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HPLC-MS method for simultaneous quantification of the antiretroviral agents rilpivirine and cabotegravir in rat plasma and tissues

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ABSTRACT

The antiretroviral agents rilpivirine (RPV) and cabotegravir (CAB) are approved as a combined treatment regimen against human immunodeficiency virus (HIV). To fully understand the biodistribution of these agents and determine their concentration levels in various parts of the body, a simple, selective and sensitive bioanalytical method is essential. In the present study, a high performance liquid chromatography method with mass spectrometry detection (HPLC-MS) was developed for simultaneous detection and quantification of RPV and CAB in various biological matrices. These included plasma, skin, lymph nodes, vaginal tissue, liver, kidneys and spleen, harvested from female Sprague Dawley rats. The suitability of the developed method for each matrix was validated based on the guidelines of the International Council for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) on bioanalytical method validation. Analytes were extracted from biological samples employing a simple one-step protein precipitation method using acetonitrile. Samples were analysed using an Apex Scientific Inertsil ODS-3 column (4.6 mm × 250 mm, 5 μm particle size), maintained at 40 °C, on a HPLC system coupled with a single quadrupole MS detector. RPV was detected at a mass-to-charge ratio (*m/z*) of 367.4 and CAB at 406.3. Separation was achieved using isocratic elution at 0.3 mL/min with a mixture of acetonitrile and 0.1% (v/v) trifluoroacetic acid in water (81:19, v/v) as the mobile phase. The run time was set at 13 min. The presented method was selective, sensitive, accurate and precise for detection and quantification of RPV and CAB in all matrices. The developed and validated bioanalytical method was successfully employed for *in vivo* samples with both drugs simultaneously.

1. Introduction

Human immunodeficiency virus (HIV) continues to be a major global public health issue [1], with approximately 38 million people across the world living with HIV at the end of 2019 [2]. Developments in antiretroviral therapy (ART) have dramatically increased the life expectancy and quality of life of infected individuals [3]. However, well established treatment regimens require daily oral medication [4], which can be challenging for some user groups [5]. The recent approval of a combined treatment regimen utilising prolonged-release suspensions of the antiretroviral agents rilpivirine (RPV) [6] and cabotegravir (CAB) [7] for monthly intramuscular injections was a great step forward towards improved HIV treatment regimens. A further important aspect is that HIV requires lifelong treatment, as current ART options can only effectively control viral replication in the systemic blood circulation. Drug permeation to specific viral reservoirs, such as lymph nodes, is limited and therapy interruption leads to viral rebounds [8]. To fully understand the biodistribution of antiretroviral agents and determine their concentration levels in various parts of the body, a simple bioanalytical method is essential. Since RPV and CAB are approved for combined administration, the method should be selective for simultaneous detection and quantification of both drugs in the presence of possible matrix interference. It should furthermore be sensitive enough for quantification of drug concentrations in the range of therapeutically relevant levels, the concentrations required to inhibit 90% of *in vitro* viral replication (IC90). Due to the high plasma protein binding of both RPV and CAB, the IC90 is protein binding adjusted. For RPV, an IC90 of 12 ng/mL is generally recognised [9]. For CAB, studies conducted in

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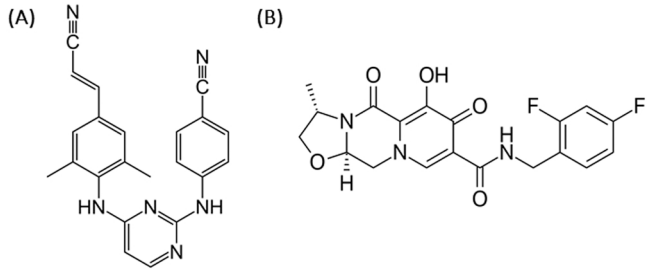

Fig. 1. Skeletal structural formulas of (A) rilpivirine and (B) cabotegravir.

macaque models have suggested that a concentration four times higher than the previously acknowledged IC90 of 166 ng/mL is more accurate, that is a 4IC90 of 664 ng/mL [10].

The skeletal structural formulas of RPV and CAB are displayed in Fig. 1. The chemical name of RPV is 4-[[4-[[4-[(E)− 2-cyanoethenyl]− 2,6-dimethylphenyl]amino]− 2-pyrimidinyl] amino] benzonitrile ($C_{22}H_{18}N_6$). RPV has a molecular weight of 366.43 g/mol, a log P > 4.16 and a pKa of 5.6 (basic pyrimidine moiety). It is a white to slightly yellow crystalline powder which is practically insoluble in aqueous media over a wide range of pH values [6]. The chemical name of CAB is (3 S,11aR)-N-[(2,4-Difluorophenyl)methyl]− 6-hydroxy-3-methyl5, 7-dioxo-2,3,5,7,11,11a-hexahydro[1,3]oxazolo [3,2-a]pyrido[1,2-*d*] pyrazine-8-carboxamide ($C_{19}H_{17}F_2N_3O_5$). CAB has a molecular weight of 405.35 g/mol and two pKa values of pKa1 = 7.7 (OH group) and pKa2 = 11.1 (NH group). It is a white to almost white crystalline powder which is practically insoluble in aqueous media below pH 9 and slightly soluble above pH 10 [7]. It has a log P value of 0.16 [11].

Various techniques have been described in the literature for the analytical quantification of RPV and CAB in *in vitro* and *in vivo* sample matrices. For the quantification of RPV in *in vitro* matrices, high performance liquid chromatography (HPLC) in combination with ultraviolet (UV) or diode array detection has been commonly employed [12], [13], [14], [15]. Further, UV detection has been used for analysis of *in vivo* matrices such as rat plasma, vaginal tissue or lymph nodes [16]. However, the ranges of the described methods did not cover the IC90 of RPV. To achieve higher sensitivity, HPLC or ultraperformance liquid chromatography (UPLC) were coupled with mass spectrometry (MS) detection. One method employed a simple MS method [17], whereas all other described methods used MS/MS detection {Merging Citations}, thus achieving lower limits of quantification (LLOQ) below the IC90 of RPV. For CAB, one method employing UV detection has been described [11], while the other methods focused on MS/MS detection [18–21], [22], [23], [24], [25]. Reversed phase (RP) C18 columns were used throughout the described methods as a stationary phase and the mobile phase was commonly acidified with suitable acids or buffers. Several of the described analytical assays employed UV detection or, in most cases, less cost effective MS/MS detection for the quantitative determination of RPV and CAB in plasma or individual drugs in single tissue matrices. However, none of the described methods focus on different tissue matrices or the simultaneous quantification of RPV and CAB, an important aspect considering their approval for combined administration. Here, for the first time, the development and validation of a HPLC-MS method for simultaneous detection and quantification of RPV and CAB in various biological sample matrices harvested from Sprague Dawley rats (plasma, skin, lymph nodes, vaginal tissue, liver, kidneys, spleen) is described.

2. Materials and Methods

2.1. Materials

Acetonitrile (HPLC grade, >99%) was purchased from Sigma-Aldrich (Dorset, UK) and trifluoroacetic acid (reagent grade, >99%) from Honeywell (Seelze, Germany). RPV free base and RPV prolonged-release suspension for injection were supplied by Janssen Pharmaceutica (Beerse, Belgium). CAB free acid and CAB prolonged-release suspension for injection were supplied by ViiV Healthcare Ltd. (Brentford, UK). Ultrapure water was obtained from an Elga Option Purelab water purification system (High Wycombe, UK). An Apex Scientific Intersil ODS-3 column (particle size 5 μm) was purchased from GL Sciences Inc. (Tokyo, Japan). All other reagents were of analytical grade and purchased from standard commercial suppliers.

2.2. Instrumentation and chromatographic conditions

To facilitate simultaneous separation, detection and quantification of RPV and CAB, an RP-HPLC method was developed on an Agilent 1260 Infinity II series system consisting of a quaternary pump, a multisampler, a multicolumn thermostat and a diode array detector (Agilent Technologies UK Ltd., Stockport, UK). This was coupled with a single quadrupole API 6400 MS detector (Agilent Technologies UK Ltd., Stockport, UK). The column used was an Apex Scientific Inertsil ODS-3 column (4.6 mm internal diameter, 250 mm length, 5 μm particle size; GL Sciences Inc., Tokyo, Japan), preceded by a Phenomenex® SecurityGuard™ HPLC guard cartridge (Phenomenex, Torrance, CA, USA) of matching chemistry. The temperature of the column was maintained at 40 °C. Separation was achieved using isocratic elution. A mixture of acetonitrile and 0.1% (v/v) trifluoroacetic acid in water (81:19, v/v) was used as the mobile phase. The flow rate was kept at 0.3 mL/min. The aqueous mobile phase was filtered through a 0.45 μm membrane filter and degassed by sonication for 30 min prior to use. The injection volume was 15 μL and the run time was set at 13 min. Samples were diverted to the MS detector after 5.5 min. For analyte MS detection, samples were introduced and ionised by electrospray ionisation in positive ion mode (+). Analysis was performed in single ion monitoring mode, with RPV detected at a mass-to-charge ratio (*m/z*) of 367.4 and CAB at 406.3. The capillary voltage was kept at 4 kV, the drying gas temperature at 300 °C, the drying gas flow at 11 L/min and the nebuliser pressure at 15 psi. Nitrogen was maintained at 100 psi and used as the source vapour. Mobile phase was implemented as a washing solution of the autosampler needle after each injection. After each sample run, 15 μL blank acetonitrile was injected with a run time of 5 min. Chromatographic data acquisition and analysis was performed using Agilent OpenLAB® Software.

2.3. Preparation of stock solutions, working standards and quality control samples

Individual stock solutions of RPV and CAB in acetonitrile were prepared at a concentration of 1 mg/mL. These were combined to obtain a stock solution containing both analytes at a concentration of 500 μg/mL. A serial dilution in acetonitrile was performed to produce working standards ranging from 0.05 μg/mL to 100 μg/mL (0.05, 0.25, 0.5, 1, 5, 10, 30, 50, 100 μg/mL).

Blank rat plasma was obtained from healthy female Sprague Dawley rats, aged 10–12 weeks. They were acclimatised to laboratory conditions for a minimum of seven days. After culling using a CO_2 chamber followed by cervical dislocation, blood was collected into heparinised tubes following cardiac puncture. Plasma was separated from blood by centrifugation at 2000 g at 4 °C for 10 min and stored in aliquots of 90 μL in 1.5 mL Eppendorf® tubes at − 20 °C until further use. After defrosting at room temperature, plasma aliquots were spiked with 10 μL of the working standards to obtain reference standards in a range from 5 ng/mL to 10,000 ng/mL (1 in 10 dilution of working standards) and quality control (QC) samples at concentrations of 25 ng/mL (low QC), 5000 ng/mL (medium QC) and 10,000 ng/mL (high QC). Calibration standards and QC samples were prepared from separate stock solutions to avoid biased estimations. Plasma standards were vortexed for 10 s to ensure even distribution of drug throughout the sample prior to

extraction.

After cardiac puncture, the following blank organs were harvested from healthy female Sprague Dawley rats after culling: skin, lymph nodes (different sites), vaginal tissue, liver, kidneys and spleen. Organs were rinsed in phosphate buffered saline (PBS, pH 7.4) to remove blood and other contaminants, blotted with paper towels, disrupted mechanically and stored in 2 mL Eppendorf® tubes at − 20 °C until further use. Blank tissue matrices were prepared by weighing out approximately 100 mg tissue into 2 mL Eppendorf® tubes. After addition of 50 μL purified water, blank matrices were homogenised in a Qiagen TissueLyser LT (UK Qiagen Ltd. Manchester, UK) at 50 Hz for 10 min and centrifuged at 14,000 g at 4 °C for 1 min. Matrices were then spiked with 10 μL of the working standards to obtain calibration standards. All standards were vortexed for 10 s prior to extraction.

Animal handling was approved by the Committee of the Biological Services Unit, Queen's University Belfast. The procedures were conducted under Procedure Project Licence number PPL 2903 and Procedure Individual Licence numbers PIL 1892, 1747, 2059 and 2056 according to the policy of the Federation of European Laboratory Animal Science Associations and the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes with implementation of the principles of the 3Rs (replacement, reduction and refinement).

2.4. Analyte extraction

For protein precipitation and analyte extraction from reference and QC standards, 900 μL acetonitrile was added to each standard. Plasma samples were vortexed for 15 min, whereas tissue samples were homogenised in the TissueLyser LT at 50 Hz for 20 min. After centrifugation at 14,000 g at 4 °C for 10 min, the supernatants were transferred into disposable glass culture tubes. These were placed into a Zymark TurboVap® LV Evaporator Workstation (McKinley Scientific, Sparta, NJ, USA) to evaporate the extracts under a stream of nitrogen (5 psi) at 35 °C for 40 min. The residues were reconstituted in 100 μL aliquots of mobile phase. Each reconstituted sample was vortexed for 10 s and transferred into a 0.5 mL Eppendorf® tube. After centrifugation at 14,000 g at 4 °C for 10 min for sample purification, supernatants were transferred into Agilent® HPLC vials containing Agilent® 250 μL vial inserts. Validation was performed based on detected concentrations in the reconstituted standards, in ng/mL. In the case of tissue matrix standards, this allowed for further calculation of the drug concentration in ng/g tissue.

2.5. Bioanalytical method validation

The developed method was validated based on the International Council for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines on bioanalytical method validation [26]. For plasma samples, a full method validation was performed, including selectivity, matrix effect, recovery, calibration curve, range (LLOQ to upper limit of quantification (ULOQ)), accuracy, precision, carry-over, dilution integrity, stability and reinjection reproducibility. A partial validation was performed for each tissue matrix, including selectivity, calibration curve, range, accuracy and precision, with a reduced number of sources and repetitions compared to plasma samples due to limited matrix availability.

2.5.1. Selectivity

Selectivity is the ability to quantify an analyte in the presence of potential interfering substances in a blank biological matrix [26]. Blank plasma and tissue matrices from six different sources were extracted and the resulting chromatograms were compared to samples spiked with analytes to investigate possible interference with endogenous substances.

2.5.2. Calibration curve, range, limit of detection and lower limit of quantification

The calibration curve demonstrates the correlation between the analyte concentration and the response [26]. Calibration standards comprise the calibration curve and span the calibration range from the LLOQ to the ULOQ. An adequate regression model should be used for describing the concentration-response relationship [26].

The calibration curves for bioanalytical samples were generated by analysing a blank sample, eight different concentration levels for plasma samples and nine different concentration levels for tissue samples. The peak areas were plotted against the analyte concentrations (excluding the blank samples). A least squares linear regression analysis was performed, and slope and y-intercept were determined. Calibrations curves for tissue samples were split in two (low concentrations and high concentrations) to maintain linearity throughout and allow for accurate quantification of low concentrations. For plasma standards, five independent runs were performed on three different days. Three of these runs were performed within one day. One representative plot was collated and individual responses from each run were back-calculated to confirm their accuracy (± 15% of the nominal concentration). A similar procedure was performed for tissue samples, however, only three independent runs were performed for each matrix.

While the limit of detection (LOD) of a procedure is the lowest amount of an analyte that can be detected but not necessarily quantified, the LLOQ is the lowest amount possible that can be precisely and accurately quantified [27]. The LOD each matrix was calculated based on the residual standard deviation (S_{res}) of the regression line and the slope of the calibration plot (S).

2.5.3. Accuracy and precision

Accuracy is the closeness of agreement between the value found and the true value. The degree of variation between a series of measurements is called precision [27]. For bioanalytical method validation, accuracy and precision are determined by analysing QCs within each run (within-run) and in different runs (between-run) [26]. For plasma samples, within-run accuracy and precision were calculated based on the analysis of five replicates each of low, medium and high QCs. The between-run accuracy was calculated based on three different runs. For tissue samples, only the between-run values were assessed. The accuracy was expressed as the percentage recovery (RE) of the found concentration (found value), calculated using the calibration plot and compared to the nominal concentration of the measured standard (true value). Precision was reported as the coefficient of variation (CV) of the measured concentrations. For this purpose, the mean concentration ($\overline{X}$), standard deviation (SD) and CV for each concentration were calculated.

2.5.4. Carry-over

Carry-over is defined as an alteration of a measured concentration due to residual analytes from a preceding sample [26]. Carry-over was assessed by injecting a blank sample after a plasma calibration standard at the ULOQ and examining the response.

2.5.5. Matrix effect and recovery

The matrix effect of a biological sample is the alteration of an analyte response by interfering components in the matrix [26]. The recovery (extraction efficiency) is the percentage of a known analyte amount that is carried through the processing steps [26]. To assess the matrix effect and recovery, blank plasma samples (90 μL) were extracted and reconstituted with working standards of low and high QCs (three replicates each). For evaluation of the matrix effect, these were compared to working standards of the same concentration. To calculate the recovery, responses were compared to plasma standards spiked prior to extraction.

2.5.6. Dilution integrity

Dilution integrity confirms that a sample dilution procedure for

highly concentrated samples does not impact the accuracy and precision of the measured analyte concentration [26]. To assess the dilution integrity of plasma samples, dilution QCs with RPV and CAB at a concentration of 50,000 ng/mL were prepared and extracted (five replicates). After reconstitution, 10 μL of the reconstituted samples were diluted with 90 μL acetonitrile (dilution factor 1 in 10) and analysed. Accuracy and precision were calculated.

2.5.7. Stability

Stability evaluations ensure that concentrations of bioanalytical samples are stable during preparation, processing, analysis and storage [26]. The freeze-thaw matrix stability, bench top (short-term) matrix stability, processed sample stability and long-term matrix stability were assessed using three replicates of low and high QCs each. For the freeze-thaw matrix stability, QCs were frozen and thawed in three cycles with at least 12 h between each thawing cycle before analysis. For bench top (short-term) matrix stability, QCs were kept on the bench top under the same conditions and for the same duration as study samples (up to four hours) before further processing. The processed sample stability (on-instrument stability) was ensured by placing processed samples into the autosampler 24 h prior to injection to simulate a possible run time. The long-term stability of the analytes during storage at − 20 °C was investigated by freezing QCs for four weeks.

2.5.8. Reinjection reproducibility

Reinjection reproducibility of bioanalytical samples should be evaluated if samples might have to be reinjected due to instrument interruptions or other reasons [26]. Low and high QCs were reinjected twice with an interval of 12 h between each injection and accuracy and precision were determined.

2.6. Application to an in vivo model

The suitability of the developed and validated bioanalytical method for detection and quantification of RPV and CAB in different biological matrices after intramuscular application was demonstrated in an *in vivo* model in healthy, female Sprague Dawley rats ($n = 4$). Animals were 10–12 weeks old and weighed 260 ± 23 g when commencing the experiment. They were acclimatised to laboratory conditions for a minimum of seven days prior to the experiment. Commercially available prolonged-release nanosuspensions of RPV (300 mg/mL, Janssen Pharmaceutica, Beerse, Belgium) and CAB (200 mg/mL, ViiV Healthcare Ltd., Brentford, UK) were diluted with water for injection to achieve a concentration of 25 mg/mL each. Each animal received one intramuscular injection (50 μL containing 1.25 mg drug) into the right posterior thigh (RPV) and one injection into the left posterior thigh (CAB). Blood samples (200 μL) were collected *via* tail vein bleeds into heparinised tubes (10 μL) after 24 h. To determine the drug biodistribution, animals were then culled using a CO_2 chamber followed by cervical dislocation. After blood collection *via* cardiac puncture, the following tissues were excised: abdominal skin, axillary lymph nodes, vaginal tissue, liver, kidneys and spleen. Blood samples were centrifuged at 2000 g at 4 °C for 10 min to separate plasma from blood. Plasma was stored at − 20 °C until analyte extraction. Harvested organs were washed with PBS (pH 7.4), blotted with paper towels and stored at − 20 °C until further processing. Prior to storage, livers, kidneys and spleens were mechanically disrupted. Analyte extraction followed the same procedures as employed during method validation. For plasma samples, aliquots of 100 μL were extracted. Organs were weighed out into aliquots of approximately 100 mg and exact weights were recorded. Skin and vaginal tissue samples were mechanically disrupted using a scalpel or surgical scissors prior to weighing. Sample extracts with concentrations exceeding the method ranges were diluted accordingly (dilution factor 1 in 10).

The *in vivo* study was approved by the Committee of the Biological Services Unit, Queen's University Belfast. The study was conducted under Procedure Project Licence number PPL 2903 and Procedure Individual Licence numbers PIL 1892, 1747, 2059 and 2056 according to the policy of the Federation of European Laboratory Animal Science Associations and the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes with implementation of the principles of the 3Rs (replacement, reduction and refinement).

2.7. Statistical analysis

Calculation of means, SD, least squares linear regression analysis, correlation analysis, percentage recovery, CV, LOD and LLOQ were performed using Microsoft Office 365 ProPlus Excel (Microsoft Corporation, Redmond, WA, USA).

3. Results and Discussion

3.1. Chromatographic conditions

A commonly used technique for the rapid detection and quantification of compounds is RP-HPLC analysis [28]. For detecting the analytes, RP-HPLC systems can be paired with different detectors, such as UV, diode array or MS detectors. The presented chromatographic method was initially developed using UV detection with a wavelength of 257 nm. The developed method was then translated to MS detection to achieve higher selectivity and sensitivity. An Inertsil ODS-3 column (4.6 ×250 mm, 5 μm) was selected. The aqueous and the organic parts of the mobile phase and their respective ratios were chosen and adjusted based on peak separation, symmetry, area and a desired run time of below 15 min. Acetonitrile was selected as the organic part of the mobile phase due to the solubility of the analytical compounds. As a starting point, water was employed as the aqueous phase. This mobile phase composition was able to elute both compounds within a maximum run time of 10 min. However, even at different ratios, RPV and CAB could not be fully separated. Based on a previously published method [16] the aqueous mobile phase was acidified with 0.1% (v/v) trifluoroacetic acid which lowered the pH to approximately 2. This lowered pH does not influence the charge of CAB compared to water at pH 7, nevertheless, as the pKa of the basic pyrimidine moiety of RPV is 5.6, an acidified mobile phase leads to protonation of RPV. This allows for the separation of CAB and RPV. The peak shape and symmetry of CAB were not considerably influenced by the ratio of the mobile phase. RPV on the other hand showed peak splitting when high percentages of aqueous phase were used and peak tailing at high percentages of organic phase. A possible explanation for this is the presence of further basic nitrogen containing functional groups (secondary amines) in the molecule. Peak splitting might have occurred due to partly protonated secondary amine groups at high percentages of 0.1% (v/v) trifluoroacetic acid in water. In contrast, at low percentages these basic groups might interact with partly uncapped, negatively charged silanol groups of the C18 column, leading to peak tailing. Thus, the mobile phase ratio was set at acetonitrile: 0.1% (v/v) trifluoroacetic acid in water (81:19). The flow rate was adjusted based on the Van Deemter equation, a hyperbolic function that predicts an optimum velocity at which a maximum efficiency can be reached [29]. The equation considers the pathways of molecules within the column (independent of the flow rate), longitudinal diffusion within the mobile phase (decreasing with increasing velocity) and mass transfer between the stationary and the mobile phase (proportional to the velocity). Taking this into account, using a particular flow rate maximises the resolving power of a column [29]. During method development, resolution could be increased considerably by decreasing the flow rate. However, to keep elution times in a practical range (below 15 min), the flow rate was settled at 0.3 mL/min. The temperature was set at 40 °C, as at 35 °C and 45 °C the peak symmetry was impaired. Increasing the injection volume enables the analysis of lower concentrations, but it can also lead to an overload of the column. The injection volume was set at

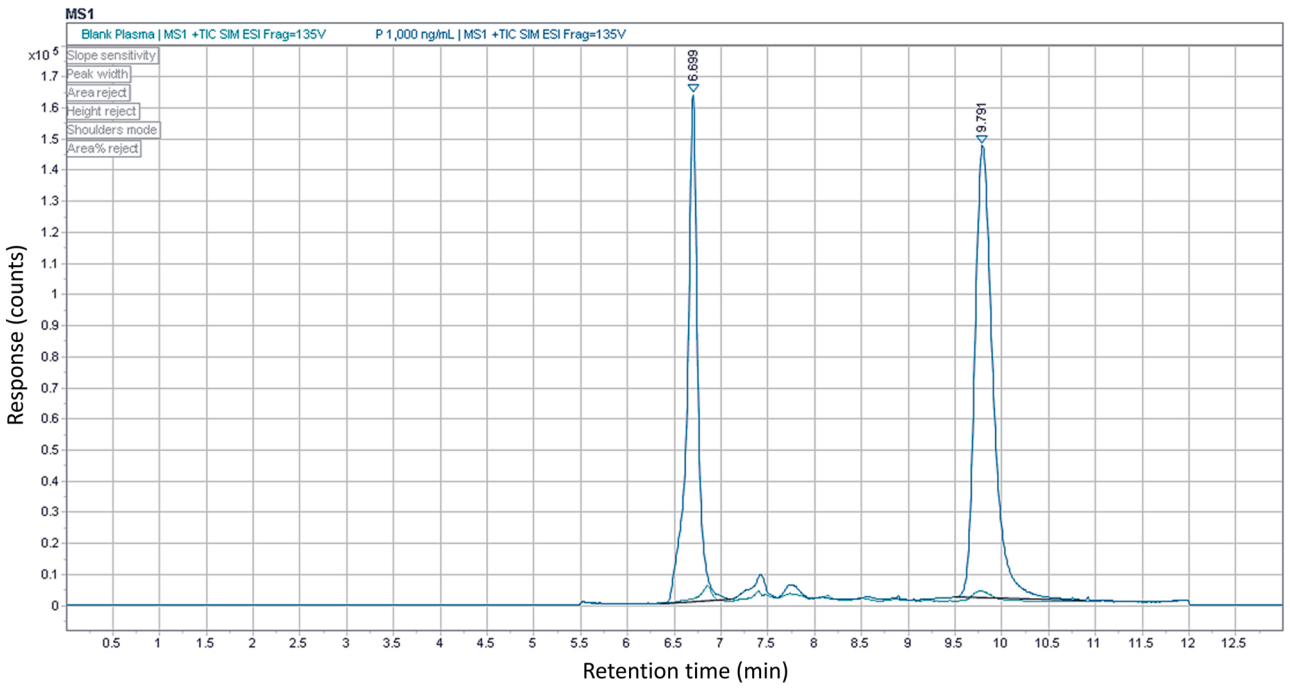

Fig. 2. Chromatograms of blank plasma and plasma spiked with rilpivirine (RPV) and cabotegravir (CAB) (1000 ng/mL each).

Table 1
Representative calibration plot characteristics of rilpivirine (RPV) and cabotegravir (CAB) in different biological matrices (harvested from female Sprague Dawley rats), including the ranges, slopes, y-intercepts and limits of detection (LODs).

Matrix	Range [ng/mL]	RPV			CAB		
		Slope	y-intercept	LOD [ng/mL]	Slope	y-intercept	LOD [ng/mL]
Plasma	5–10,000	1212.94	54,557.50	4.77	1799.25	80,862.24	4.93
Skin	5–500	2098.38	222,010.80	2.12	3274.31	210,711.37	2.96
	500–10,000	1853.15	351,057.87		1764.20	1036,980.52	
Lymph nodes	5–500	2762.83	67,990.84	1.16	2422.35	46,949.18	1.10
	500–10,000	2325.73	368,913.84		2053.35	295,755.17	
Vaginal tissue	5–500	2572.77	147,904.38	1.42	1980.13	246,509.83	2.90
	500–10,000	1683.93	686,064.90		1854.75	374,723.75	
Liver	5–500	2996.99	123,987.97	1.73	2789.73	166,463.18	3.10
	500–10,000	1453.50	926,501.52		2072.67	516,076.74	
Kidneys	5–500	1637.43	69,027.69	1.84	3649.64	374,410.75	3.15
	500–10,000	1573.67	64,752.80		1713.28	1392,985.03	
Spleen	5–500	2031.41	86,659.52	1.90	2730.21	173,129.74	3.19
	500–10,000	1566.91	383,622.21		1831.19	708,343.00	

15 μL as this resulted in increased peak areas compared to 10 μL without distortion, which could be observed at 20 μL.

3.2. Analyte extraction

Prior to sample analysis, RPV and CAB were extracted from rat plasma and tissue samples. Both drugs are highly protein bound, with > 99% in rats for RPV [6] and > 99.9% for CAB [7]. Proteins can be simply precipitated by addition of an organic solvent such as acetonitrile. After centrifugation to concentrate the precipitated proteins as a pellet at the bottom of a vial, the supernatant can either be immediately injected into the RP-HPLC system or further concentrated and purified. A high ratio of acetonitrile to plasma/ tissue samples (900 μL acetonitrile: 100 μL or 100 mg sample) was chosen to maximise the extraction efficiency. The benefits of a double extraction, that is addition of organic solvent followed by centrifugation and removal of supernatant followed by a second extraction cycle, were investigated. However, there were no apparent improvements in extraction efficiency compared to single extraction, and as double extraction is more time-consuming, single extraction was employed. If immediately injecting the supernatant, the analyte concentrations in the samples are considerably diluted. As relatively low concentrations, especially for RPV, were anticipated in the *in vivo* study, the supernatants were evaporated, and samples were reconstituted in a volume equivalent to the initial sample volume. This approach also further purified the samples and avoided protein interference detected at the retention time of RPV when immediately injecting the supernatant. Sample reconstitution was performed with mobile phase. Reconstitution in only acetonitrile led to a reduced and highly variable extraction efficiency due to the presence of remaining hydrophilic plasma components in the precipitate that trapped the hydrophobic drugs. The use of mobile phase (19% (v/v) aqueous) circumvented this problem. The outlined sample extraction procedure resulted in highly purified samples with minimal noise and interference. It also extended the column lifetime, which was further protected by using a guard column of matching chemistry.

3.3. Bioanalytical method validation

The developed bioanalytical method was validated according to the ICH guidelines [26]. Full method validation was performed for rat

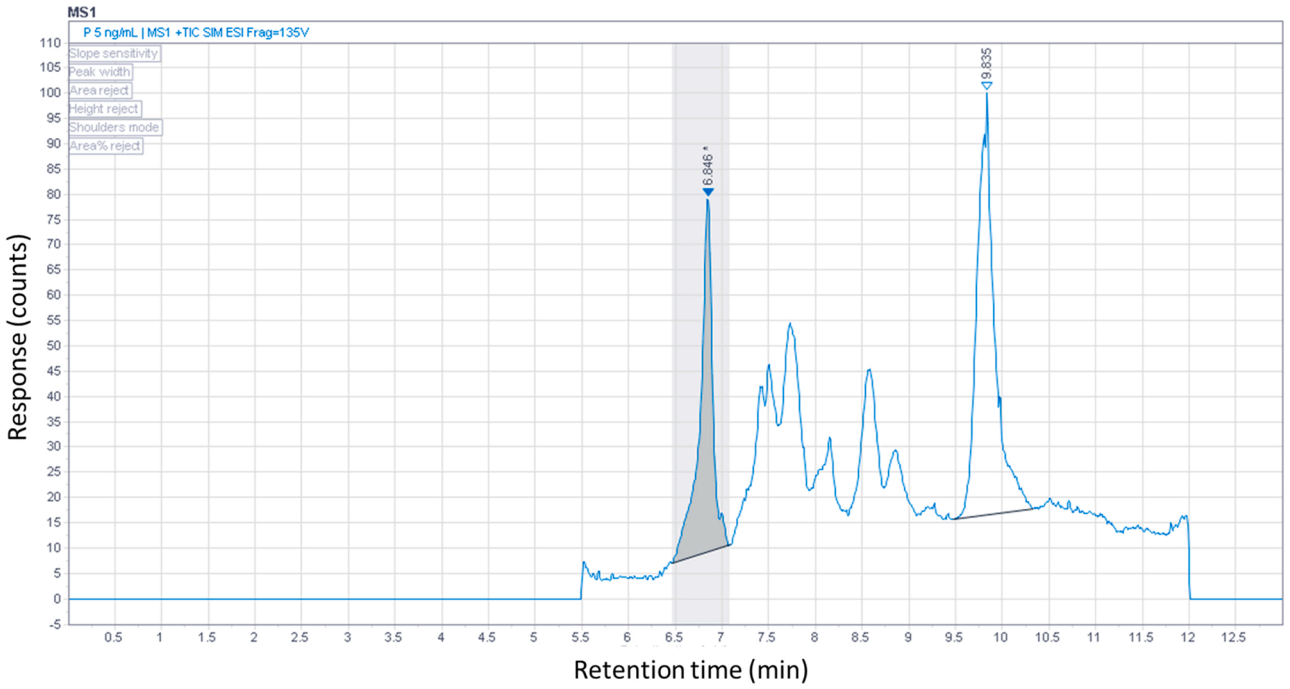

Fig. 3. Chromatograms in plasma with rilpivirine (RPV) and cabotegravir (CAB) at LLOQ (5 ng/mL each).

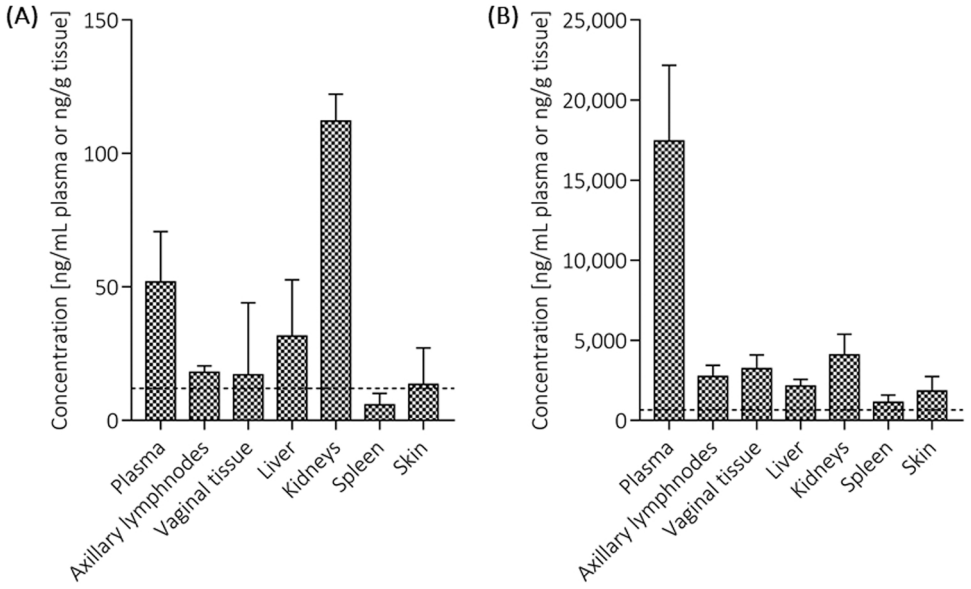

Fig. 4. Concentration levels of (A) rilpivirine (RPV) and (B) cabotegravir (CAB) in plasma [ng/mL] and different tissues [ng/g] excised from healthy, female Sprague Dawley rats 24 h after intramuscular injection of 5 mg/kg RPV and 5 mg/kg CAB long-acting nanosuspensions (means ± SD, $n = 4$). Therapeutically relevant concentrations are indicated by the dashed black lines (RPV: IC90 = 12 ng/mL; CAB: 4IC90 = 664 ng/mL).

plasma to ensure the reliability of analytical results. According to the guidelines, when changing from one matrix within a species to another, a partial method validation may be sufficient and the extent depends on the nature of the method [26]. As the extraction efficiency and response varied between different tissues without linear correlation to plasma samples, three independent calibration runs across the full range were performed for each tissue, that is skin, lymph nodes, vaginal tissue, liver, kidneys and spleen. However, further assessments that would be required for a full validation were omitted due to the limited availability of tissue matrices.

3.3.1. *Selectivity*

The developed bioanalytical method was selective for plasma and tissue matrices, with the responses of RPV and CAB clearly separated from matrix interference. Exemplary chromatograms of blank plasma and plasma spiked with RPV and CAB (1000 ng/mL) are displayed in Fig. 2.

3.3.2. *Calibration curve, range, limit of detection and lower limit of quantification*

The calibration curves were generated by analysing eight different concentration levels for plasma samples and nine different concentration levels for tissue samples. For plasma samples, the method showed

Table 2
Accuracy and precision for the quantification of rilpivirine (RPV) and cabotegravir (CAB) in different biological matrices (harvested from female Sprague Dawley rats). For plasma samples, within-run and between-run accuracy and precision were determined. For all other matrices, only between-run values were determined. Displayed are nominal concentrations (C_N) covering the range of the methods (low, medium and high quality control samples), calculated concentrations ($C_C \pm SD$, $n = 5$ for within-run, $n = 3$ for between-run), accuracy expressed as percentage recoveries (RE) and precision expressed as coefficient of variation (CV).

Accuracy/ precision		C_N [ng/mL]	RPV			CAB		
			C_C [ng/mL]	RE [%]	CV [%]	C_C [ng/mL]	RE [%]	CV [%]
Plasma	Within-run	25	25.5 ± 2.3	102.1	9.1	24.6 ± 2.2	98.3	9.0
		5000	5201.8 ± 356.4	104.0	6.9	5138.2 ± 174.7	102.8	3.4
		10,000	10,259.3 ± 478.3	102.6	4.7	9890.0 ± 330.5	98.9	3.3
	Between-run	25	25.6 ± 2.4	102.2	9.5	26.6 ± 2.6	106.2	9.6
		5000	5011.5 ± 241.4	100.2	4.8	4981.1 ± 144.2	99.6	2.9
		10,000	9597.6 ± 859.6	96.0	9.0	9964.1 ± 346.6	99.6	3.5
Skin	Low Range	25	25.1 ± 0.3	100.5	1.2	25.4 ± 0.9	101.7	3.6
		100	100.4 ± 3.9	100.4	3.8	101.1 ± 3.4	101.1	3.3
		500	500.0 ± 49.7	100.0	9.9	499.9 ± 25.0	100.0	5.0
	High Range	500	496.5 ± 56.3	99.3	11.3	459.4 ± 46.4	91.9	10.1
		5000	4938.4 ± 33.5	98.8	0.7	5037.6 ± 24.5	100.8	0.5
		10,000	10,025.0 ± 296.4	100.3	3.0	9988.7 ± 135.7	99.9	1.4
Lymph nodes	Low Range	25	25.2 ± 0.2	100.6	0.7	25.4 ± 0.5	101.5	1.9
		100	100.3 ± 0.6	100.3	0.6	100.0 ± 0.6	100.0	0.6
		500	499.9 ± 2.6	100.0	0.5	500.0 ± 1.8	100.0	0.4
	High Range	500	464.5 ± 3.1	92.9	0.7	505.6 ± 8.6	93.7	1.7
		5000	4989.1 ± 298.9	99.8	6.0	4702.7 ± 124.5	100.4	2.6
		10,000	10,001.7 ± 128.4	100.0	1.3	9439.3 ± 178.4	99.9	1.9
Vaginal tissue	Low Range	25	24.7 ± 2.1	98.7	8.6	25.7 ± 0.7	102.9	2.7
		100	100.0 ± 4.4	100.0	4.4	99.7 ± 8.9	99.7	8.9
		500	500.0 ± 9.5	100.0	1.9	500.1 ± 3.9	100.0	0.8
	High Range	500	444.4 ± 14.5	88.9	3.3	517.2 ± 9.5	93.0	1.8
		5000	5011.8 ± 311.9	100.2	6.2	5163.7 ± 137.8	101.1	2.7
		10,000	9989.8 ± 414.5	99.9	4.1	10,407.5 ± 197.5	99.8	1.9
Liver	Low Range	25	25.1 ± 0.8	100.5	3.1	25.2 ± 1.0	100.8	4.1
		100	99.3 ± 4.0	99.3	4.1	98.6 ± 7.2	98.6	7.3
		500	500.1 ± 15.1	100.0	3.0	500.2 ± 6.6	100.0	1.3
	High Range	500	479.1 ± 31.1	95.8	6.5	504.6 ± 8.8	100.9	1.7
		5000	4945.2 ± 114.0	98.9	2.3	4944.8 ± 95.9	98.9	1.9
		10,000	10,024.6 ± 492.8	100.2	4.9	10,017.9 ± 1039.8	100.2	10.4
Kidneys	Low Range	25	25.5 ± 2.7	102.1	10.6	24.1 ± 3.4	96.5	14.2
		100	99.5 ± 6.3	99.5	6.3	101.1 ± 5.1	101.1	5.0
		500	500.1 ± 13.2	100.0	2.6	499.9 ± 10.1	100.0	2.0
	High Range	500	523.1 ± 13.7	104.6	2.6	470.3 ± 21.5	94.1	4.6
		5000	4952.4 ± 51.8	99.0	1.0	4947.6 ± 125.2	99.0	2.5
		10,000	10,020.4 ± 398.5	100.2	4.0	10,019.4 ± 58.5	100.2	0.6
Spleen	Low Range	25	25.1 ± 1.5	100.3	6.0	25.3 ± 2.3	101.2	9.0
		100	100.3 ± 6.6	100.3	6.5	99.4 ± 0.6	99.4	0.6
		500	500.0 ± 24.8	100.0	5.0	500.2 ± 14.3	100.0	2.9
	High Range	500	458.7 ± 32.1	91.7	7.0	453.5 ± 21.4	90.7	4.7
		5000	4970.8 ± 156.1	99.4	3.1	5012.6 ± 372.1	100.3	7.4
		10,000	10,009.7 ± 102.3	100.1	1.0	9985.4 ± 965.6	99.9	9.7

Table 3
Recovery and matrix effect of plasma (harvested from female Sprague Dawley rats) on the response of rilpivirine (RPV) and cabotegravir (CAB). Assessed were accuracy and precision. Displayed are nominal concentrations (C_N) (low and high quality control samples), calculated concentrations (CC ± SD, n = 3), accuracy of the extraction efficiency expressed as percentage recoveries (RE), precision expressed as coefficient of variation (CV) and the matrix effect (ME ± SD, n = 3).

Recovery/ Matrix effect	C_N [ng/mL]	C_C [ng/mL]	RE [%]	CV [%]	ME [%]
RPV	25	26.6 ± 1.8	106.9	6.9	105.6 ± 4.2
	10,000	9532.1 ± 934.3	95.3	9.8	105.8 ± 13.5
CAB	25	22.2 ± 0.7	89.0	3.2	109.3 ± 4.6
	10,000	10,539.4 ± 242.3	105.4	2.3	93.6 ± 2.1

linearity over the full calibration range. Calibrations curves for tissue samples were split in two (low concentrations and high concentrations) to maintain linearity throughout and allow for accurate quantification of low concentrations. For plasma standards, five independent runs were performed on three different days. Three of these runs were performed within one day. One representative plot was collated for each drug. Individual responses from each run were back-calculated to confirm their accuracies. These ranged from 90.2% to 110.7% of the nominal concentrations (within in the limits of ± 15%). A similar procedure was performed for tissue samples, however, only three independent runs were performed for each matrix. Each run was split into two graphs ranging from 5 ng/mL to 500 ng/mL and from 500 ng/mL to 10,000 ng/mL to allow for accurate quantification of low and high concentrations. Correlation coefficient values (R^2) ranged from 0.9998 to 1.0000. Characteristics of the collated calibration curves for each matrix, including ranges, slopes, y-intercepts and LODs, are presented in Table 1. The LODs of the developed method were calculated based on the residual standard deviations of the regression lines. The LLOQs were set at 5 ng/mL based on the lowest concentrations accurately determined within the range of the method (Figs. 3 and 4).

Table 4
Dilution integrity (dilution 1 in 10), freeze-thaw matrix stability, bench top (short-term) matrix stability, processed sample (on-instrument) stability, long-term matrix stability and reinjection reproducibility of rilpivirine (RPV) and cabotegravir (CAB) in plasma (harvested from female Sprague Dawley rats). Assessed were accuracy and precision (low and high quality control samples). Displayed are nominal concentrations (C_N), calculated concentrations ($C_C \pm SD$, $n = 5$ for dilution integrity, $n = 3$ for stabilities and reinjection reproducibility), accuracy expressed as percentage recoveries (RE) and precision expressed as coefficient of variation (CV).

Plasma	C_N [ng/mL]	RPV			CAB		
		C_C [ng/mL]	RE [%]	CV [%]	C_C [ng/mL]	RE [%]	CV [%]
Dilution integrity	5000	5510.1 ± 301.6	110.2	5.5	5171.7 ± 253.9	100.6	5.1
Freeze-thaw stability	25	23.6 ± 3.1	94.2	13.3	23.3 ± 1.0	93.3	4.4
	10,000	9247.6 ± 424.2	92.5	4.6	9967.7 ± 952.7	99.7	9.6
Bench top stability	25	25.7 ± 2.9	102.9	11.1	24.7 ± 1.9	98.7	7.6
	10,000	10,410.6 ± 575.4	104.1	5.5	9772.5 ± 212.6	97.7	2.2
On-instrument stability	25	24.7 ± 2.4	99.0	9.9	24.4 ± 2.4	97.5	9.9
	10,000	9894.1 ± 658.4	98.9	6.7	10,625.1 ± 463.7	106.3	4.4
Long-term stability	25	25.7 ± 2.4	103.0	9.2	23.6 ± 2.0	94.3	8.7
	10,000	8661.8 ± 153.4	86.6	1.8	9087.2 ± 221.6	90.9	2.4
Reinjection reproducibility	25	24.7 ± 3.3	98.8	13.5	25.3 ± 0.7	101.3	2.7
	10,000	9387.8 ± 489.7	93.9	5.2	9528.1 ± 44.5	95.3	0.5

3.3.3. Accuracy and precision

For plasma samples, within-run and between-run accuracy and precision were determined. As multiple matrices were analysed and the LLOQs varied between matrices, only three QCs were analysed, that is low, medium and high QC. For RPV, the percentage recovery varied between 96.0% and 104.0% and the coefficient of variation ranged from 4.7% to 9.5% The percentage recovery for CAB varied from 98.3% to 106.2% and the coefficient of variation from 2.9% to 9.6% (Table 2). According to the ICH guidelines [26], the overall accuracy should be within ± 15% of the nominal concentration and the precision should not exceed a coefficient of variation of 15%. All results were well within these limits, indicating a high accuracy and precision of the method. For tissue matrices, a partial validation was performed. Therefore, only the between-run accuracy and precision were determined. None of the values exceeded the outlined specifications, indicating that the methods were accurate and precise for the quantification of RPV and CAB in skin, lymph nodes, vaginal tissue, liver, kidneys and spleen (Table 2).

3.3.4. Carry-over

Carry-over was assessed by injecting a blank acetonitrile sample after a plasma calibration standard at the ULOQ and examining the response. No relevant response could be observed in the blank sample, indicating a minimum risk for carry-over. However, to eliminate any risks of possible carry-over during sample analysis, a blank acetonitrile sample was injected after each bioanalytical sample with a run time of 5 min

3.3.5. Matrix effect and recovery

The matrix effect of plasma on the responses of RPV and CAB and their extraction efficiency (recovery) during the extraction process were assessed by extracting blank plasma samples and reconstituting them with working standards of low and high QCs. For the matrix effect, the responses were compared to working standards. For the recovery, the responses were compared to plasma standards spiked prior to extraction. The accuracy did not exceed the limits of ± 15% and the coefficient of variation was not greater than 15% (Table 3) as required by the ICH guidelines [26].

3.3.6. Dilution integrity

To assess the dilution integrity of plasma samples, dilution QCs with RPV and CAB at a concentration of 50,000 ng/mL were prepared and extracted. After reconstitution, 10 µL of the reconstituted samples were diluted with 90 µL acetonitrile (dilution factor 1 in 10) and analysed. The recovery of RPV was 110.2% with a precision of 5.5%. For CAB, the recovery was 100.6% with a precision of 5.1% (Table 4). The values were within the limits stated by the ICH guidelines (accuracy ± 15% and coefficient of variation below 15%) [26].

3.3.7. Stability

During an *in vivo* study, samples may need to be stored for a certain amount of time until sample analysis. Thus, stability under storage conditions and, additionally, under conditions to be expected during sample processing and analysis need to be assessed. The freeze-thaw matrix stability, bench top (short-term) matrix stability, processed sample (on-instrument) stability and long-term matrix stability were assessed for plasma samples using three replicates of low and high QCs each. Values for accuracy and precision were to be within ± 15% and below 15%, respectively [26]. RPV and CAB were both stable under all conditions tested (Table 4).

3.3.8. Reinjection reproducibility

Low and high QCs of RPV and CAB in plasma samples were reinjected twice with an interval of 12 h between each injection, and accuracy and precision were determined to evaluate if samples could be reinjected in the case of instrument interruptions or other reasons. All values (Table 4) were within the required limits for accuracy and precision (± 15% and below 15%, respectively) [26].

3.4. Application to an in vivo model

The suitability of the developed and validated bioanalytical method for detection and quantification of RPV and CAB was demonstrated by application to an *in vivo* model. Plasma and tissue samples were taken 24 h after intramuscular injection of commercially available RPV and CAB prolonged-release nanosuspensions in healthy, female Sprague Dawley rats. RPV and CAB were present in all samples analysed. Detected concentration levels stayed within the ranges of the bioanalytical methods. Plasma samples were analysed twice, once undiluted for detection and quantification of RPV and once after dilution (1 in 10) for analysis of CAB. Therapeutically relevant RPV and CAB concentration levels above the IC90 of 12 ng/mL for RPV and 4IC90 of 664 ng/mL for CAB were detected in all samples but spleens (RPV).

4. Conclusion

Since the antiretroviral agents RPV and CAB are approved for combined administration, a novel, selective and sensitive HPLC-MS method for the simultaneous detection and quantification of both analytes in rat plasma and different tissue matrices was developed. The method was successfully validated for all matrices following the ICH guidelines for validation of bioanalytical methods. Analytes were extracted from biological samples employing a simple one-step protein precipitation method using acetonitrile. Analysis was conducted within 13 min and method ranges included therapeutically relevant concentration levels. Suitability of the developed method for *in vivo* application was

demonstrated in an investigational *in vivo* study in female Sprague Dawley rats. The presented method can be employed for future studies to enhance the understanding of the pharmacokinetics and biodistribution of RPV and CAB. This is an important aspect especially regarding the development and characterisation of novel drug delivery systems for targeting of specific viral reservoirs, such as lymph nodes.

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CRediT authorship contribution statement

Inken K. Ramöller: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualisation, Writing – original draft. **Marco T. A. Abbate**: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing. **Lalitkumar K. Vora**: Conceptualization, Investigation, Methodology, Writing – review & editing. **Aaron R. J. Hutton**: Investigation. **Ke Peng**: Investigation. **Fabiana Volpe-Zanutto**: Conceptualization, Methodology. **Ismaiel A. Tekko**: Methodology. **Kurtis Moffatt**: Methodology. **Alejandro J. Paredes**: Conceptualization, Supervision. **Helen O. McCarthy**: Project administration, Supervision. **Ryan F. Donnelly**: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

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

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References

[1] World Health Organization, Fact sheet HIV/AIDS, (2019). ⟨https://www.who.int/en/news-room/fact-sheets/detail/hiv-aids⟩ (accessed October 29, 2019).
[2] World Health Organization, Fact sheet HIV/AIDS, Geneva, 2020. ⟨https://www.who.int/news-room/fact-sheets/detail/hiv-aids⟩ (accessed October 16, 2020).
[3] R.N.O. Cobucci, P.H. Lima, P.C. de Souza, V.V. Costa, M. da, C. de, M. Cornetta, J. V. Fernandes, A.K. Gonçalves, Assessing the impact of HAART on the incidence of defining and non-defining AIDS cancers among patients with HIV/AIDS: a systematic review, J. Infect. Public Health 8 (2015) 1–10, https://doi.org/10.1016/j.jiph.2014.08.003.
[4] World Health Organization, Consolidated guidelines on the use of antiretroviral drugs for treating and preventing HIV infection: recommendations for a public health approach, 2nd ed., Geneva, 2016.
[5] M. Barnhart, Long-Acting HIV treatment and prevention: closer to the threshold, Glob. Health Sci. Pract. 5 (2017) 182–187, https://doi.org/10.9745/GHSP-D-17-00206.
[6] European Medicines Agency CHMP, Assessment report - Rekambys - Procedure No. EMEA/H/C/005060/0000, (2020). ⟨https://www.ema.europa.eu/en/documents/assessment-report/rekambys-epar-public-assessment-report_en.pdf⟩ (accessed June 16, 2021).
[7] European Medicines Agency CHMP, Assessment report - Vocabria - Procedure No. EMEA/H/C/004976/0000, (2020). ⟨https://www.ema.europa.eu/en/documents/assessment-report/vocabria-epar-public-assessment-report_en.pdf⟩ (accessed June 16, 2021).
[8] M.M. Elsheikh, Y. Tang, D. Li, G. Jiang, Deep latency: a new insight into a functional HIV cure, EBioMedicine 45 (2019) 624–629, https://doi.org/10.1016/j.ebiom.2019.06.020.
[9] P. Patel, S.L. Ford, H. Crauwels, K. Han, S. Rossenu, M. Neyens, S. Griffith, K. J. Hudson, D. Margolis, M. Baker, P. Williams, W. Spreen, Pharmacokinetics of cabotegravir (CAB) and rilpivirine (RPV) long-acting (LA) injectables in HIV-infected individuals through 48 weeks in the FLAIR and ATLAS phase 3 studies, Open Forum Infect. Dis. 6 (2019) S865, https://doi.org/10.1093/OFID/OFZ360.2173.
[10] T. Whitfield, A. Torkington, C. van Halsema, Profile of cabotegravir and its potential in the treatment and prevention of HIV-1 infection: evidence to date, HIV/AIDS - Res. Palliat. Care 8 (2016) 157–164, https://doi.org/10.2147/HIV.S97920.
[11] T.A. Kulkarni, A.N. Bade, B. Sillman, B.L.D. Shetty, M.S. Wojtkiewicz, N. Gautam, J.R. Hilaire, S. Sravanam, A. Szlachetka, B.G. Lamberty, B.M. Morsey, H.S. Fox, Y. Alnouti, J.E.M. McMillan, R.L. Mosley, J. Meza, P.L. Domanico, T.Y. Yue, G. Moore, B.J. Edagwa, H.E. Gendelman, A year-long extended release nanoformulated cabotegravir prodrug, Nat. Mater. 19 (2020) 910–920, https://doi.org/10.1038/s41563-020-0674-z.
[12] S. Ghosh, S. Bomma, V.L. Prasanna, S. Vidyadhar, D. Banji, S. Roy, Method development and validation of rilpivirine in bulk and tablet doses form by RP-HPLC method, Res. J. Pharm. Technol. 6 (2013) 240–243.
[13] S. Venkatesan, N. Kannappan, S.S. Mannemala, Stability-indicating HPLC method for the simultaneous determination of HIV tablet containing emtricitabine, tenofovir disoproxil fumarate, and rilpivirine hydrochloride in pharmaceutical dosage forms, Int. Sch. Res. Not. 2014 (2014) 1–9, https://doi.org/10.1155/2014/849149.
[14] A. Date, P. Shibata, C.J.Destache Bruck, Development and validation of a simple and isocratic reversed-phase HPLC method for the determination of rilpivirine from tablets, nanoparticles and HeLa cell lysates, Biomed. Chromatogr. 29 (2015) 709–715, https://doi.org/10.1002/bmc.3346.
[15] D. Karunakranth, A.K. Midha, R.S. Babu, D. Kishore, Development and validation of HPLC method for simultaneous estimation of emtricitabine, rilpivirine and tenofovir disoproxil fumarate tablet dosage form, Indian, J. Res. Pharm. Biotechnol. 6 (2018) 8–15.
[16] M.T.C. McCrudden, E. Larrañeta, A. Clark, C. Jarrahian, A. Rein-Weston, S. Lachau-Durand, N. Niemeijer, P. Williams, C. Haeck, H.O. McCarthy, D. Zehrung, R. F. Donnelly, Design, formulation and evaluation of novel dissolving microarray patches containing a long-acting rilpivirine nanosuspension, J. Control. Release 292 (2018) 119–129, https://doi.org/10.1016/j.jconrel.2018.11.002.
[17] M. Shibata, M. Takahashi, M. Yoshino, T. Kuwahara, T. Nomura, Y. Yokomaku, W. Sugiura, Development and application of a simple LC-MS method for the determination of plasma rilpivirine (TMC-278) concentrations, J. Med. Investig. 60 (2013) 35–40, https://doi.org/10.2152/jmi.60.35.
[18] A. Gupta, S. Guttikar, Y. Patel, P.S. Shrivastav, M. Sanyal, Reliable LC-MS/MS assay for the estimation of rilpivirine in human plasma: application to a bioequivalence study and incurred sample reanalysis, Drug Test. Anal. 7 (2015) 290–299, https://doi.org/10.1002/dta.1665.
[19] P.K. Prathipati, S. Mandal, C.J. Destache, Simultaneous quantification of tenofovir, emtricitabine, rilpivirine, elvitegravir and dolutegravir in mouse biological matrices by LC–MS/MS and its application to a pharmacokinetic study, J. Pharm. Biomed. Anal. 129 (2016) 473–481, https://doi.org/10.1016/j.jpba.2016.07.040.
[20] Y. Zheng, R. Aboura, S. Boujaafar, G. Lui, D. Hirt, N. Bouazza, F. Foissac, J. M. Treluyer, S. Benaboud, I. Gana, HPLC-MS/MS method for the simultaneous quantification of dolutegravir, elvitegravir, rilpivirine, darunavir, ritonavir, raltegravir and raltegravir-β-D-glucuronide in human plasma, J. Pharm. Biomed. Anal. 182 (2020), 113119, https://doi.org/10.1016/j.jpba.2020.113119.
[21] A.V. Raju, A.R. Nemala, Development and validation of a LC-MS/MS method for the determination of rilpivirine in sprague dawley rat serum and its application to pharmacokinetic study, Asian J. Biomed. Pharm. Sci. 3 (2013) 23–29. ⟨http://www.jbiopharm.com⟩. accessed June 10, 2021.
[22] A.R. Kirtane, O. Abouzid, D. Minahan, T. Bensel, A.L. Hill, C. Selinger, A. Bershteyn, M. Craig, S.S. Mo, H. Mazdiyasni, C. Cleveland, J. Rogner, Y.-A. L. Lee, L. Booth, F. Javid, S.J. Wu, T. Grant, A.M. Bellinger, B. Nikolic, A. Hayward, L. Wood, P.A. Eckhoff, M.A. Nowak, R. Langer, G. Traverso, Development of an oral once-weekly drug delivery system for HIV antiretroviral therapy, Nat. Commun. 9 (2018) 1–12, https://doi.org/10.1038/s41467-017-02294-6.
[23] S.L. Letendre, A. Mills, D. Hagins, S. Swindells, F. Felizarta, J. Devente, C. Bettacchi, Y. Lou, S. Ford, K. Sutton, J.S. Shaik, H. Crauwels, R. D'Amico, P. Patel, Pharmacokinetics and antiviral activity of cabotegravir and rilpivirine in cerebrospinal fluid following long-acting injectable administration in HIV-infected adults, J. Antimicrob. Chemother. 75 (2020) 648–655, https://doi.org/10.1093/jac/dkz504.
[24] P. Courlet, S. Alves Saldanha, M. Cavassini, C. Marzolini, E. Choong, C. Csajka, H. F. Günthard, P. André, T. Buclin, V. Desfontaine, L.A. Decosterd, Development and validation of a multiplex UHPLC-MS/MS assay with stable isotopic internal standards for the monitoring of the plasma concentrations of the antiretroviral drugs bictegravir, cabotegravir, doravirine, and rilpivirine in people living with HIV, J. Mass Spectrom. 55 (2020) 9, https://doi.org/10.1002/jms.4506.
[25] J.S.B. Shaik, S.L. Ford, Y. Lou, Z. Zhang, K.K. Bakshi, A.R. Tenorio, C. Trezza, W. R. Spreen, P. Patel, A phase 1 study to evaluate the pharmacokinetics and safety of cabotegravir in patients with hepatic impairment and healthy matched controls, Clin. Pharmacol. Drug Dev. 8 (2019) 664–673, https://doi.org/10.1002/cpdd.655.
[26] European Medicines Agency, ICH guideline M10 on bioanalytical method validation, (2019). ⟨https://www.ema.europa.eu/en/documents/scientific-guideline/draft-ich-guideline-m10-bioanalytical-method-validation-step-2b_en.pdf⟩ (accessed February 17, 2021).
[27] European Medicines Agency, ICH Harmonized tripartite guideline - Validation of analytical procedures: Text and methodology, Q2(R1), (2005). ⟨https://www.ich.

org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf) (accessed May 7, 2019).
[28] G.A. Shabir, A practical approach to validation of HPLC methods under current good manufacturing practices, J. Valid. Technol. 1 (2004) 29–37.
[29] J.J. van Deemter, F.J. Zuiderweg, A. Klinkenberg, Longitudinal diffusion and resistance to mass transfer as causes of nonideality in chromatography, Chem. Eng. Sci. 5 (1956) 271–289, https://doi.org/10.1016/0009-2509(56)80003-1.