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# Development, validation and clinical implementation of a UPLC-MS/MS bioanalytical method for simultaneous quantification of cabotegravir and rilpivirine E-isomer in human plasma

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ABSTRACT

A reversed phase ultra-high performance liquid chromatography method was developed for the simultaneous quantification of cabotegravir (CAB) and the E-isomer of rilpivirine (RPV) in human EDTA plasma, also considering RPV E-isomer instability. Because of the instability of RPV (and CAB) in all light conditions, the (RPV Z-isomer/total RPV)-isomer ratio of RPV was determined for each stock, calibration curve standard, quality control sample and patient sample. [$^{2}H_3$]-CAB and [$^{13}C_6$]-RPV were used as internal standard. Sample preparation involved protein precipitation of plasma using methanol. An HSS T3 column with a guard column (set at 40 °C) was used for analyte separation. The mobile phase components were 65 % 0.1 % formic acid in water (A) and 35 % 0.1 % formic acid in acetonitrile (B) and the flow rate was 0.5 mL/min. Detection was performed with tandem mass spectrometry (MS/MS) in a total runtime of 3.0 min. The assay was validated over the concentration range of 0.0500 – 10.0 mg/L for CAB and 0.00300 – 3.00 mg/L for RPV. The average within-day and between-day accuracies for the assay in plasma were 101 % and 101 % for CAB and 97.6 % and 98.5 % voor RPV, respectively. Within-day and between-day precision in coefficients of variations (CV) were 5.0 %. Extraction recovery was 99 % and 102 % for CAB and its internal standard and 95 % and 97 % for RPV and its internal standard. As our aim was that the (Z-isomer RPV/total RPV) response ratio in patient samples had to be less than 10 % to give reliable results, the (Z-isomer RPV/total RPV) response ratio in stocks, calibration curve standards and internal quality control samples were also taken into account being maximal 0.9 % and 2.3 % respectively. This assay has been successfully used in our Therapeutic Drug Monitoring (TDM) service for people living with HIV on long-acting injectable therapy with CAB/RPV and will also be used in future pharmacokinetic studies.

## 1. Introduction

Combination antiretroviral therapy (cART) for treatment of HIV provides durable viral suppression, which is associated with enhanced life expectancy and reduced mortality in people living with HIV [1]. Today, the optimal first-line regimens as cART consists of two NRTIs in combination with a third active drug [2]. However, these first-line regimens require lifelong daily oral therapy which can be burdensome and potentially affecting treatment adherence. This is further complicated by (self)-stigma and the need for discretion. As a consequence, sub-optimal adherence to oral administration of cART has been recognized as a significant patient-related risk factor for subtherapeutic exposure and risk of virological failure and development of viral resistance [3]. To solve these problems, research has been done in investigating the options for simplifying the antiretroviral therapy. One of these options includes the development of long-acting injectable regimens and therefore the first long-acting injectable with the combination cabotegravir (CAB) and rilpivirine (RPV) entered the market in 2020 [4, 5].

CAB belongs to the class of integrase strand transfer inhibitors (INSTIs) and is structurally related to the robust oral INSTI dolutegravir, which has a higher barrier to resistance than first-generation INSTIs. RPV is a potent second-generation nonnucleoside reverse transcriptase inhibitor (NNRTI) and has a better tolerability compared to another

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widely used NNRTI, efavirenz. Both, CAB and RPV are also available as short-acting oral formulations, but are now combined in a long-acting intramuscular injectable as a two-drug ART regimen administered every 1–2 months for the maintenance of virologic suppression in people living with HIV.

Therapeutic drug monitoring (TDM) is a widely used tool in clinical practice to improve therapeutic and safety outcomes in the management of people livening with HIV [6]. Measuring antiretroviral drug concentrations can be useful in a range of clinical scenarios, i.e. unexpected viral load increase, unexplained side effects, the presumption of drug-drug interactions and in specific situations in which pharmacokinetics may be altered, such as in pregnancy. With oral antiretroviral drugs, therapeutic drug monitoring also provides insightful information if non-adherence is suspected, but this is not the case with long-acting injectables. However, problems can also arise when setting the injection, where TDM does add value. In addition, for the long-acting CAB and RPV the week 8 RPV plasma trough concentration was shown to be statistically associated with higher risk of virological failure if one of the following factors was also present: pro-viral RPV resistance-associated mutations (RAMs), HIV-1 subtype A6/A1 and baseline BMI $>$ 30 kg/m$^2$ [7]. It is expected that an increasing number of people living with HIV will be taking the long-acting injectable with CAB and RPV, and based on these experiences the criteria regarding the clinical application of therapeutic drug monitoring of these long-acting injectables has yet to be evaluated.

These clinical applications together with a substantial amount of research that will be conducted with long-acting injectables leads to the need for an accurate, precise and validated quantification method. This method should be efficient and practical for routine use, and therefore ideally both CAB and RPV will be combined in one assay method. Current methodologic approaches for measuring CAB and RPV concentrations uses liquid chromatographic-tandem mass spectrometric (LC-MS/MS) techniques, but most of the recently published assays require a separate measurement method. Only three other published assays measured CAB and RPV simultaneously, but each of these assays has its limitations. One of the shortcomings of the current assays is ignoring the two different RPV isomers: the E-isomer and the Z-isomer. The Z-isomer is formed when RPV is exposed to all light conditions and is considered an impurity. The E-isomer is the desired drug molecule and therefore considered to be the active isomer based on previous research [8]. To have an insight in the RPV degradation process, the aim was to measure both isomers in a combined method as well.

Therefore, in this paper we present an accurate, simple and rapid UPLC-MS/MS method for the simultaneous quantification of CAB and RPV (E-isomer). The Z-isomer is not quantified, but its response is used to calculate the (response Z isomer/response Z-isomer + response E-isomer) ratio as a tool for percentage degradation of the E-isomer due to light exposure.

To our knowledge this is the first described method combining these two drugs with the two isomers of RPV in one assay, which leads to increased laboratory efficiency, since both substances are administered together. Moreover, in this paper we present a practicable tool for monitoring degradation of both highly light sensitive compounds.

## 2. Materials and methods

### 2.1. Chemicals and materials

CAB ($C_{19}H_{17}F_2N_3O_5$, cas.nr. 1051375-10-0, C050100, lot. 3-MFI-108-1, purity 97 %) and its internal standard [$^2H_3$]-CAB (C050102, lot. 11-JES-8–3, purity 98 %) were purchased from Toronto Research Chemicals. RPV ($C_{22}H_{18}N_6$, cas.nr. 500287-72-9, HY-10574, lot. 08014, purity 99.88 %) was purchased from MedChemExpress and its internal standard [$^{13}C_6$]-RPV (C2526, lot. GV-ALS-15–056-P1, purity 96,4 %) was purchased from Alsachim (Illkirch Graffenstaden, France). Formic acid (98–100 %), acetonitrile U-LC/MS, methanol U-LC/MS and DMSO were purchased from Merck (Darmstadt, Germany). All water used was obtained with a Veolia Purelab flex 4 system from Veolia (Ede, The Netherlands). Drug-free human $K_2$EDTA anti-coagulated whole blood was obtained from Sanquin (Nijmegen, The Netherlands) and was centrifuged for 5 min with 4300 g at room temperature. The collected EDTA-plasma was stored at −40 °C.

Because of light instability of the compounds, all weighing was performed in a room with no direct light. Also all preparations were executed in a laboratory fume hood with no direct light and with use of amber polypropylene tubes and bio vials or autosampler vials with glass insert.

### 2.2. Preparation of stock solutions

Two separate CAB stock solutions containing 1000 mg/L of CAB in DMSO and the internal standard stock solution containing 1000 mg/L of [$^2H_3$]-CAB in DMSO were made in amber polypropylene tubes. Also two separate RPV stock solutions containing 250 mg/L of RPV in DMSO and the internal standard stock solution containing 1000 mg/L of [$^{13}C_6$]-RPV in methanol were made in amber polypropylene tubes. All stocks were stored at −40 °C.

Precipitation solution was made in a 50 mL amber polypropylene tube by adding 10.0 µL [$^2H_3$]-CAB stock solution and 2.50 µL [$^{13}C_6$]-RPV stock solution to 50 mL of methanol and stored at 4 °C.

### 2.3. Preparation of standards and internal quality control samples

The CAB and RPV concentration range in plasma was set at 0.0500–10.0 mg/L and 0.00300–3.00, respectively. The first CAB and RPV stock solutions were diluted in amber- polypropylene tubes with blank EDTA-plasma to achieve seven standards of - and 0.003 (LLOQ RPV), 0.0500 and 0.0150 (LLOQ CAB), 0.100 / 0.0300, 0.500 / 0.150, 1.00 / 0.300, 5.00 / 1.50 and 10.0 / 3.00 mg/L (ULOQ) for CAB and RPV respectively.

The QC samples were prepared from the second CAB and RPV stock solutions in amber polypropylene tubes resulting in concentrations of 0.0750 / 0.00750, 0.375 / 0.100 and 7.50 / 2.00 mg/L in EDTA-plasma, for CAB and RPV respectively, designated as QC Low, Medium and High.

Details of preparing standards and QC samples are shown in Table 1. The standards and QC samples were aliquoted in 1.5 mL amber polypropylene bio vials and stored at −40 °C.

### 2.4. Sample preparation

Patient samples consisting of EDTA-anticoagulated whole blood

**Table 1**
Preparation of standards and quality control samples.

| St | Volume blank plasma (mL) | Volume per solution | CAB (mg/L) | RPV (mg/L) |
|---|---|---|---|---|
| 1 | 9.78 | 100 µL CAB en 120 µL RPV stock 1 | 10.0 | 3.00 |
| 2 | 3.00 | 3,00 mL St1 | 5.00 | 1.50 |
| 3 | 4.50 | 500 µL St 1 | 1.00 | 0.300 |
| 4 | 4.50 | 500 µL St 2 | 0.500 | 0.150 |
| 5 | 4.95 | 50 µL St 1 | 0.100 | 0.0300 |
| 6 | 4.95 | 50 µL St 2 | 0.0500 | 0.0150 |
| 7 | 4.95 | 50 µL St 3 | - | 0.00300 |
| 0 | 5.00 | 0 | 0 | 0 |

| QC | Volume blank plasma (mL) | Volume per solution | CAB (mg/L) | RPV (mg/L) |
|---|---|---|---|---|
| H | 9.845 | 75 µL CAB +80 µL RPV stock 2 | 7.50 | 2.00 |
| M | 4.75 | 250 µL QC H | 0.375 | 0.100 |
| QCW | 9.885 | 75 µL CAB +30 µL RPV stock | | |
| L | 4.95 | 50 µL QCW | 0.0750 | 0.00750 |

were centrifuged for 5 min at 1900 g. The resulting EDTA plasma was transferred into amber polypropylene tubes at −40 °C until further use.

### 2.5. Protein precipitation method

After thawing, each calibration curve standard, quality control sample and patient sample was mixed on a multi-tube vortex at 2500 rpm for 5 min and centrifugated for 5 min at 1900g. Subsequently, sample work-up was performed in two steps.

First, 150 μL precipitation reagent was pipetted into the first ten 1.5 mL amber bio vials. Then 50 μL of plasma was added, followed by direct closing of the bio vial. This was repeated for the next ten bio vials until all plasma's were pipetted. All bio vials were mixed on a multi-tube vortex at 2500 rpm for 5 min and centrifugated for 5 min at 18,620g.

Second, hundred microliters of the supernatant was transferred into an amber vial with a 200 μL glass insert, followed by direct closing with a screw cap with pre-slit septum. All vials were centrifugated for 5 min at 4856g before placing them in the autosampler.

A simplified work-up without a second step: 150 μL of precipitation reagent was pipetted into the first twelve wells of a 700 μL round polypropylene 96 well plate. Then 50 μL of plasma was added, followed by protecting the well plate row against light with aluminium foil. This was repeated for the next twelve wells until all plasma's were pipetted. The well plate was closed off with a round plug cap mat. The plate was mixed on a ThermoMixer (Eppendorf) at 2500 rpm for 2 min at room temperature. Notice: this whole process had to be finished for all samples within 20 min. The well plate was centrifugated for 5 min at 4856g before placing it in the autosampler.

### 2.6. UPLC-MS/MS analysis

The Acquity H-class UPLC system consisted of a QSM solvent delivery pump, a flow-through needle (FTN) autosampler, a CO column oven and a Xevo TQ-s micro Tandem Mass Spectrometer. Two μL of the sample was injected onto a HSS T3 column (100 × 2.1 mm ID; particle size,1.8 μm) with a HSS C18 column guard column (10 × 2.1 mm ID; particle size,1.8 μm) and the column oven temperature set at 40 °C. The mobile phase components were 65 % 0.1 % formic acid in water (A) and 35 % 0.1 % formic acid in acetonitrile (B). Flow rate was 0.5 mL/min and the total run time was 3.0 min. Samples were kept at room temperature in the autosampler during analysis. The needle was washed post-injection for 6 s with a mixture of water, acetonitrile and formic acid (60: 40: 1, v/v/v). The Xevo TQ-s micro mass spectrometer operated in the positive electrospray ionization mode (ESI) using multiple reaction monitoring (MRM). The capillary voltage was 0.5 kV, source temperature 150 °C, desolvation temperature 500 °C and the desolvation gas flow was set to 1000 L/hr. Argon was used as collision gas, nitrogen was used as de-solvation and nebulizer gas. The analyte and internal standard were optimized on the [M+H] ion and we chose one MRM transition for qualification. Proposed mass-to-charge ratio (*m/z*) fragmentation patterns for quantifying CAB and RPV are shown in Fig. 1. Cone voltage and collision energy were optimized for both components (Table 2). With every injection only between 1.2 and 3.0 min the eluent was allowed into the source of the Tandem Mass Spectrometer. Analytical runs were controlled and processed by Masslynx software version 4.1 (All by Waters, Etten-Leur, The Netherlands).

### 2.7. Validation procedures

The validation of the assay in plasma was based on the most recent versions of the guidelines on bioanalytical method validation of the

**Table 2**
Analyte and internal standard specific mass spectrometric parameters and optimized mass spectrometer setting.

| Component | Retention time (min) | Positive ion mode MRM transition Trace (*m/z*) | Cone voltage (V) | Collision energy (eV) |
|---|---|---|---|---|
| CAB | 2.39 | 405.95 > | 58 | 24 |
| Quantifier | 2.39 | 126.78 | 58 | 24 |
| CAB | 2.39 | 405.95 > | 58 | 24 |
| Qualifier | | 262.86 | | |
| [$^{2}H_3$]-CAB | | 409.00 > | | |
| | | 126.78 | | |
| RPV | 1.69/1.87* | 367.06 > | 58 | 56 |
| Quantifier | 1.69/1.87* | 127.69 | 58 | 36 |
| RPV | 1.69/1.87* | 367.06 > | 58 | 56 |
| Qualifier | | 194.90 | | |
| [$^{13}C_6$]-RPV | | 373.10 > | | |
| | | 127.67 | | |

* retention time Z-RPV/E-RPV

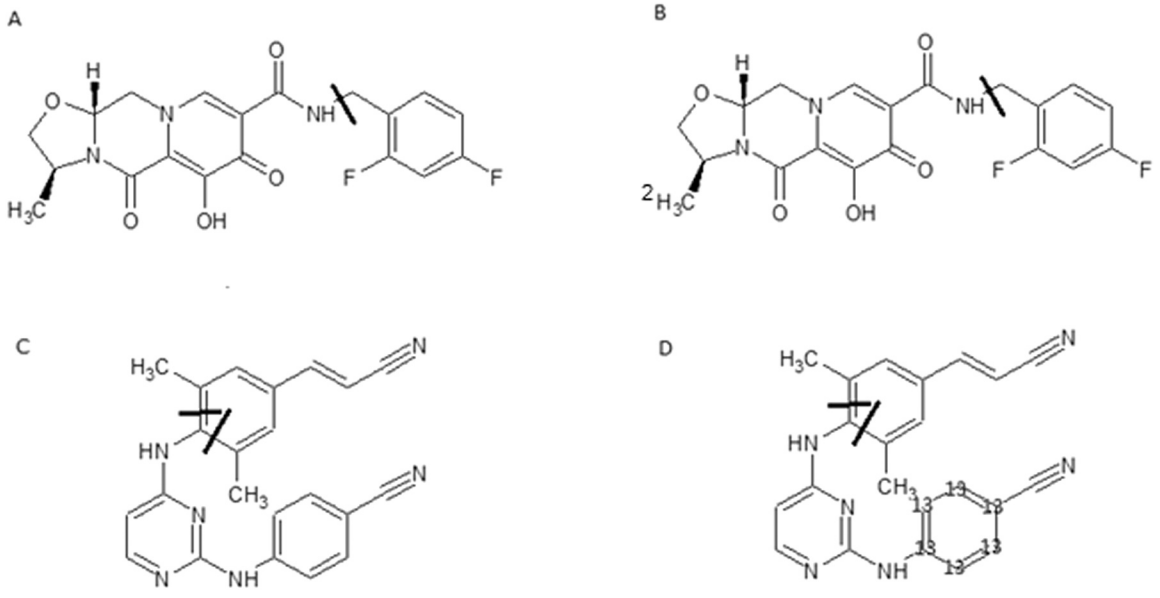


**Fig. 1.** Proposed mass-to-charge ratio (*m/z*) fragmentation patterns for CAB (A). and CAB-$^{2}H_3$ (B) and B: RPV (C) and RPV-$^{13}C_6$ (D).

European Medicines Agency [9] and the FDA [10].

#### 2.7.1. Selectivity

Blank EDTA-plasma of six HIV patients who did not receive CAB nor RPV were evaluated for interference by endogenous substances.

The presence of interfering components was accepted if the response was less than 20 % of the LLOQ for CAB and E-isomer of RPV and less than 5 % of their internal standards.

#### 2.7.2. Cross-talk

Cross-talk was investigated by first, the interference of their internal standards on CAB and RPV and second, the interference of CAB and RPV on their internal standards. For the first, five replicate blank samples with internal standard were analyzed. For the second, five replicate samples at a concentration of ULOQ were analyzed without internal standards.

The presence of interference was accepted if first, the response for CAB and E-isomer of RPV in the blank was less than 20 % of the LLOQ and second, the response for the internal standards of CAB and E-isomer of RPV in the ULOQ sample was less than 5 % of their internal standards in the LLOQ.

#### 2.7.3. Carry-over

Carry-over was assessed by injecting blank samples after the ULOQ. Carry-over in the blank sample, following the ULOQ, had to be less than 20 % of the LLOQ for CAB and E-isomer of RPV and 5 % of their internal standards.

#### 2.7.4. Accuracy and precision

In order to determine accuracy and within-day and between-day precision of the method, five replicates of validation samples of CAB and RPV in EDTA-plasma at the LLOQ, the three QC samples and ULOQ were analysed during three different days. To analyse these samples, six calibration concentration levels were used in duplicate for CAB and seven calibration concentration levels were used in duplicate for RPV, in addition to the blank sample which was not incorporated in the calibration line. The calibration curve was constructed as a plot of the analyte concentration versus peak area ratio of cabotegravir and rilpivirine E-isomer to their internal standards (IS). The method of least squares was used to determine which regression model fitted the calibration data best. For each replicate measurement, the concentrations measured in the LLOQ, the three QC samples and ULOQ samples were divided by the nominal concentrations. To assess accuracy, the mean ratio of measured concentrations versus nominal concentrations (n = 15) was calculated and multiplied by 100.

One-way Analysis of variance (ANOVA) was used to assess the within-day and between-day precision at each of the five concentrations of the validation samples, using the run day as the classification variable. The error mean square or mean square within groups (ErrMS), the day mean square or mean square among groups (DayMS), and the grand mean (GM) of all 15 measurements across the three run days were obtained from the ANOVA. The estimate of the within-day and between-day precision at every concentration were calculated as follows:

Within-day precision = $((\text{ErrMS})^{0.5}/\text{GM}) \times 100$ %.

Between-day precision = $([(\text{DayMS-ErrMS})/n]^{0.5}/\text{GM}) \times 100$ %.

in which $n$ is the number of replicate measurements within each day.

For the LLOQ, the percent deviation from the nominal concentration (accuracy) and the relative standard deviation (precision) had to be less than 20 % and for the three QC samples and ULOQ both these measures had to be less than 15 %.

#### 2.7.5. Dilution integrity

Dilution integrity was investigated for samples with concentrations above the established calibration range by analyzing five replicate samples with CAB and RPV at a concentration of 1.5 times the ULOQ. The percent deviation between the mean concentrations after dilution as compared to the nominal values and the relative standard deviations in measurement of each diluted sample had to be less than 15 %.

#### 2.7.6. Matrix effect

The matrix factor (MF) was calculated for CAB and RPV E-isomer and their internal standards by calculating the ratio of the peak area in the presence of matrix to the peak area in the absence of matrix. The IS-normalized MF was then calculated by dividing the MF of the analyte by the MF of the IS for the analyte. This was done at QC High and Low concentration levels for six different lots of blank EDTA-plasma from individual donors in duplicate. The relative standard deviation of the IS-normalized MF calculated for both concentrations from the six lots had to be less than 15 %.

#### 2.7.7. Recovery

Total extraction recovery for the analytes were defined as the ratio of the peak area of the analytes and their internal standard (IS) in the extracted samples with those of the corresponding extracts of the blank spiked with the analyte post-extraction. This was done in duplicate at a QC High and Low concentration, and for this range our aim was a recovery that was over 70 % and constant over the studied concentration range.

#### 2.7.8. Hemolyzed and lipemic plasma

The effect of hemolyzed plasma, with a H-Index [11] of about 600 was investigated by analyzing five replicate samples with CAB and RPV E-isomer at a QC Low concentration.

The effect of lipemic plasma, with a concentration of 150 and 300 mg/dL purified soya bean oil (Intralipid 20 % ®, Fresenius Kabi, The Netherlands) was investigated by analyzing five replicate samples with CAB and RPV E-isomer at a QC Low concentration. The percent deviation between the mean concentrations as compared to the nominal values and the relative standard deviations in measurement of each condition had to be less than 15 % for CAB and RPV E-isomer.

#### 2.7.9. Stability

The stability of the CAB and RPV stock solutions at −40°C was tested when fresh stocks were made. Our aim was that the percent deviation from the nominal concentration of the new made stocks had to be less than 5 %.

The stability during sample handling for the analyte in EDTA-plasma was verified by subjecting samples to three freeze-thaw cycles, testing the stability at room temperature in light and dark, 4–8 °C and −40°C. The stability during sample handling for the analyte in EDTA-whole blood was verified by testing the stability at room temperature in light and dark and at 4–8 °C. All this was performed in duplicate at three different concentration levels (QC High, Medium and Low) in spiked samples.

Also three freeze-thaw cycles and the stability at room temperature in light and dark was tested on EDTA-plasma from three different patients and the stability at room temperature in light and dark in their whole blood.

The stability of processed samples in the autosampler was also tested. All processed samples obtained at the first day of assessment of accuracy and precision were re-analysed after seven days in vials and after two days in the 96-well plate in the dark at room temperature. For each sample, the percentage of concentrations obtained after seven and two days in the autosampler compared to the nominal concentration was calculated. For the LLOQ, the percent deviation from the initially measured concentration (accuracy) and the relative standard deviation (precision) had to be less than 20 % and for ULOQ, QC High, Medium and Low both these measures had to be less than 15 %.

#### 2.7.10. Additional validation of simplified protein precipitation method

The simplified protein precipitation method was cross validated by analysing five replicates of samples of CAB and RPV in EDTA-plasma at

the LLOQ, the three QC samples and ULOQ during one day, in order to determine accuracy and within-day precision of the method. The criteria were the same as described by 2.7.4.

## 3. Results

### 3.1. Selectivity

Plasma of six HIV patients without CAB or RPV medication were evaluated and found to be free from potential endogenous or other interferences. Chromatograms of CAB and RPV at the LLOQ level and its blank are shown in Fig. 2.

### 3.2. Cross-talk

Cross-talk in the blank sample with internal standards proved not to be greater than 4.0 % of the LLOQ and in the ULOQ sample without internal standard not to be greater than 1.4 % of the IS for CAB and not to be greater than 2.4 % of the LLOQ and in the ULOQ sample without internal standard 0 % of the IS for RPV E-isomer.

### 3.3. Carry-over

Carry-over in the blank sample following the high concentration calibrator proved not to be greater than 2.2 % of the LLOQ for CAB, 5.3 % of the LLOQ for RPV E-isomer and 0 % for both internal standards.

### 3.4. Accuracy and precision

The calibration curve for CAB and RPV E-isomer (peak area ratios versus concentrations) were constructed using a weighting factor of $1/x^2$ and were fitted quadratically. Regression coefficients ($r^2$) of all three calibration curves during validation of CAB and RPV E-isomer in EDTA-plasma were 0.9989 ± 0.0008 and 0.9995 ± 0.0006 respectively.

The results of analysis of five replicates of EDTA-plasma LLOQ, QC Low, Medium, High and ULOQ samples on three different days for CAB and RPV E-isomer are presented in Table 3.

For all calibration curve standards and validation samples the (RPV Z-isomer/total RPV) response ratio proved not to be greater than 2.3 %.

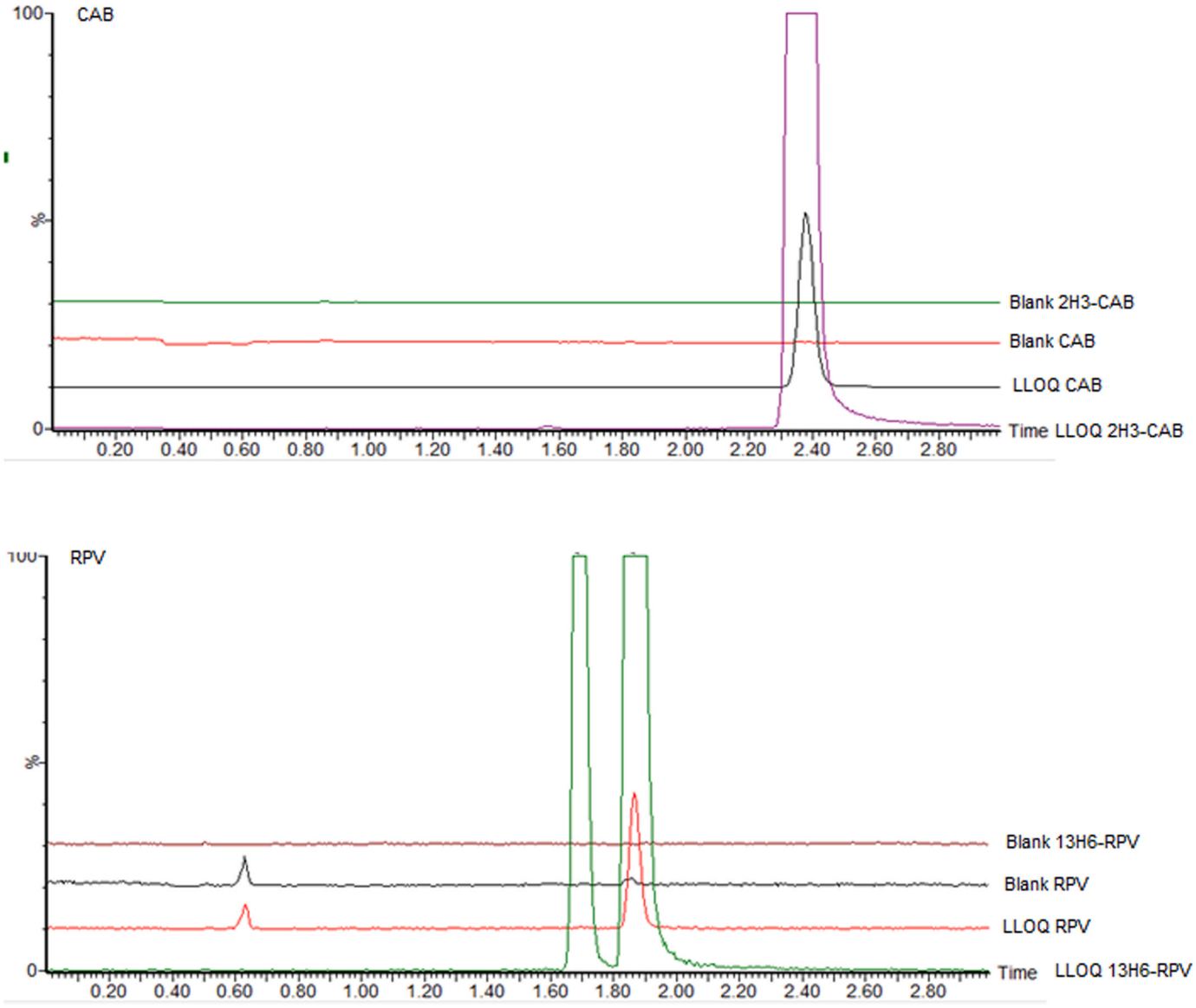


**Fig. 2.** MRM chromatograms of a LLOQ sample with IS, blank without IS.

**Table 3**
Accuracy and precision of CAB and E-RPV determination in EDTA-plasma.

| CAB Validation sample | Nominal Concentration (mg/L) | Within-day Accuracy (n = 5) (%) | Within-day imprecision (RSD%) | Between-day Accuracy (n = 15) (%) | Between-day imprecision (RSD%) |
|---|---|---|---|---|---|
| LLOQ | 0.05005 | 104 | 1.82 | 102 | 2.09 |
| QC Low | 0.07530 | 104 | 1.41 | 103 | 1.35 |
| QC M | 0.3765 | 103 | 4.33 | 102 | 0.00 |
| QC H | 7.530 | 95.7 | 2.18 | 98.9 | 2.77 |
| ULOQ | 10.01 | 96.0 | 1.35 | 99.0 | 2.60 |
| Average | | 101 | | 101 | |

| E-RPV Validation sample | Nominal Concentration (mg/L) | Within-day Accuracy (n = 5) (%) | Within-day imprecision (RSD%) | Between-day Accuracy (n = 15) (%) | Between-day imprecision (RSD%) | Maximum (Z-RPV/total RPV) response ratio (%) |
|---|---|---|---|---|---|---|
| LLOQ | 0.003001 | 98.8 | 2.70 | 100 | 0.35 | 2.3 |
| QC Low | 0.007506 | 98.4 | 2.62 | 99.6 | 1.11 | 2.0 |
| QC M | 0.1001 | 96.5 | 4.70 | 97.5 | 0.00 | 0.99 |
| QC H | 2.002 | 96.1 | 0.88 | 96.7 | 0.49 | 1.4 |
| ULOQ | 3.001 | 98.4 | 1.26 | 98.6 | 0.00 | 0.46 |
| Average | | 97.6 | | 98.5 | | |

### 3.5. Dilution integrity

The percent deviations between the mean concentrations of 1.5 times ULOQ after two and five time dilution to the nominal concentrations were 3.37 % and −2.05 % for CAB and 0.28 % and −3.79 % for RPV E-isomer, respectively. The relative standard deviations after dilution were 1.02 % and 1.47 % for CAB and 1.31 % and 2.11 % for RPV E-isomer.

### 3.6. Matrix effect

The mean IS-normalized MF calculated for QC Low and QC High from the six lots is 1.04 and 0.982 for CAB and 0.978 and 0.991 for RPV E-isomer, respectively. The relative standard deviation of the MF calculated for QC Low and QC High from the six lots is 0.83 % and 1.60 % for CAB and 1.59 % and 0.75 % for RPV E-isomer, respectively.

### 3.7. Recovery

Recovery of CAB in duplicate at QC Low concentration was 103 % and at QC High concentration 95 %. For its internal standard [$^{2}H_{3}$]-CAB the recovery was 102 %.

Recovery of RPV E-isomer in duplicate at QC Low concentration was 95 % and at QC High concentration 94 %. For the internal standard [$^{13}C_{6}$]-RPV E-isomer the recovery was 97 %.

### 3.8. Hemolyzed and lipemic plasma

The percent deviation between the mean concentration of QC Low in hemolyzed plasma to the nominal concentration were 0.10 % and −1.18 % for CAB and RPV E-isomer, respectively. The relative standard deviations in hemolyzed plasma were 0.48 % and 1.70 %.

The percent deviations between the mean concentration of QC Low in lipemic plasma with 150 mg/dL and 300 mg/dL Intralipid to the nominal concentration were −0.07 % and 1.42 % for CAB and −3.51 % and −1.42 % for RPV E-isomer, respectively. The relative standard deviations in lipemic plasma were 0.86 % and 0.70 % for CAB and 2.88 % and 2.00 % for RPV E-isomer.

### 3.9. Stability

The results of the stability of CAB and RPV in various solutions and at various circumstances in the three spiked QC samples and patient samples in duplicate are presented in Table 4.

The results of analysis of three replicates of the two CAB and RPV E-isomer stock solutions were 100 % (offset), 102 % and 102 % for CAB and 100 % (offset), 97.8 % and 96.9 % for RPV E-isomer. Processed samples of CAB and RPV in plasma were 101 % and 98.9 % after 7 days in vials at room temperature in the dark. Due to evaporation two samples could not be re-analyzed after 7 days.

Processed samples of CAB and RPV in plasma were 100 % and 96.2 % after 2 days in the 96-well plate at room temperature in the dark.

### 3.10. Additional validation of simplified protein precipitation method

The results of analysis of five replicates of EDTA-plasma LLOQ, QC Low, Medium, High and ULOQ samples on one day for CAB and E-RPV are presented in Table 5.

### 3.11. Clinical application of the analytical method

After the analytical method was validated, requests for measurement of CAB/RPV drug concentrations for TDM purposes in clinical practice could be processed. Table 6 shows the results of the first clinical requests within our laboratory with limited demographic information of the patients. The analyzed CAB and RPV concentrations had a range of 0.423–4.77 mg/L and 0.0328–0.317 mg/L, respectively, so within the calibration ranges of the two compounds. Two out of the first eight requests had a Z/Z + E ratio above our aimed cut-off of < 10 %, i.e. 14 % and 15 %.

## 4. Discussion

In this paper we report the successful development, validation and application of an UPLC-MS/MS method for the measurement of CAB and both isomers of RPV simultaneously. To our knowledge, this is the first LC-MS/MS method which is able to quantify CAB and the active E-isomer while the use the response of the E- and Z-isomer of RPV in (RPV Z-isomer * 100 / RPV Z-isomer + RPV E-isomer) response ratio provides a tool for percentage degradation of the E-isomer due to light exposure. With this comprehensive report of the method, we show the challenges and solutions working with the extreme light sensitive compounds CAB and RPV.

### 4.1. Chromatography

As RPV is the most light sensitive compound of the two and alters from RPV E-isomer to its Z-isomer under the influence of light, it is important to separate the two isomers chromatographically. When the response of both isomers can be measured, a (RPV Z-isomer * 100 / RPV Z-isomer + RPV E-isomer) response ratio can be calculated as a tool for percentage degradation of stock solutions, calibration curve standards,

**Table 4**
Stability data at various conditions for cabotegravir and rilpivirine E-isomer in stock solution, spiked plasma and whole blood.

| CAB Matrix | Condition | Time interval | Mean accuracy (%) |
|---|---|---|---|
| Stock solutions (n = 3) | -40°C | 4.5 months | 101 |
| Spiked plasma (n = 6) | Room temperature light | 1, 2, 3 h | 95.7, 87.9, 79.9 |
| | Room temperature dark | 3 days | 99.8 |
| | 4 °C (dark) | 7 days | 102 |
| | -40°C | 7 months | 100 |
| | freeze-thaw cycles | yes | 102 |
| Spiked whole blood (n = 6) | Room temperature light | 2, 4 h | 99.6, 93.4 |
| | Room temperature dark | 3 days | 103 |
| | 4 °C (dark) | 7 days | 97.8 |
| Patient plasma (n = 6) | Room temperature light | 0.5, 1, 2 h | 97.1, 91.3, 81.5 |
| | Room temperature dark | 24 h | 99.9 |
| | freeze-thaw 3 cycles | yes | 97.5 |
| Patient whole blood (n = 6) | Room temperature light | 0.5, 1, 2, 4 h | 99.5, 97.8, 92.7, |
| | Room temperature dark | 24 h | 86.4 |
| | | | 101 |
| **E-RPV Matrix** | **Condition** | **Time interval** | **Mean accuracy (%)** |
| Stock solutions (n = 3) | -40°C | 12 months | 97.4 |
| Spiked plasma (n = 3) | Room temperature light | 1, 2, 3 h | 90.3, 76.4, 65.2 |
| | Room temperature dark | 3 days | 95.1 |
| | 4 °C (dark) | 7 days | 96.4 |
| | -40°C | 7 months | 109 |
| | freeze-thaw cycles | yes | 95.9 |
| Spiked whole blood (n = 3) | Room temperature light | 2,4 h | 90.8, 80.9 |
| | Room temperature dark | 3 days | 96.3 |
| | 4 °C (dark) | 7 days | 95.2 |
| Patient plasma (n = 3) | Room temperature light | 0.5, 1, 2 h | 92.5, 81.1, 66.0 |
| | Room temperature dark | 24 h | 99.5 |
| | freeze-thaw 3 cycles | yes | 96.6 |
| Patient whole blood (n = 3) | Room temperature light | 0.5, 1, 2, 4 h | 96.6, 91.5, 81.9, |
| | Room temperature dark | 24 h | 74.5 |
| | | | 94.5 |

**Table 5**
Accuracy and precision of CAB and E-RPV cross validation in EDTA-plasma.

| CAB Validation sample | Nominal Concentration (mg/L) | Within-day Accuracy (n = 5) (%) | Within-day imprecision (RSD%) |
|---|---|---|---|
| LLOQ | 0.05021 | 99.6 | 0.77 |
| QC Low | 0.07530 | 99.0 | 1.51 |
| QC M | 0.3765 | 96.9 | 5.88 |
| QC H | 7.530 | 102 | 1.91 |
| ULOQ | 10.04 | 103 | 1.35 |
| Average | | 100 | |

| E-RPV Validation sample | Nominal Concentration (mg/L) | Within-day Accuracy (n = 5) (%) | Within-day imprecision (RSD%) | Maximum (Z-RPV/total RPV) response ratio (%) |
|---|---|---|---|---|
| LLOQ | 0.003001 | 101 | 1.27 | 0.75 |
| QC Low | 0.007506 | 96.0 | 1.89 | 1.6 |
| QC M | 0.1001 | 92.3 | 5.37 | 1.5 |
| QC H | 2.002 | 93.2 | 5.32 | 2.0 |
| ULOQ | 3.001 | 95.4 | 7.79 | 1.0 |
| Average | | 95.6 | | |

**Table 6**
Therapeutic Drug Monitoring results of the first clinical requests using the described analysis method along with the available demographic information of the patients.

| Gender | Age | CAB conc (mg/L) | RPV conc (mg/L) | (Z-RPV/total RPV) response ratio (%) |
|---|---|---|---|---|
| Male | 34 | 1.564 | 0.3185 | 14* |
| | | 1.973 | 0.2667 | 3.1 |
| Male | 31 | 2.200 | 0.06981 | 3.8 |
| Male | 50 | 0.4234 | 0.03280 | 15* |
| Female | 47 | 1.095 | 0.1129 | 1.1 |
| Male | 33 | 1.652 | 0.07523 | 0.69 |
| | | 4.769 | 0.2642 | 1.0 |

* Above cut-off limit of 10 % for Z vs. Z+E-isomer

quality control samples and patient samples. Therefore the HSS T3 column was chosen. With the column oven temperature set at 40 °C, the mobile phase of 65 % 0.1 % formic acid in water (A) and 35 % 0.1 % formic acid in acetonitrile (B) and the flow rate of 0.5 mL/min the total run time was 3.0 min to elute RPV Z-isomer, RPV E-isomer and CAB.

### 4.2. Preparation of standards and internal quality control samples

The concentrations of QC High was 7.50 mg/L for CAB and 2.00 mg/L for RPV, which do not met the EMA an FDA criterium of >75 % of the ULOQ. As this is a shortcoming in our assay, we don't expect it to have great impact on the validation results.

### 4.3. Sample pre-treatment

Because of light instability of the compounds, all weighing was performed in a room with no direct all light conditions and also all preparations were executed in a fume-hood with no direct light. For the preparations amber polypropylene tubes, bio vials or autosampler vials with glass insert were used. An alternative is to use a room and a fume-hood with yellow light to prevent degradation of both compounds.

During sample pre-treatment dilution of the samples was only 4 times before placing them in the sample manager, to make sure that the Z-isomer of RPV, which could be 500 times lower than the E-isomer, also could be detected.

During validation it became apparent that when pipetting out of amber tubes or bio vials it was difficult to solely pipet plasma (the first pipetting step) or supernatant (the second pipetting step) and avoid any residue. Calibration curve standards, quality control samples and patient samples had to be stored in amber bio vials and tubes to protect the plasma from light, so the first pipetting step could not be altered. For the second step, an additional centrifugation step (5 min at 4856g) before placing the vials in the autosampler helped to prevent injecting protein on the column.

An additional validation was carried out with protein precipitation performed in a 700 μL round 96-well plate to avoid the second pipetting step. Since 96-well plates do not offer protection from light, pipetting and mixing had to be done within 20 min before centrifuging and placing it in the (dark) sample manager.

For both protein precipitation methods the (RPV Z-isomer * 100 / RPV Z-isomer + RPV E-isomer) response ratio proved not to be greater than 2.3 %, which means that degradation was limited during sample pre-treatment.

### 4.4. Clinical sample handling

Because of the instability of both compounds in whole blood there is

a need to protect a patient's blood sample after it has been drawn. The current advice is to wrap the tube in aluminium foil directly after drawing. This way the blood is stable for 24 h, during which the sample can be transported to the lab. In the lab the blood sample is centrifuged for 5 min at 1900 g and the resulting EDTA-plasma pipetted into amber polypropylene tubes and stored at −40 °C until further use.

### 4.5. Additional parameters

When CAB and RPV was to be quantified in a patient sample our aim was that the (Z-isomer RPV/total RPV) response ratio had to be less than 10 % to give a reliable result. This meant that some boundaries had to be set for the (Z-isomer RPV/total RPV) response ratio in our stocks, calibration curve standards and internal quality control samples.

Since for all RPV stock solutions the (RPV Z-isomer/total RPV) response ratio proved not to be greater than 0.9 %, the maximum (Z-isomer RPV/total RPV) response ratio for stock solutions was limited to 1.0 %.

For all calibration curve standards and validation samples the (RPV Z-isomer/total RPV) response ratio proved not to be greater than 2.3 % during validation, so this was limited to 2.5 %.

The [$^{13}C_6$]-rilpivrine stock solution contained both isomers direct after dissolving, as is show in Fig. 2. For all calculations only the [$^{13}C_6$]-RPV E-isomer was used.

### 4.6. Comparison with other published methods

To our knowledge, only three other methods has been described in the literature which measures both long acting injectable drugs (CAB/RPV) together in one assay, summarized in Table 7. However, our method has added value over all these previously described methods. One of the methods measures indeed both drugs in one assay, but in rat plasma instead of human plasma. The other assay measures both drugs in dried blood spots (DBS) rather than human plasma and the authors in the paper describe that there is a potential uncertainty in estimating plasma concentrations from DBS. The paper by Courlet et. Al. is most similar to what we were striving for with our method: CAB and RPV measurements in one single UHPLC-MS/MS assay. However, this assay has the shortcoming of not being able to identify the ratio of the RPV Z-isomer to total RPV in plasma, for which we found a solution in our method. Measuring the ratio of RPV Z-isomer to total RPV allows a better understanding of sample quality because both RPV and CAB are very light-sensitive and the Z-isomer of RPV, which is considered as an impurity, arises from the E-isomer under the influence of light, while the E-isomer of RPV is the active isomer.

### 4.7. Current clinical experiences

Since the introduction of the CAB/RPV long acting injectable option for people living with HIV, the application for measuring concentrations of CAB and RPV is increasingly frequent. Physicians have several reasons to closely monitor the drug concentrations of their patients on the long-acting injectables, even though therapy adherence is no longer an issue. The described LC-MS/MS assay has already been applied to patient's samples obtained for clinical purposes as part of our TDM service, results are shown in Table 6. The motives of the initial requests were diverse, i.e. uncertainty whether the injection had come subcutaneously, unexplained adverse reactions and suspected resistance. The results helped us in the interpretation of the cases, although research is still needed to determine therapeutic ranges accurately. In addition, two of the eight samples had a too high (Z-isomer RPV/total RPV) response ratio, but this was not unexpected since most of the first measured samples had been stored before and sent to our laboratory without sample handling instructions. We are confident that when samples are protected from light directly after drawing, in line with our validated stability conditions, the aimed (Z-isomer RPV/total RPV) response ratio < 10 % will easily be met, and thus the results will be reliable.

**Table 7**
An overview of previously published analysis methods measuring cabotegravir ad rilpivirine together in one assay.

| Reference | Comments |
|---|---|
| Weld ED, Parsons TL, Gollings R, McCauley M, Grinsztejn B, Landovitz RJ, Marzinke MA. Development and validation of a liquid chromatographic-tandem mass spectrometric assay for the quantification of cabotegravir and rilpivirine from dried blood spots. J Pharm Biomed Anal. 2023 May 10;228:115307 [12]. | – Blood collected as DBS: offer increased capacity and flexibility in translational applications<br>– Limitations:<br>• Differences between spotted whole blood and plasma, so exact concentration needs to be extrapolated from DBS. The application of a conversion facto to estimate plasma concentrations is therefore required and therefore there is a potential uncertainty around estimated plasma concentrations from DBS<br>• Additional analysis will still be needed to define acceptance criteria between plasma concentrations and estimated plasma concentrations from DBS<br>• DBS and estimated plasma concentrations were determined from venipuncture-collected blood; correlations with capillary blood, and appropriate conversion factors would still need to be established for fingerstick-based blood collections. |
| Courlet P, Alves Saldanha S, Cavassini M, Marzolini C, Choong E, Csajka C, Günthard HF, André P, Buclin T, Desfontaine V, Decosterd LA. 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. 2020 Jun;55(6):e4506 [13]. | – This article includes the development and validation of a simple and fast multiplex assay by ultrahigh-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) for the simultaneous determination of the latest generation ARV drugs bictegravir, cabotegravir, doravirine, and rilpivirine in human plasma<br>– Limitations:<br>• No identification of Z- and E-isomer |
| Ramöller IK, Abbate MTA, Vora LK, Hutton ARJ, Peng K, Volpe-Zanutto F, Tekko IA, Moffatt K, Paredes AJ, McCarthy HO, Donnelly RF. HPLC-MS method for simultaneous quantification of the antiretroviral agents rilpivirine and cabotegravir in rat plasma and tissues. J Pharm Biomed Anal. 2022 May 10;213:114698 [14]. | – This is an article describing a novel, selective and sensitive HPLC-MS method for the simultaneous detection and quantification of both analytes in rat plasma and different tissue matrices.<br>– This method can be employed for future studies to enhance the understanding of the pharmacokinetics and bio distribution of RPV and CAB; especially regarding the development and characterization of novel drug delivery systems for targeting of specific viral reservoirs, such as lymph nodes.<br>– Limitations:<br>• Not applicable with human plasma and therefore not useful as TDM |

## 5. Conclusion

We successfully developed a highly sensitive UPLC-MS/MS assay for the simultaneous analysis of CAB and both isomers of RPV in EDTA-plasma. This method can be used for Therapeutic Drug Monitoring and research purposes.

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## CRediT authorship contribution statement

Bevers L.A.H.: method application, first contact person, partial writing: introduction, discussion. van Ewijk - Beneken Kolmer E.W.J.: methodology, method validation, formal analysis, partial writing: abstract, methodology, results and discussion. te Brake H.M.L.: methodology, critically revising the manuscript. Burgers D.M.: clinical application, critically revising the manuscript.

## 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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