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Development and validation of a simple and isocratic reversed-phase HPLC method for the determination of rilpivirine from tablets, nanoparticles and HeLa cell lysates

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Abstract

In the present investigation, a simple and isocratic HPLC-UV method was developed and validated for determination of rilpivirine (RPV) from dosage forms (tablets and nanoparticles) and biological matrices like HeLa cell lysates. The separation and analysis of RPV was carried out under isocratic conditions using (a) a Gemini reversed-phase C₁₈ column (5 μ m; 4.6 $\times$ 150 mm) maintained at 35°C, (b) a mobile phase consisting of a mixture of acetonitrile and 25 mM potassium dihydrogen phosphate (in the ratio 50:50 v/v) at a flow rate of 0.6 mL/min and (c) atazanavir as an internal standard. The total run time was 17 min and the analysis of RPV and internal standard was carried out at 290 nm. The method was found to be linear (r^2 value > 0.998), specific, accurate and precise over the concentration range of 0.025–2 μ g/mL. The lower limit of quantification was 0.025 μ g/mL, the limit of detection was 0.008 μ g/mL and the recovery of RPV was >90%. The stability of the RPV analytical method was confirmed at various conditions such as room temperature (24 h), –20°C (7 days), three freeze–thaw cycles and storage in an autosampler (4°C for 48 h). The method was successfully applied for the determination of RPV from conventional dosage forms like tablets, from polymeric nanoparticles and from biological matrices like HeLa cell lysates.

Introduction

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) play an important role in the treatment and prevention of HIV infections (Jayaweera and Dilanchian, 2012; Sanford, 2012; Schafer and Short 2012; Putcharoen *et al.*, 2013). NNRTIs are an integral part of the combination therapy (HAART or highly active antiretroviral therapy) employed for the treatment of HIV. First-generation NNRTIs such as efavirenz and nevirapine were initially used as a part of HAART therapy (Jayaweera and Dilanchian, 2012; Sanford, 2012; Schafer and Short 2012; Putcharoen *et al.*, 2013). However, issues such as hepatotoxicity and rash (nevirapine), neuropsychiatric disorders and low genetic barrier (efavirenz), and the rapid emergence of drug-resistant strains compromised the utility of first-generation NNRTIs (Fulco and McNicholl, 2009; Azijn *et al.*, 2010; Sharma and Saravolatz, 2013). In recent years, second-generation NNRTIs such as etravirine and rilpivirine have been developed and approved for the treatment of HIV (Jayaweera and Dilanchian, 2012; Sanford, 2012; Schafer and Short 2012; Putcharoen *et al.*, 2013; Sharma and Saravolatz, 2013). Rilpivirine (investigational names TMC278 and R278474) is the most recently approved NNRTI (US

Food and Drug Administration, FDA, approval year 2011) and is more potent than first-generation NNRTIs and efavirenz (Fulco and McNicholl, 2009; Azijn *et al.*, 2010; Sharma and Saravolatz, 2013). Moreover, rilpivirine (RPV) is effective against a variety of wild-type and efavirenz/nevirapine-resistant and efavirenz-resistant viral strains and has a favorable safety profile (Fulco and McNicholl, 2009; Azijn *et al.*, 2010; Sharma and Saravolatz, 2013). The chemical name for rilpivirine hydrochloride is 4-{{[4-[(*E*)-2-cyanoethenyl]-2,6-dimethylphenyl]amino}-2-pyrimidinyl}amino}benzonitrile monohydrochloride (Fig. 1). At present, tablets containing only RPV (Eudrant®) or RPV in combination with tenofovir and emtricitabine (Complera®) are approved by the FDA for HIV treatment (Jayaweera and Dilanchian, 2012; Sanford, 2012; Schafer and Short 2012; Putcharoen *et al.*, 2013; Sharma and Saravolatz, 2013). The recommended dose of RPV is 25 mg (equivalent to 27.5 mg of rilpivirine hydrochloride) once daily, orally with meals (Jayaweera and Dilanchian, 2012; Sanford, 2012; Schafer and Short 2012; Putcharoen *et al.*, 2013; Sharma and Saravolatz, 2013). In the last few years, the potential of nanotechnology-based approaches such as nanocrystals has been explored to improve delivery of RPV for HIV treatment and prophylaxis (Baert *et al.*, 2009; van 't Klooster *et al.*, 2010). RPV nanocrystals are currently being explored in clinical trials (Spreen *et al.*, 2013). We have been involved in preclinical development of biodegradable polymeric nanoparticles of a variety of antiretroviral drugs either alone or in combination for treatment and prevention of HIV (Date *et al.*, 2012; Shibata *et al.*, 2013a, b). Recently, we have developed a thermosensitive gel containing rilpivirine nanoparticles for vaginal prophylaxis of HIV (Long *et al.*, 2013). We wanted to develop a simple but sensitive analytical method for determination of RPV from nanoparticles and cells (HeLa cells) relevant to *in vitro* cellular release studies involved in vaginal prophylaxis.

Until today, few studies have been reported on the determination of RPV (either alone or in combination with other antiretroviral drugs) from human plasma using LC-MS/MS (Else *et al.*, 2010; Mathias *et al.*, 2012; Aouri *et al.*, 2013; Burugula *et al.*, 2013; Shibata *et al.*, 2013). Although LC-MS/MS is crucial for determination of RPV from complex biological matrices like plasma, owing to cost associated with analysis, it may not be suitable for routine analysis of RPV from conventional dosage forms, nanoparticles or less complex biological matrices like cell lysates. Few HPLC methods have been reported for routine analysis of RPV from conventional dosage forms like tablets (Pendela *et al.*, 2009; Mohanareddy *et al.*, 2012) but the limit of quantification (LOQ) for RPV reported in these methods is in the microgram range. These methods employ gradient elution for RPV determination and it is not clear whether these methods could determine RPV from biological matrices such as cell lysates without any interference. Hence, in the present study, we aimed at development and validation of a simple, isocratic reversed-phase HPLC method for the determination of RPV from conventional dosage forms, nanoparticles and cell lysates.

Experimental

Materials

Rilpivirine and the internal standard (IS), atazanavir, were purchased from Sequoia Chemicals (Pangbourne, UK). Acetonitrile (HPLC grade), methanol (HPLC grade), potassium di-hydrogen phosphate (HPLC grade), dimethyl sulfoxide (DMSO, ACS grade), ethyl acetate (ACS grade) and 0.45 µm filters were purchased from Fisher Science (Hanover Park, IL, USA). Resomer 752H (PLGA 75:25) was purchased from Boehringer Ingelheim (Ridgefield, CT, USA). Complera® tablets were purchased from Creighton University Medical Center Pharmacy (Omaha, NE, USA). Pluronic F127 was received as a gift sample from BASF (Edison, NJ, USA). Ultrapure water was prepared in-house using a ELGA Purelab Ultra system (ELGA, Woodridge, IL, USA). All other chemicals and solvents were of analytical grade or equivalent. All the solvents were filtered through 0.45 µm filter and were further degassed for 10 min using a bath sonicator before initiating the experiments.

Cell culture

Human cervical (HeLa) cells were purchased from American Tissue Culture Collection (ATCC, Manassas, VA, USA). HeLa cells were maintained in Dulbecco's modified Eagle media (DMEM, MediaTech Inc., Manassas) supplemented with 10% fetal bovine serum (FBS, Hyclone Inc., UT, USA), 4 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (MP Biomedical Inc., Solon, OH, USA) and maintained in a logarithmic growth phase. All cells were grown at 37°C and 5% CO₂.

Analytical matrices

HeLa cell lysates and PLGA nanoparticles served as analytical matrices for the determination of RPV. For obtaining HeLa cell lysates, HeLa cells (1×10^5 cells/well) were seeded onto 12-well plates and cultured overnight. The following day, media was removed, cells were washed and 0.1 mL of MPER reagent (Fisher Science, IL, USA) was added for cell lysis and plates were incubated at 37°C for 20 min. After this, 0.5 mL of acetonitrile containing a specific amount of IS was added into each well and the contents were centrifuged at 14,000 rpm for 15 min. The supernatant was used for the calibration curves and validation unless stated otherwise. For PLGA (Resomer® 752H) nanoparticles, blank PLGA nanoparticles were prepared using the emulsion-solvent evaporation method described earlier (Date *et al.*, 2012; Long *et al.*, 2013). The blank PLGA nanoparticles contained Resomer 752H and Pluronic F127 as a stabilizer. For preparation of PLGA nanoparticles matrix, 20 µL of PLGA nanoparticles was diluted with 980 µL of acetonitrile containing a specific amount of IS. This solution was used for validation and calibration curves.

Stock solution and calibration curve solutions

Stock solutions of RPV (1 mg/mL) and IS (1 mg/mL) were prepared by dissolving a specific amount of RPV or IS in acetonitrile. For preparation of calibration and working solutions, RPV stock solution was diluted with acetonitrile to achieve intermediate RPV stock solutions of concentration 50, 100, 200, 400, 800, 1200, 1600, 2000 and 4000 ng/mL,

whereas IS stock solution was diluted to obtain a concentration of 50,000 ng/mL. For preparation of calibration curves in the different matrices, 0.5 mL each of 50, 100, 200, 400, 800, 1200, 1600, 2000 and 4000 ng/mL RPV intermediate stock solution was added to 0.5 mL of analytical matrix containing IS to obtain a final concentration range of 25–2000 ng/mL. For quality control (QC), samples were prepared in a similar manner to obtain RPV solutions of concentration 50 ng/mL (QC2), 600 ng/mL (QC3) and 1800 ng/mL (QC4).

Chromatographic instrument details

Chromatographic experiments were carried out on a Shimadzu Prominence HPLC system (Shimadzu Corporation, Lenexa, KS, USA) equipped with LC-20A binary pump, DGU-20A online degasser, SIL-20A autosampler with refrigeration control, CTO-20A thermostated column oven, SPD-20A UV–vis detector and CBM-20A system controller.

Chromatographic separation was carried out at 35°C using a Gemini C₁₈ reversed-phase column (Phenomenex, Torrance, CA) with 5 µm particle size, 4.5 mm internal diameter and 150 mm length, and a Security Guard HPLC guard column containing C₁₈ security guard cartridge (4 × 3.0 mm i.d.; Phenomenex, Torrance, CA, USA). Chromatograms were processed with Class-VP-7.3 software (Shimadzu Corporation).

Chromatographic conditions

Isocratic mode was used for chromatographic analysis during all the experiments. The mobile phase was composed of a mixture of acetonitrile (ACN) and 25 mM KH₂PO₄ solution at a ratio of 50:50 (v/v). The final composition was selected after checking resolution of the standard, internal standard and influence of biological matrix (HeLa cell lysate). The flow rate was 0.6 mL/min, injection volume was 20 µL and analysis of RPV and IS was carried out at 290 nm. The temperature of autosampler and column oven was set at 4 and 35°C respectively. The total run time for all the analyses was 17 min.

Method validation

The HPLC method was validated as per the FDA Guidelines on Bioanalytical Method Validation (US Food and Drug Administration, 2001). Various parameters such as selectivity, carry-over effect, linearity, lower limit of quantification (LLOQ), limit of detection (LOD), accuracy and precision, recovery and stability were evaluated for the method validation.

Selectivity—The specificity of the method was evaluated to confirm that components of analytical matrices did not interfere with analysis of RPV and IS. In order to ensure selectivity, analytical matrices (HeLa cell lysate and PLGA nanoparticles) were prepared as described in the earlier section except that IS and RPV were not added in preparation of any of the matrices. The chromatograms of these matrices were compared with the chromatograms of analytical matrices spiked with RPV and IS.

Carry-over effect—Carry-over effect was studied by injecting the analytical matrices spiked with maximum concentration of RPV (2 µg/mL) and IS followed by injection of blank analytical matrices (without RPV and IS). For the preparation of blank analytical matrices, blank PLGA nanoparticles (no RPV) were prepared as per the procedure described

in the earlier section and 20 μ L of blank PLGA nanoparticles were dissolved in 980 μ L of acetonitrile. In the case of HeLa cells, control HeLa cells (no RPV treatment) were lysed using 0.1 mL of MPER reagent and 0.5 mL of acetonitrile was added to the lysed cells followed by injection of blank analytical matrices (without RPV and IS). The chromatograms of blank analytical matrices were evaluated to ensure the absence of any measurable peak at the retention time of RPV and IS.

Linearity, LLOQ and LOD—The LOD and LLOQ for RPV were determined on the basis of signal-to-noise ratio. For the linearity, calibration curve samples were prepared in triplicate in different analytical matrices. Calibration curves were obtained by plotting response (ratio of peak area of RPV and IS) against the different concentrations of RPV by the least square linear regression. Linearity was evaluated by visual analysis of graphs, calculation of the correlation coefficient, back-calculation of concentrations of the calibration curve samples and analysis of the response factor (ratio between the response and nominal concentration of each calibration curve sample).

Precision, accuracy and recovery—Intra-day ($n = 5$) and inter-day ($n = 5$) accuracy and precision were determined for all the analytical matrices using four quality control samples (LLOQ, QC2, QC3 and QC4). The relative standard deviation (RSD) for intra- and inter-day assays determined the precision, whereas the measured concentrations yielded accuracy. The RPV recovered from HeLa cell lysates was determined for the four quality control samples (LLOQ, QC2, QC3 and QC4) and for IS. The percentage recovery was calculated by comparing the concentrations of the spiked samples with the concentration of the nonextracted samples.

Stability—The stability of RPV in different matrices was determined at 2 different levels (QC2 and QC4). The stability was determined (a) at room temperature (20°C) after 24 h, (b) at –20°C after 7 days, (c) after three freeze–thaw cycles (storage at –20°C for 24 h followed by thawing at 20°C) and (d) in the autosampler (4°C) after 48 h.

Method applicability

Determination of RPV from PLGA nanoparticles—RPV-loaded PLGA nanoparticles were prepared using emulsion-solvent evaporation method described earlier (Date *et al.*, 2012; Long *et al.*, 2013). The theoretical content of RPV in the PLGA nanoparticles dispersion was 0.05% w/v. For determination of RPV from PLGA nanoparticles, 20 μ L of RPV loaded PLGA nanoparticles were dissolved with 480 μ L of acetonitrile and added to 0.5 mL of acetonitrile containing a specific amount of IS. The percentage RPV content in PLGA nanoparticles was determined ($n = 3$).

Determination of RPV from Complera tablets—Complera® tablets contain 27.5 mg of RPV hydrochloride (equivalent to 25 mg of RPV). For the analysis of RPV from tablets, each tablet was crushed in the mortar and pestle and mixed thoroughly to obtain a homogenous powder. The crushed powder was transferred to a 50 mL volumetric flask and volume was made to 50 mL with methanol. The contents of the volumetric flask were subjected to bath-sonication for 10 min. From the volumetric flask, 1 mL of mixture was

transferred to a micro-centrifuge tube and contents were centrifuged at 14,000 rpm for 15 min. The supernatant was collected and diluted to an appropriate concentration with acetonitrile (containing a specific amount of IS). The percent RPV content in tablets was determined ($n = 3$).

Determination of intracellular concentrations of RPV in HeLa cells—HeLa cells were seeded into 12-well plates at a density of 100,000 cells/well and were allowed to attach to the wells overnight. RPV solution and RPV loaded PLGA nanoparticles were added to the wells such that the starting concentration of the RPV was 5 µg per well. The intracellular concentration of RPV in the HeLa cells was evaluated after 1, 2, 4 and 6 h. At a specific analysis time point, all the media from the wells was removed and cells were washed with PBS to get rid of any remaining RPV. HeLa cells were lysed using 0.1 mL of MPER solution and acetonitrile (0.5 mL) was added to the wells for extraction of RPV from the cells. The concentration of RPV in HeLa cell lysate was analyzed by HPLC. All of the experiments were carried out in triplicate.

Results and discussion

Development and optimization of analytical method for determination of RPV from different matrices

In the present investigation, atazanavir was used as an internal standard (IS) owing to some structural similarities to RPV (Fig. 1). Atazanavir also has a fair absorption at the wavelength (290 nm; data not shown) chosen for the analysis of RPV. Preliminary studies were carried out to identify chromatographic conditions for determination of RPV and IS. Various mobile phase compositions such as methanol–water, acetonitrile–water, methanol–potassium dihydrogen phosphate and acetonitrile–potassium dihydrogen phosphate solution were tested for their ability to resolve RPV and IS. It was observed that mobile phase containing acetonitrile and potassium dihydrogen phosphate solution mixtures yielded good resolution of RPV and IS with acceptable run time. Increase in the concentration of acetonitrile and concentration of potassium dihydrogen phosphate in the solution reduced the run time and resolution between RPV and IS. Further optimization of the chromatographic conditions was performed using HeLa cell lysates spiked with RPV and IS. For identifying optimal chromatographic conditions to achieve proper resolution between RPV and IS and to eliminate any interference from HeLa cell lysates, various trials were taken. In these trials, the effects of mobile phase composition (mobile phases containing different ratios of acetonitrile–25 mM potassium dihydrogen phosphate solution; 70:30 to 45:55), flow rate (1–0.5 mL/min) and temperature of column oven (25–40°C) were evaluated. After several trials, the optimal conditions identified were (a) mobile phase acetonitrile–25 mM potassium dihydrogen phosphate solution (50:50) and (b) flow rate 0.6 mL/min and temperature of column oven 35°C. Using these chromatographic conditions, the retention time obtained for RPV was 12.9 ± 0.03 min and for IS was 15.43 ± 0.04 min (Fig. 2a). The total run time was 17 min.

Validation of analytical method

The HPLC method was validated for the selected matrices (PLGA nanoparticles and HeLa cell lysates) as per guidelines on validation of bioanalytical method by FDA (US Food and Drug Administration, 2001).

Selectivity and carry-over effect—Various matrices (PLGA nanoparticles and HeLa cell lysate) were tested to confirm the absence of interference from any endogenous substances in the analysis of RPV and IS. For this purpose, chromatograms of blank HeLa cell lysate and blank PLGA nanoparticles (dissolved in acetonitrile) were compared with chromatograms of blank HeLa cell lysate and blank PLGA nanoparticles spiked with RPV and IS (Fig. 2b and c). The chromatogram of blank PLGA nanoparticles did not show any peaks (data not shown) whereas the chromatogram of HeLa cell lysate showed several peaks from endogenous substances. However, the developed HPLC method could successfully resolve RPV and IS without any interference from the endogenous substances present in the HeLa cell lysates (Fig. 2c). For determining carry-over effect of the HPLC column, blank HeLa cell lysate and blank PLGA nanoparticles were injected after finishing the HPLC run of analytical matrices spiked with maximum concentration of RPV (2 µg/mL) and IS. No carry-over effect was observed for both the matrices (data not shown).

Linearity, LLOQ and LOD—For the linearity, calibration curves for RPV were obtained for all the matrices (PLGA nanoparticles and HeLa lysates) over the concentration range of 0.025–2 µg/mL. The detector response was linear over the tested concentration ranges for both the matrices and the r^2 value ranged from 0.9989 to 0.9994. The equations for the nine-point standard curve for determination of RPV from PLGA nanoparticles and HeLa cell lysates ($n = 5$) are shown in Table 1. The individual concentrations of the calibration curve back-calculated from the regression equation did not exceed the accepted limit of $\pm 15\%$ of theoretical concentrations ($\pm 20\%$ for LLOQ).

For all the matrices tested in this investigation, the LLOQ was 0.025 µg/mL (or 25 ng/mL). Recently, Aouri *et al.* (2013) reported an LC-MS method for determination of RPV and elvitegravir combination and they reported LLOQ of 5 ng/mL RPV. Although the current HPLC-UV method is not as sensitive as their method, we believe that it is sufficiently sensitive for determination of RPV from dosage forms and less complex biological matrices. In the present investigation, the LOD obtained for RPV was 8 ng/mL.

Precision, accuracy and recovery—The inter- and intra-day accuracy and precision values for RPV analysis from both the analytical matrices were determined using four quality control samples, viz. 0.025 µg/mL (LLOQ), 0.05 µg/mL (QC2), 0.6 µg/mL (QC3) and 1.8 µg/mL (QC4). The results are shown in Table 2. The percentage accuracy (bias) values ranged from –8.69 to + 6.54 (intra-day) and from –4.16 to + 10.17 (inter-day), whereas intra-day precision values ranged from 3.3 to 13.1 for intra-day and from 2.9 to 12.2 for inter-day analysis. The accuracy and precision values were in accordance with the limits set by the FDA ($< 15\%$). The results of recovery studies are shown in Table 3. The percent recovery of RPV ranged from 94.1 to 103.7%.

Stability—The stability of RPV in both the matrices was determined at a variety of conditions. The results are shown in Table 4. RPV was found to be stable at different conditions. Hence, analysis of RPV can be satisfactorily carried out using the method reported in the present investigation.

Method applicability

The applicability of developed method was determined by analyzing RPV content in the developed PLGA nanoparticles and commercial RPV tablets (Complera®), and by analyzing intracellular concentration of RPV in HeLa cells treated with RPV solution or RPV nanoparticles for 1–6 h. The results are shown in Table 5 and Fig. 3. The percentage RPV content in PLGA nanoparticles and Complera® tablets was within the acceptable limits ($\pm 5\%$). The other drugs present in Complera® tablets (emtricitabine and tenofovir disoproxil fumarate) as well as tablet excipients did not interfere with the RPV analysis. Hence, the present HPLC method can be used for routine analysis of RPV from PLGA nanoparticles and tablets. The intracellular concentrations of RPV in HeLa cells treated with RPV solution and RPV nanoparticles were comparable ($p > 0.05$) for the duration of the study (Fig. 3). No interference from endogenous substances was observed while analyzing RPV from HeLa cell lysates.

Conclusion

In the present investigation, a simple isocratic HPLC-UV method was developed and validated for determination of RPV from tablets, PLGA nanoparticles and biological matrices like HeLa cell lysates. The method enabled determination of RPV up to a concentration of 25 ng/mL. The method can be conveniently used for routine analysis of RPV from conventional dosage forms and from less complex biological matrices such as cell lysates, without any interference from the other components of analytical matrices.

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Abbreviations used

HAART	highly active antiretroviral therapy
NNRTI	non-nucleoside reverse transcriptase inhibitors
RPV	rilpivirine

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Rilpivirine **Atazanavir**

Figure 1.
Structure of rilpivirine and atazanavir (IS).

Figure 2.

Chromatograms of: (a) mixture of rilpivirine (RPV) at LLOQ (0.25 $\mu\text{g/mL}$) and IS (25 $\mu\text{g/mL}$); (b) blank HeLa cell lysate; and (c) HeLa cell lysate spiked with RPV (2 $\mu\text{g/mL}$) and IS (25 $\mu\text{g/mL}$); HPLC analysis was carried out at 290 nm. Chromatogram of HeLa cell lysates spiked with RPV and IS did not show any interference from the endogenous substances in the separation and analysis of RPV and IS.

Figure 3.

Intracellular RPV concentrations in HeLa cells treated with RPV solution and RPV nanoparticles ($n = 3$); HeLa cells were treated with a nontoxic concentration of RPV solution and RPV nanoparticles (equivalent to 5 $\mu\text{g}/\text{well}$) for 1, 2, 4 and 6 h. HeLa cells were lysed and RPV was extracted and analyzed at 290 nm for intracellular RPV concentrations.

Table 1

Results for calibration curves for determination of rilpivirine (RPV) from different matrices ($n = 3 \pm$ standard error)

	PLGA nanoparticles	HeLa lysates
Regression equation ^a	$y = 0.3340 (\pm 0.01385)x - 0.0025 (\pm 0.002)$	$y = 0.31820 (\pm 0.03406)x - 0.0033 (\pm 0.00214)$
r^2	0.9994	0.9989

^a y = Ratio of peak areas of RPV to IS; x = concentration of RPV ($\mu\text{g/mL}$).

Table 2

Intra- and inter-day accuracy and precision of RPV HPLC analysis from PLGA nanoparticles and HeLa cell lysates

Matrix	Nominal concentration (µg/mL)	Intra-day (<i>n</i> = 5)			Inter-day (<i>n</i> = 5)		
		Mean measured concentration (µg/mL)	Precision (%)	Accuracy (% bias)	Measured Concentration (µg/mL)	Precision (%)	Accuracy (% bias)
PLGA nanoparticles	0.025	0.026	12.2	+3.84	0.024	11.3	-4.16
	0.05	0.052	8.2	+4.0	0.048	6.2	-4.05
	1	0.98	4.4	-2.04	1.05	6.4	+4.76
	1.8	1.86	3.3	+3.22	1.84	2.9	-2.17
HeLa cell lysate	0.025	0.023	13.1	-8.69	0.028	12.2	+10.71
	0.05	0.051	6.7	+1.96	0.053	5.7	+5.66
	1	1.07	5.4	+6.54	1.1	4.2	+9.09
	1.8	1.76	4.1	-2.27	1.83	3.7	+1.63

Table 3Percentage recovery of RPV from PLGA nanoparticles and HeLa cell lysates ($n = 5$)

Matrix	Nominal Concentration ($\mu\text{g/mL}$)	Recovery (%)	RSD (%)
PLGA nanoparticles	0.025	97.1	6.2
	0.05	98.9	3.1
	1	103.7	2.9
	1.8	105.3	3.8
	IS	99.1	2.8
HeLa lysates	0.025	94.1	8.6
	0.05	102.9	5.1
	1	96.4	4.6
	1.8	102.7	4.1
	IS	98.7	3.6

Table 4Stability of RPV in PLGA nanoparticles and HeLa cell lysates at different storage conditions ($n = 3$)

Condition	Nominal concentration (µg/mL)	PLGA nanoparticles		HeLa lysates	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Room temperature for 24 h	0.05	98.1	5.2	96.9	4.8
	1.8	103.4	3.7	97.6	4.1
−20°C for 7 days	0.05	103.4	6.1	101.4	5.1
	1.8	98.9	3.1	102.9	3.7
Freeze–thaw cycles	0.05	97.7	6.2	97.4	4.7
	1.8	102.4	2.9	96.4	6.1
Autosampler after 48 h	0.05	102.6	2.4	102.4	1.9
	1.8	99.1	2.8	102.6	4.1

Table 5Assay of RPV from marketed RPV formulation (Complera® tablets) and PLGA nanoparticles ($n = 3$)

Matrix	Theoretical RPV Content	Amount of RPV found	% RPV content	RSD (%)
RPV loaded PLGA nanoparticles	0.5 mg/mL	0.51 ± 0.3 mg/mL	102.3	3.1
Complera® tablets	27.5 mg	28.65 ± 0.7 mg	104.2	2.7