formulations. We realized that a major drawback associated with single stage MS detectors is their relatively low sensitivity and selectivity [21–23]. Therefore, due to its superior selectivity and sensitivity we employed LC-MS/MS methods as these have recently been developed for the quantification of PIs and NNRTIs in plasma [3,24–27], dried blood spots [28,29], peripheral blood mononuclear cells [30–33], and hair [34] in humans. To date, no bio-analytical assays have been validated for the simultaneous quantification of all four nanoformulated anti-HIV drugs of interest in mouse serum and tissues such as liver, kidney, lung, and spleen. More elaborate extraction procedures are usually required for the analysis of tissue and sera samples, due to the complexity of the endogenous components in biological fluids. Our collective goal was to maximize the assay sensitivity through improving MS, chromatographic, and sample preparation without compromising assay accuracy and precision. The pH and the composition of the aqueous mobile phase were investigated to increase the retention and the signal intensity of all four analytes. Fig. 1 shows the retention time and signal response of the four analytes plotted against the composition and the pH of the mobile phase. The retention time of RTV, EFV, and ATV was generally resistant to changes in the pH and the type of buffer used, with the exception that the retention time of ATV decreased markedly when 0.2% formic acid (FA) was used as an aqueous mobile phase. In contrast, the retention time of IDV increased with increasing the mobile phase pH in the pH range of 3–5. Increasing the pH above 5 and up to pH 9 did not affect the retention time of IDV. As shown in Fig. 1B, signal intensity of all four analytes was also strongly dependent on mobile phase pH and composition. PIs had the highest MS signal using NH_4HCO_3 buffer at pH 7–8, whereas the NNRTI, EFV, had the highest MS signal using 0.2% acetic acid. NH_4AC at pH 4 was selected because it offered a compromise between the signal of PIs and EFV. Another difference in the MS response between PIs and EFV was that the signal of PIs was 10–100-fold higher in positive than in negative ionization mode, whereas EFV had similar signal intensity in both ionization modes, which was consistent with previous findings [3,24–37]. A typical extracted ion chromatogram from a blank serum sample and a serum sample at the LLOQ level is shown in Fig. 2. The retention times of RTV, IDV, ATV, EFV and IS at the final chromatography conditions were 2.9, 1.8, 2.8, 3.2 and 4.1 min, respectively. Lower limit of detection (LOD) values for all three PIs were 0.02 ng/mL, whereas the LOD for EFV was 0.1 ng/mL. The LLOQs for PIs and EFV were 0.2 ng/mL and 0.5 ng/mL, respectively, corresponding to an amount of 2 pg of PIs and 5pg of EFV in the 10 μL injection volume. These LLOQ values are lower than or similar to those reported in previous LC-MS/MS methods [24,30,37]. The wide range of doses associated with the various formulations developed in this project resulted in a marked variation in serum and tissue concentrations of the analytes. Therefore, it was important to develop a method that is valid for as wide of a dynamic range as possible. The dynamic range of PIs was 5000 (0.2–1000 ng/mL) and 2000 for EFV (0.5–1000 ng/mL).

Several protein precipitation, SPE, LLE, and salting out techniques were investigated to maximize extraction recovery and minimize matrix effect from the various matrices. High recoveries (>90%) of all four analytes in plasma were previously reported using ACN protein precipitation [3,17,19]. We applied the same ACN protein precipitation procedure to tissues, but the extraction recoveries of all analytes from tissues were lower than those from serum (Table 2). Therefore, we investigated the use of various protein precipitation procedures including acidic (5% FA) and alkaline (5% NH_4OH), alone or in combination with ACN (5% FA or 5% NH_4OH in ACN). Furthermore, we applied SPE methods using different types of SPE cartridges including C18, C8, C2, Phenyl, Amino, Cyano, DiOH, Silica cartridges, as well as CBA ion exchange cartridges. Additionally, we used hybrid SPE cartridges (Supelco Hybrid SPE), which combines protein precipitation with 1% FA in ACN, and phospholipids clean-up via passing through the Hybrid SPE cartridges that

contain proprietary zirconia coated silica particles, in order to efficiently remove phospholipids from samples. Phospholipids are known to cause ion-suppression in the ESI source [38,39]. Furthermore, LLE with acetic ether, ethyl acetate, or n-butanoland salting out procedures with Mg_2SO_4 were applied. Finally, combination of LLE or SPE with protein precipitation and salting out procedures were investigated. Table 3 summarizes recoveries of the four analytes in mouse liver using several different sample preparation techniques. As illustrated by Table 3, none of the mentioned extraction procedures improved the extraction recovery from tissues using ACN protein precipitation. The relatively low extraction recovery in tissues was due to matrix effect, as determined from peak area ratio of samples spiked pre-/post-extraction, rather than low extraction efficiencies (data not shown). This is expected due to the complexity of these tissue matrices compared to serum.

Despite the low extraction recoveries of EFV and IDV in mouse tissues, the recovery was consistent throughout the dynamic range of all analytes in all matrices (Table 2). Extraction recoveries were determined at each QC level for all analytes in all matrices. Therefore, ACN-protein precipitation was used for the extraction from tissues and serum, despite the relatively low recoveries in tissues. Finally extraction recoveries of analytes contained in nona-ART were similar to those of the non-formulated analytes (data not shown).

3.2. Method validation

This assay was validated for the quantification of RTV, IDV, and ATV, in the range of 0.2–1000 ng/mL, and EFV in the range of 0.5–1000 ng/mL in mouse serum, liver, kidney, lung, and spleen. Calibration curves of all analytes in all matrices were linear in this range with correlation coefficients > 0.99. Inter-day accuracy and precision data for all analytes in serum and liver are summarized in Table 4. Similarly, the inter-day accuracy and precision obtained in other tissues (kidney, lung, and spleen) were also less than 15% (data not shown). In addition, the intra-day accuracy and precision (data not shown) for all analytes in serum and tissues were less than 15% at all concentration levels.

The selectivity of the method was verified by the lack of interfering peaks from endogenous compounds in blank matrices, which co-elute with any of the analytes or IS. The carry-over effect was also examined and found to be < 20% of the LLOQ for all analytes. All four antiretroviral drugs and IS were stable for at least 3 months in the $-20\text{ }^{\circ}\text{C}$ freezer, for 48 hrs in the $4\text{ }^{\circ}\text{C}$ autosampler, and for 24 hrs on the laboratory bench at room temperature (data not shown).

3.3. Animal studies

This method is currently being used to support all the preclinical PK and biodistribution studies as part of the efforts to develop nanoformulations with targeted-, sustained- and enhanced-delivery of ART. Fig. 3 shows the serum concentration vs. time profile resulting from the *i.v.* administration of nanoART containing RTV, ATV, and EFV to mice ($n=3$). Table 5 shows PK parameters for these 3 drugs using noncompartmental analysis. The AUC in serum of these drugs were increased 1.5- to 4-fold compared to equimolar doses of the unformulated drugs, whereas no significant differences of the half-lives, clearance, and steady-state volume of distribution were observed between unformulated and nanoART drugs. Fig. 4 shows the biodistribution of nanoART containing RTV, ATV, and EFV in liver, kidney, lung, and spleen tissues. RTV and ATV were detected in the tested tissues up to 14 days after dose administration, whereas EFV levels in most tissues were lower than LLOQ 7 days after dose administration. The AUC of individual antiviral drugs in mouse tissues following nanoART administration were also higher than those obtained from unformulated drugs (Table 5). The most marked increase in AUC_{tissue} (1.7–15-fold) was

observed with ATV. Collectively, these data indicate that higher serum and tissue levels were attained and sustained for a longer period of time using nanoART formulations.

4. Conclusion

A quantitative assay is described for the simultaneous quantification of nanoART in mouse serum, liver, kidney, lung and spleen tissues. This UPLC-MS/MS method provides high sensitivity and selectivity for quantitative profiling of four commonly used antiretroviral drugs in a relatively short run-time using a one-step sample preparation. The method is used to support the PK, biodistribution, and other preclinical studies as part of the efforts to develop nanoformulations with targeted-, sustained- and enhanced-delivery of ART. Several candidate nanoformulations, which demonstrated sustained serum and tissue PK profiles, are being now tested in a range of nanotoxicologic and drug efficacy models of human HIV-1 disease.

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Abbreviations

UPLC	ultra performance liquid chromatography
LC-MS/MS	liquid chromatography-tandem mass spectrometry
RTV	ritonavir
IDV	indinavir
ATV	atazanavir
EFV	efavirenz
IS	internal standard
MeOH	methanol
ACN	acetonitrile
QC	quality control

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Fig. 1.

Effect of mobile phase pH and composition on (A) retention time and (B) signal sensitivity of analytes. The mobile phase consisted of 70% mobile phase A (5% acetonitrile (ACN) in methanol (MeOH)) and 30% mobile phase B at a total flow rate of 0.3 mL/min. The composition of the various buffers used in mobile phase B included: AA: 0.2% (v/v) acetic acid, FA: 0.2% (v/v) formic acid, AF: 7.5 mM ammonium formate, AM: 7.5 mM ammonium acetate, AB: 7.5 mM ammonium bicarbonate, or AC: 7.5 mM ammonium carbonate.

Fig. 2. Representative LC-MS/MS chromatogram of (A) blank serum and (B) blank serum spiked with IS and analytes at the LLOQ level under the final chromatography and detection conditions.

Fig. 3. Serum concentration vs. time profile of ritonavir (RTV), atazanavir (ATV) and efavirenz (EFV) in male mice following a single *i.v.* bolus dose of nanoART containing 0.31 mg RTV, 0.9 mg ATV, and 1.25 mg EFV.

Fig. 4.

Tissue distribution profile of ritonavir (RTV), atazanavir (ATV) and efavirenz (EFV) in male mice following a single *i.v.* bolus dose of nanoART containing 0.31 mg RTV, 0.9 mg ATV, and 1.25 mg EFV.

Table 1

MRM transitions and MS parameters for all analytes and internal standard (IS).

	MRM transition	Declustering potential (V)	Entrance potential (V)	Collision energy (eV)	Cell exit potential (V)
Ritonavir	721.3 → 140.0	70	10	87	6
Indinavir	614.4 → 97.1	101	10	81	16
Atazanavir	705.4 → 168.2	111	10	79	8
Efavirenz	313.9 → 243.6	-55	-10	-24	-13
IS	629.4 → 447.2	56	10	23	10

Table 2

Recoveries (%) of four antiretroviral drugs in mouse serum and tissues from 25 QC samples at 5 QC levels for each analyte using ACN-protein precipitation (n=25).

	Serum	Liver	Kidney	Lung	Spleen
Ritonavir	96.4 ± 6.3	101.3 ± 5.9	93.3 ± 4.7	94.3 ± 3.8	94.8 ± 5.0
Indinavir	90.4 ± 10.4	87.0 ± 7.9	48.5 ± 9.9	49.6 ± 10.6	50.6 ± 11.5
Atazanavir	96.1 ± 5.5	115.8 ± 6.8	107.9 ± 10.3	105.4 ± 5.8	109.4 ± 9.0
Efavirenz	97.2 ± 8.0	44.3 ± 7.1	45.9 ± 6.3	44.5 ± 7.9	46.7 ± 7.4

Table 3

Recoveries (%) of four antiretroviral drugs at 100 ng/mL in mouse liver using different sample preparation techniques.

	Protein precipitation				SPE			
	ACN	5% FA-ACN	5% NH ₄ OH-ACN	Supelclean (LC18)	Bond Elut (DiOH)	Supelco Hybrid		
Ritonavir	94.9 ± 3.1	74.3 ± 1.4	81.8 ± 2.9	46.2 ± 4.6	59.9 ± 5.0	25.9 ± 4.2		
Indinavir	85.8 ± 8.9	65.9 ± 10.5	73.8 ± 4.0	42.1 ± 7.5	34.1 ± 0.4	39.1 ± 4.9		
Atazanavir	94.5 ± 8.6	78.0 ± 3.2	80.8 ± 0.8	107.7 ± 6.7	84.1 ± 6.2	37.0 ± 4.4		
Efavirenz	52.8 ± 3.3	47.8 ± 2.6	47.1 ± 0.9	24.0 ± 4.3	21.4 ± 1.4	29.0 ± 0.7		

Table 4
Summary of the inter-day accuracy and precision of antiretroviral drug assay in mouse serum and liver (n=15).

Nominal Conc.	QC1		QC2		QC3		QC4		QC5	
	LLOQ ^a	%R.S.D.	Low ^b	%R.S.D.	Medium (50 ng/mL)	%R.S.D.	High (800 ng/mL)	%R.S.D.	ULOQ (1000 ng/mL)	%R.S.D.
Serum										
Ritonavir	0.19	11.54	0.51	6.93	46.39	4.05	759.2	4.19	927.8	3.44
Indinavir	0.20	11.07	0.50	7.33	47.64	2.95	764.9	3.74	936.9	3.43
Atazanavir	0.18	13.66	0.52	10.49	46.46	3.87	759.4	4.85	931.8	4.38
Efavirenz	0.48	11.91	1.05	8.68	48.64	6.62	772.9	3.34	975.2	4.89
Liver										
Ritonavir	0.19	14.04	0.50	9.18	50.13	2.30	741.6	2.70	912.9	2.38
Indinavir	0.20	13.93	0.46	10.85	50.91	3.21	764.5	2.96	946.3	2.64
Atazanavir	0.20	12.15	0.53	6.92	48.70	3.43	747.6	3.50	904.2	2.37
Efavirenz	0.51	11.98	1.05	6.52	51.27	5.76	789.1	3.24	959.5	6.61

^a 0.2ng/mL for ritonavir, indinavir, and atazanavir; 0.5 ng/mL for efavirenz.

^b 0.5ng/mL for ritonavir, indinavir, and atazanavir; 1.0ng/mL for efavirenz.

Table 5
Pharmacokinetic parameters for 3 antiretroviral drugs using noncompartmental analysis (n=3).

Parameter	RTV		ATV		EFV	
	NanoART	Free	NanoART	Free	NanoART	Free
AUC _{all} (ng.min/mL)	5529.3 ± 2229.1	1554.4 ± 547.0	24078.4 ± 3678.5	13957.0 ± 3496.2	26249.0 ± 4104.7	18997.2 ± 3496.1
AUC _{INF} (ng.min/mL)	5530.0 ± 2229.0	1557.4 ± 547.0	24088.3 ± 3671.2	13962.1 ± 3495.2	26250.2 ± 4108.0	19003.6 ± 3493.9
AUC _{%Extrap} (%)	0.02 ± 0.006	0.02 ± 0.008	0.06 ± 0.05	0.05 ± 0.03	0.01 ± 0.006	0.04 ± 0.02
Cl (L/hr)	0.15 ± 0.04	0.14 ± 0.06	0.08 ± 0.01	0.04 ± 0.01	0.10 ± 0.02	0.03 ± 0.01
Lambda _z (1/hr)	0.029 ± 0.001	0.020 ± 0.002	0.028 ± 0.003	0.026 ± 0.002	0.039 ± 0.009	0.027 ± 0.002
t _{1/2} -Lambda _z (hr)	24.04 ± 0.40	35.36 ± 3.60	25.33 ± 2.82	27.48 ± 2.27	19.37 ± 3.65	26.04 ± 2.07
V _{ss} (L)	0.65 ± 0.45	2.97 ± 1.22	3.10 ± 0.90	1.67 ± 0.68	0.60 ± 0.23	0.22 ± 0.05
AUC _{liver} (µg.min/mL)	1123.4 ± 274.8	631.4 ± 61.1	28638.0 ± 11933.0	2392.6 ± 801.2	5025.6 ± 1033.8	4391.4 ± 745.1
AUC _{kidney} (µg.min/mL)	851.3 ± 389.7	454.4 ± 61.4	1599.9 ± 374.8	956.2 ± 484.4	5154.0 ± 483.4	3767.2 ± 979.0
AUC _{lung} (µg.min/mL)	355.1 ± 109.1	140.8 ± 39.4	2045.3 ± 607.6	269.1 ± 136.2	1966.6 ± 387.9	1030.4 ± 250.3
AUC _{spleen} (µg.min/mL)	880.8 ± 117.6	246.3 ± 91.8	11224.3 ± 7719.2	737.3 ± 301.6	2900.2 ± 790.3	2140.7 ± 807.0