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Development and validation of LC-MS/MS methods for the pharmacokinetic assessment of the PROTACs bavdeglutamide (ARV-110) and vepdegestrant (ARV-471)

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ABSTRACT

Chemically induced, targeted protein degradation with proteolysis targeting chimeras (PROTACs) has shown to be a promising pharmacological strategy to circumvent the poor "druggability" of intracellular targets. However, the favorable pharmacology comes with complex molecular properties limiting the oral bioavailability of these drugs. To foster the translation of PROTACs into the clinics it is of high importance to establish sensitive bioanalytical methods that enable the assessment of absorption, bioavailability, and disposition of PROTACs after oral dosing.

In this study, two highly sensitive LC-MS/MS methods (LLOQ = 0.5 ng/mL) were developed and validated for the quantification of bavdeglutamide (ARV-110) and vepdegestrant (ARV-471) in rat plasma. Plasma samples were processed by protein precipitation and separated on a C18 column over a gradient of acetonitrile and water with 0.1 % formic acid. Selected reaction monitoring in positive ESI mode was applied to quantify ARV-110 and ARV-471.

Both methods showed linearity, accuracy, and precision as well as matrix effects and carry-over within the predefined acceptance criteria. High stability of the compounds in plasma was demonstrated at long-term storage for seven weeks at −20 °C, three freeze-thaw cycles, up to 20 min at room temperature, and as extracts in the autosampler. The plasma concentration-time curves after intravenous and intraduodenal bolus single-dose administrations in rats could be successfully quantified at clinically relevant doses per body weight. The highly sensitive bioanalytical assays presented in this work enable the application of a broad spectrum of *in vivo* studies to elucidate the oral absorption, bioavailability, and disposition of PROTACs.

1. Introduction

In 2001, Sakamoto *et al.* first introduced the concept of chemically induced, targeted protein degradation by chimeric molecules [1]. More than a decade later, the field rapidly gained traction after the so-called proteolysis targeting chimeras (PROTACs) first showed *in vivo* protein degradation [2,3]. Following the *in vivo* proof-of-concept studies, PROTACs rapidly advanced into the clinic where bavdeglutamide (ARV-110) and vepdegestrant (ARV-471) are well tolerated and currently evaluated in phase II trials in humans [4]. PROTACs act in an intracellular catalytic mode of action and knock out entire target proteins. Thereby, all potentially pathogenic functions of the protein of interest are permanently eliminated as opposed to only transient inhibition of enzymatic or signaling functions by conventional drug molecules [5–7]. These pharmacological features could mark a paradigm shift in cancer treatment as they enable a prolonged effect at very low, sub-stoichiometric doses. It could also provide new therapeutic options for targets formerly considered "undruggable" due to their inaccessible binding pockets, or their susceptibility towards drug resistances, such as the androgen receptor (AR) or the estrogen receptor (ER) in prostate or breast cancer

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patients, respectively [8–10]

The novel pharmacology of PROTACs requires certain molecular features that are typically not associated with oral drug delivery. As depicted in Fig. 1, ARV-110 and ARV-471 both consist of a ligand targeting the CRBN ligase connected via a short and rigid piperazine-based linker to a warhead targeting the AR and ER, respectively. The three required molecular building blocks - ligand, linker and warhead, result in PROTACs that generally exhibit molecular masses in the range of 700 – 1200 Da and high lipophilicity often surpassing a logP of 5.0, thus exceeding the Rule of Five (Ro5) molecular space associated with acceptable oral absorption and bioavailability [11]. Drug compounds occupying this molecular space typically have limited solubility and dissolution in the intestinal lumen in combination with low epithelial cell membrane permeability [12]. Therefore, one of the main barriers to harnessing the potential of PROTACs and enabling their translation into clinics is achieving sufficient oral absorption [13].

An additional hurdle related to the inherently low intestinal absorption of this class of drugs is the difficulty in evaluating them in conventional *in vitro* and *in vivo* drug absorption models, which pose a major challenge in pre-clinical biopharmaceutical testing of drug candidates. This is because the low intestinal permeability and solubility in combination with the propensity of highly lipophilic PROTACs to bind to material surfaces and cells often result in matrix concentrations well below the LLOQ of many analytical methods [14–17]. While LC-UV methods are limited by their comparably high detection limits, mass spectrometry (MS) analysis of PROTACs using electrospray ionization (ESI) tends to result in complex mixtures of protonated, often doubly charged ions and in-source fragments [17].

It stands to reason that highly sensitive and rapid bioanalytical assays are imperative to advance translational and clinical research on PROTACs. In this study, our main objective was to develop and validate two highly sensitive, robust, and rapid quantitative LC-MS/MS methods for ARV-110 and ARV-471 in rat plasma. Furthermore, we applied the methods to determine plasma concentration-time profiles of the two PROTACs after single-dose intravenous (IV) injection and intraduodenal dosing. We herein present the first LC-MS/MS method and plasma profiles including bioavailability data for ARV-471 in rat plasma. Moreover, an LC-MS/MS method with increased sensitivity compared to a previously published method for ARV-110 [18] as well as plasma profiles in rat plasma are shown. These methods will be highly beneficial to guide translational research and consolidate the understanding of PROTACs and their ADME properties.

2. Materials and methods

2.1. Study drugs and other chemicals

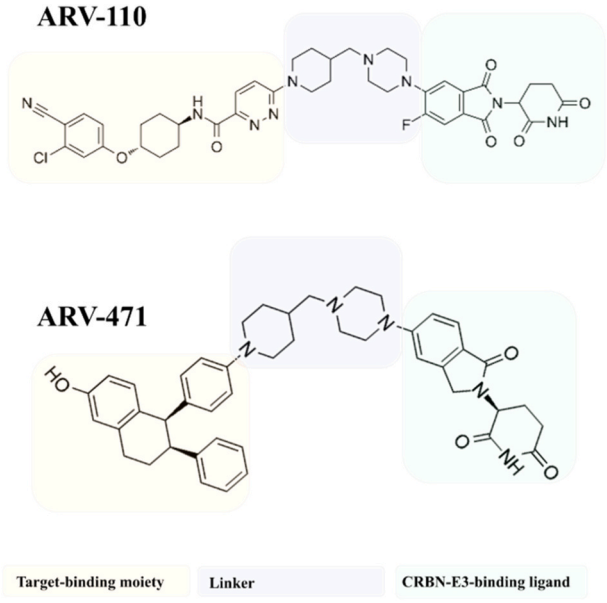
The study drugs ARV-110 (purity > 98 %) and ARV-471 (purity > 95.4 %) were purchased from Excenen PharmaTech Co., Ltd. (Shanghai, China). The internal standards (IS) warfarin (purity 97.2 %) and verapamil (purity 99.0 %) were supplied by Sigma-Aldrich (St. Louis, MO, USA). Rat blank plasma was sourced from five individual untreated rats and purchased from Innovative Research, Inc. (Novi, Michigan, USA). For the LC-MS/MS analysis, deionized water was purified through an ELGA Purelab Flex Water Purification System (Wycombe, UK). LC/MS grade acetonitrile (AcN) and methanol (MeOH) as well as polyethylene glycol 400 (PEG400) were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Formic acid (FA) (HPLC grade) and dimethyl sulfoxide (DMSO) (HPLC grade) as well as Kolliphor EL, D-α-tocopherol polyethylene glycol 1000 succinate (TPGS), sodium chloride (NaCl) and potassium dihydrogen phosphate (KH_2PO_4) were supplied by Sigma-Aldrich.

2.2. Animals

The animal study was approved by the local ethics committee for animal research (5.8.18–00250/2022) in Uppsala, Sweden. For this study, a total of 16 male Sprague Dawley rats (NTac) from Taconic (Ejby, Denmark) with body weights in the range of 270 – 340 g (age 7–11 weeks) were used. All animals were allowed to acclimatize for at least 1 week in the Animal Department prior to the start of the experiment and they were allowed water and food ad libitum. Housing conditions were 21–22 °C at a 12 h–12 h light–dark cycle. Animals were evaluated for general welfare twice daily by the Animal Department staff.

2.3. LC-MS/MS instrumentation and operating conditions

ARV-110 and ARV-471 were analyzed using an ACQUITY-UPLC I-



	ARV-110	ARV-471
Molecular Mass	812.3 Da	723.9 Da
logP	3.6	5.9
Topological Polar Surface Area (TPSA)	181.2 Å^2	96.4 Å^2
Hydrogen Bond Donors	2	2
Hydrogen Bond Acceptors	12	7
Rotatable Bonds	9	7
pKa		
- strongest acid	11.74	9.95
- strongest base	9.29	9.27

Fig. 1. Molecular structures of bavdeglutamide (ARV-110) and vepdegestrant (ARV-471) and a selection of physicochemical descriptors calculated with the Chemicalize software (Chemaxon, Hungary). The PROTACs are built from three molecular moieties: protein of interest-binding moiety (yellow), linker (blue) and E3-ligase-binding moiety.

Class system (Waters, Manchester, UK) coupled to a Xevo-TQSmicro tandem quadrupole mass spectrometer (Waters, Manchester, UK) via an ESI interface operated in positive ionization mode. A BEH C18 column (1.7 μm, 2.1 ×50 mm) (Waters, Manchester, UK) was used at 40 °C with a gradient of mobile phase (A) consisting of 0.1 % FA in water and (B) 0.1 % FA in acetonitrile over 4.0 min. The gradient elution procedure is shown in Table 1.

The autosampler temperature was maintained at 6 °C and a sample volume of 5 μL was injected. A pre- and post-run needle wash with a methanol:water (90:10, v/v) mixture was performed before and after every injection for 7 s each. Quantitative analysis was performed with tandem mass spectrometry using selected reaction monitoring (SRM) in positive ionization mode at the respective transitions from the $[M+H]^+$ ions to their product ions at *m/z* 812.6 → 452.3 (ARV-110) and *m/z* 724.7 → 396.4 (ARV-471). Specific MS parameters are displayed in Table 2.

2.4. Preparation of the stock solution, IS solutions, and working standards

Stock solutions were prepared by weighing 0.750 mg of ARV-110 and ARV-471 and dissolving them in 750 μL DMSO to obtain separate solutions at 1.000 mg/mL. From the stock solution, working standard solutions for the calibrators were prepared by serial dilution with methanol. The working standard solutions for the quality control (QC) samples were diluted in the same way using independently prepared stock solutions. Working solutions of the IS warfarin (200 ng/mL) and verapamil (70 ng/mL) were prepared in methanol from stock solutions at 100 μg/mL. All working solutions were prepared in amber glass vials and stored at −20 °C.

2.5. Sample preparation

The samples were prepared by spiking 36 μL of blank rat plasma with 4 μL of the respective working standard solution to provide separate calibration samples with concentrations of 0.5, 2, 5, 10, 50, 200, 1000, and 2000 ng/mL for ARV-110 and ARV-471. QC samples were prepared similarly at four concentration levels at the lower limit of quantification (LLOQ) at 0.5 ng/mL, a low QC at 1.5 ng/mL, a middle QC at 150 ng/mL, and a high QC at 1500 ng/mL. The calibration and QC samples were subsequently spiked with 4 μL of IS in MeOH. To achieve efficient precipitation of plasma proteins 72 μL of chilled (-20 °C) acetonitrile was added to each sample. The mixtures were vortexed for 30 s and subsequently centrifuged at 12,100 g at 5 °C for 10 min. The supernatant was then transferred into amber glass vials and 5 μL was injected into the LC-MS/MS system for analysis. All study samples were pre-treated in the same order using the same ratios of plasma to MeOH and AcN including the spiking of 4 μL of blank MeOH to the plasma samples as a substitute for the working standard solution.

Table 1
LC gradient conditions of ARV-110 and ARV-471.

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
ARV-110		
0.00	63.0	37.0
1.00	63.0	37.0
2.25	5.0	95.0
2.75	5.0	95.0
3.50	63.0	37.0
4.00	63.0	37.0
ARV-471		
0.00	68.5	31.5
1.25	68.5	31.5
2.00	10.0	90.0
2.50	10.0	90.0
3.50	68.5	31.5
4.00	68.5	31.5

2.6. Method validation

The method validation was conducted in accordance with the ICH M10 guideline on bioanalytical method validation and study sample analysis and the FDA guidance for bioanalytical method validation [19, 20]. The parameters validated were linearity, selectivity, matrix effect, accuracy, precision, carry-over, and stability.

2.6.1. Linearity and range

The lowest concentration of the calibration curve was selected based on the signal of the analyte-containing sample which should be at least five times higher than the noise from a blank plasma sample at the same retention time. To determine the linearity, the peak area ratio of the analyte to the IS was plotted against the nominal concentrations of the standard. The calibration curves were fitted using the least-square method with a weighing factor of $1/x^2$ for linear regression. Acceptance criteria for the linearity of the calibration curves were a maximum deviation of ±15 % or ±20 % (LLOQ) of the measured concentrations compared to the nominal concentrations. Moreover, it was aimed at calibration curves with a correlation coefficient (r^2) >0.990. At least six of the eight calibrator samples had to meet these criteria with freshly prepared calibrators on three different occasions.

2.6.2. Precision and accuracy

To evaluate the intra- and inter-day precision and accuracy, three analytical batches of QC samples at four concentration levels (LLOQ, LQC, MQC, HQC) with five replicates for each concentration level were prepared. Two of these batches were prepared and analyzed with freshly prepared calibrators for each batch at the same day while the third batch was prepared and analyzed on the following day. The accuracy was calculated as %RE by subtracting the nominal concentration from the measured concentration and expressing it as a percentage of the nominal concentration. The precision was calculated as %CV by expressing the ratio of the standard deviation of the measured concentration and the mean of the measured concentrations as a percentage. It is required that the accuracy of the QC samples is within ± 15 % for LQC, MQC, and HQC and ± 20 % for the LLOQ while the precision of the QC samples is within 15 % and 20 % respectively.

2.6.3. Selectivity and matrix effects

To evaluate the capability of the analytical assay to differentiate the analyte and the IS from endogenous matrix components, pretreated blank rat plasma from six different sources was injected. The chromatographic peak areas of the blanks at the retention times of the analytes and the IS were compared to the peak areas of the calibrator at the LLOQ and the IS in the respective SRM channels. The ratio of the integrated blank peak areas was expressed as a percentage of the LLOQ and IS signal respectively and should be less than 20 % for the LLOQ and less than 5 % for the IS peak area.

The matrix effect was determined in accordance with the recently updated ICH M10 guideline. The analytes were quantified in six different rat plasma sources spiked at the LQC and HQC concentration levels in triplicates. Accuracy and precision were calculated as described in Section 2.6.2. Values within ±15 % for the %RE and <15 % for the % CV were considered acceptable at both concentration levels.

2.6.4. Carry-over

To evaluate the effect of high-concentration residuals on the quantification of samples, a zero-calibrator sample (blank matrix containing IS) was injected three consecutive times after the highest calibrator was injected. The carry-over was calculated as the percentage of the peak area in the first zero-calibrator to the mean peak area ratio of three priorly measured calibrators at the LLOQ. The two additionally injected zero-calibrators were used to assess the decrease in residual signal upon consecutive injections. The peak area ratio of the first zero-calibrator should be less than 20 % of the mean LLOQ signal.

Table 2
Optimized mass spectrometric parameters for ARV-471, ARV-110 and their respective internal standards verapamil and warfarin.

Parameter	ARV-471	Verapamil (IS)	ARV-110	Warfarin (IS)
SRM transition	*m/z* 724.7→396.4	*m/z* 455.5→165.1	*m/z* 812.6→452.3	*m/z* 309.3→163.0
Cone voltage (V)	12	34	12	8
Collision energy (V)	34	28	36	14
Ion source temperature (°C)	150	150	150	150
Desolvation temperature (°C)	500	500	500	500
Desolvation gas flow (L/h)	1000	1000	1000	1000
Capillary Voltage (kV)	1.2	1.2	1.7	1.7
Collision cell entry potential	30.0	30.0	1.0	1.0
Collision cell exit potential	30.0	30.0	1.0	1.0

2.6.5. Stability studies

To assess the stability of the analytes in plasma, samples at the LQC and HQC concentration levels were stored under study-relevant conditions. This encompassed long-term stability, freeze-thaw stability, short-term stability, and autosampler stability as well as stability under light exposure and intermediate 1 h stability. All stability tests were carried out in the same polyethylene tubes used for in-study samples. The stressed samples were protected from light during storage unless indicated otherwise.

Long-term stability was assessed after storing plasma samples at −20 °C for 7 weeks, whereas short-term stability samples were stored at room temperature (25 °C) for 20 min as this was the maximum anticipated storage time under these conditions. An additional intermediate stability was performed by subjecting samples to room temperature (25 °C) for 60 min. The freeze-thaw stability was tested after three cycles with approximately 18 h of freezing at −20 °C and thawing for 20 min between each cycle. Five replicates at each QC level were prepared for injection into the LC-MS/MS system to assess the stability of processed samples. The samples were then analyzed immediately after preparation and after storage for 24 h at 6 °C in the autosampler to determine the recovery after storage with freshly prepared calibration curves. Additionally, the light sensitivity of the analytes in plasma samples was assessed by storing the samples at room temperature (25°C) for 20 min at the bench-top directly exposed to light. For each storage condition, one bulk QC was prepared at the respective concentration level. The bulk was then aliquoted into six individual samples, of which one was immediately analyzed as a reference sample against a freshly prepared calibration curve and five were stressed at the pre-defined conditions and subsequently analyzed. The stability was calculated as a mean accuracy value ± relative standard deviation.

The long-term stability of the 10-fold concentrated working standards for the preparation of QC and calibrator samples was evaluated for 8-week storage at −20 °C in amber glass vials. Samples at the LQC and HQC levels respectively were analyzed immediately after preparing the working solutions and after eight weeks of storage from the same working solutions. The working solutions were spiked to plasma and analyzed against freshly prepared calibrators. Stability was calculated as described above.

2.7. Application to pharmacokinetic studies

The validated LC-MS/MS methods for the compounds ARV-110 and ARV-471 were applied in a pharmacokinetic study in 16 male Sprague Dawley rats. On the study day, the rats were anesthetized using a single intraperitoneal injection of a 10 % w/v inactin (thiobutabarbital) solution (120 mg/kg). Body temperature was maintained at 37.5 ± 0.5 °C by a heating pad placed below the rat (Fig. 2). Systemic arterial blood pressure was continuously recorded by connecting an arterial catheter to a transducer operating a PowerLab system (AD Instruments, Hastings, UK). For both PROTACs, the drug formulations were prepared at a concentration of 0.5 mg/mL for the IV injection and the intra-duodenal administration. The formulation for IV injection was prepared by first dissolving the drug in DMSO, then adding polyethylene glycol 400 (PEG400) and Cremophor EL for stabilization and solubilization of the drug and filling up with saline solution. The formulation components DMSO:PEG400:KolliphorEL:saline were used at ratios of 7:25:5:63 (v/v/v/v). The formulation for intra-duodenal administration of the drug was prepared by first dissolving the drug in DMSO and then adding 50 mM phosphate buffer adjusted to pH 6.8 with 0.05 % TPGS to this solution. DMSO and phosphate buffer with TPGS were used at a ratio of 10:90 (v/v). The rats were given a single bolus dose of ARV-110 or ARV-471 (1 mg/kg) via the femoral vein or into the duodenum by guiding a

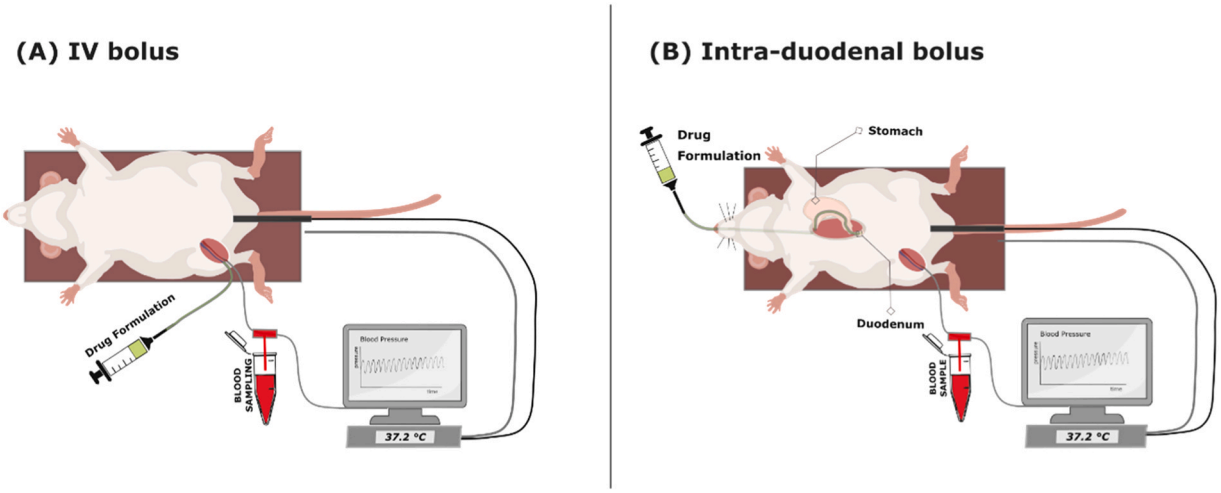


Fig. 2. Illustration of the experimental setup of a rat intravenous (IV) bolus and the intra-duodenal bolus studies with blood sampling over the course of 6 hours and continuous monitoring of blood pressure and body temperature.

polyethylene tube through the mouth, esophagus, stomach, and the pyloric sphincter into the small intestine. Blood samples (300 μL) were drawn from the femoral artery at 5 (only IV), 10, 20, 40, 60, 90, 120, 240, and 360 min and transferred into a lithium heparin-prepared tube. The administration route as well as the sampling time of the pharmacokinetic study was selected as this approach allows for a mechanistic pharmacokinetic assessment of the absorption phase of the drugs in the main region of absorption, the small intestine. All blood samples were centrifuged at 8600 rpm for 5 min with an Eppendorf MiniSpin (Eppendorf, Germany) centrifuge, and the supernatant plasma was stored until analysis at −20 °C. After every second blood sampling, the withdrawn volume was replaced by injecting 600 μL saline (0.9 % NaCl) with 70 mg/mL bovine serum albumin through the femoral artery. A simple illustration of the study setup is depicted in Fig. 2.

The 0–6 hour absolute bioavailability (F) from the intraduodenally administered formulations of ARV-110 and ARV-471 was calculated by dividing the total area under the plasma concentration-time curve in the 0–6 hour time interval ($AUC_{0-6\,h}$) following intraduodenal administration ($AUC_{0-6\,h;ID}$) by the area under the curve following an IV bolus ($AUC_{0-6\,h;IV}$), corrected by dose (Equation 1).

Equation 1:

$$F \quad (\%) = 100\% \times \left(\frac{AUC_{0-6h:ID}}{AUC_{0-6h:IV}}\right) \times \left(\frac{Dose_{IV}}{Dose_{ID}}\right)$$

3. Results and discussion

3.1. Method development

The chromatographic and mass spectrometric conditions were adjusted aiming for the highest ion abundance and sensitivity while employing a simple work-up and high throughput of samples. Owing to the chemistry of the PROTAC molecules, ionization was achieved at the highest intensity by electrospray ionization (ESI) in positive mode. Both molecules contain tertiary amines as part of their piperidine and piperazine-based linker functionalities which were readily protonated to form single-charged precursor ions $[M + H]^+$ for ARV-110 and ARV-471 at *m/z* 812.6 and 724.7 respectively. Avoiding doubly charged ions as well as in-source fragments as often reported for PROTACs did not pose a major challenge for these compounds. The most prominent product ions were found to be at *m/z* 452.3 for ARV-110 and 396.4 for ARV-471. The fragmentation of the PROTACs can be attributed to the breakage of the C-N linkage at the piperazine functionality in both molecules as depicted in Fig. 3.

Verapamil and warfarin were chosen as internal standards (IS) for ARV-110 and ARV-471 due to their proven plasma and solution stability and their relatively high lipophilicity providing similar elution behavior as the highly lipophilic PROTAC molecules [21,22]. Applying a Q1 full scan for both IS revealed that verapamil and warfarin predominantly formed single-charged $[M + H]^+$ precursor ions at *m/z* 455.5 and 309.3 respectively. The precursor ions were most abundantly fragmented into product ions of an *m/z* of 163.0 for warfarin and 165.1 for verapamil

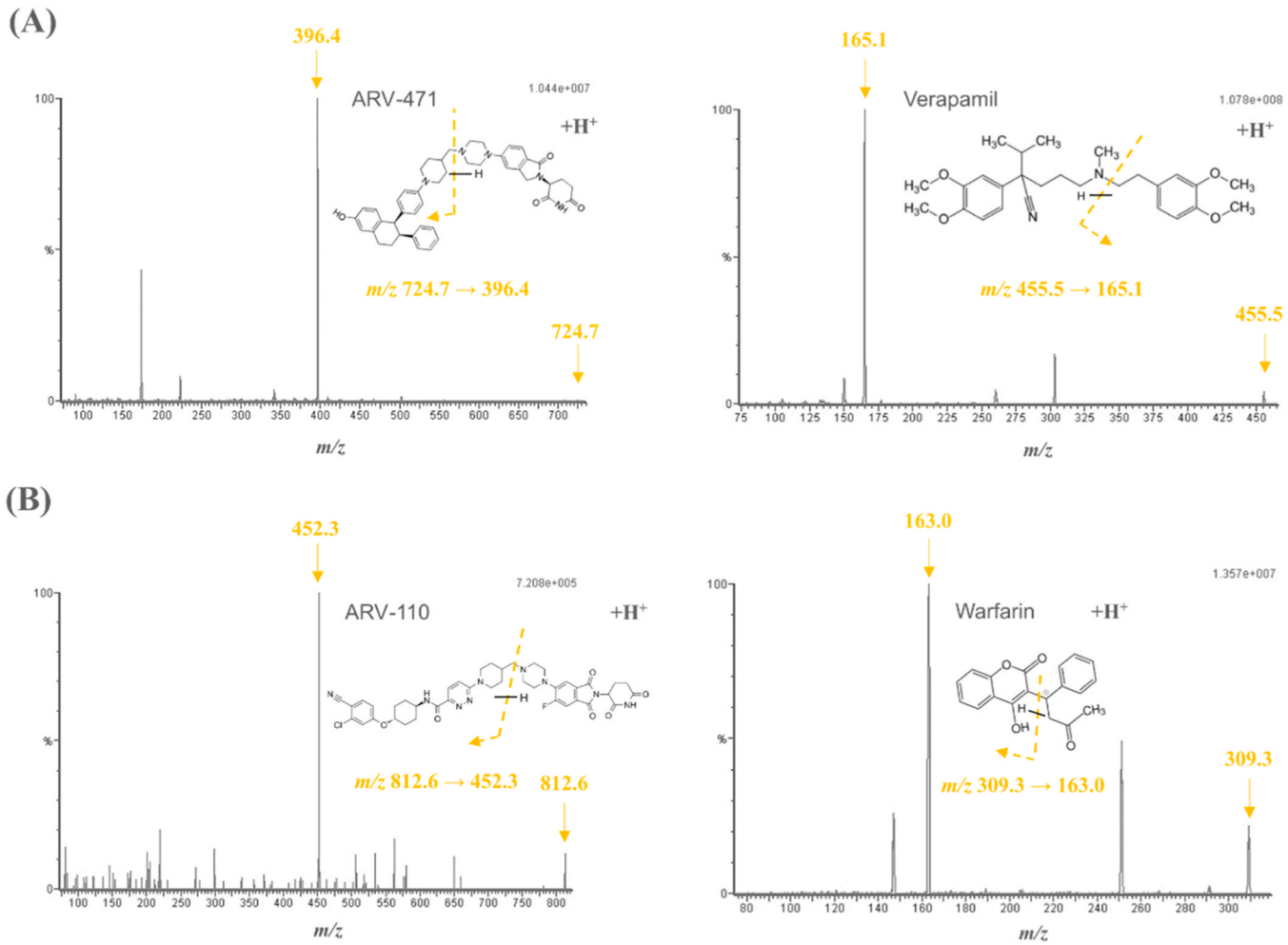


Fig. 3. Product ion spectra of ARV-471 and Verapamil (A) and ARV-110 and Warfarin (B) in positive ionization mode with the respective fragmentation sites.

(Fig. 3), similar to previous reports [23].

For the chromatographic optimization, different solvents, gradients and isocratic conditions were screened to achieve sharp peaks with high intensity and reasonably short retention times. In these trials acetonitrile proved to be superior to methanol as organic modifier as it resulted in improved peak shape with less tailing and higher area. Elution over a gradient with water and acetonitrile, both supplemented with 0.1 % formic acid, resulted in good chromatographic resolution between the analytes and background peaks with sharp peaks for ARV-110 and ARV-471 at 1.83 and 2.03 min, respectively. Both IS eluted in temporal proximity to their analytes with a retention time of 2.17 min for warfarin and 2.07 minutes for verapamil. The method's run-times were hence set to 4.0 min which allowed high sample throughput. An essential step during chromatographic optimization was the selection of favorable initial conditions. An initial gradient starting at only 5.0 % organic phase over the first minute led to extensive carry-over due to high retention of the lipophilic analytes in the column. By increasing the onset ratio of the organic phase, the carry-over could be minimized and even eliminated at a percentage of more than 45.0 % organic eluent. However, an increase in the initial organic proportion went along with a decrease in signal intensity which is why a sweet spot for high signal intensity and reasonably low carry-over was found for ARV-110 at 37.0 % and for ARV-471 at 31.5 %. These adjustments could reduce the carry-over by a factor of 40, compared to the initial gradient starting at 5.0 % organic phase, while maintaining the peak intensity to a large extent. A more extensive pre- and post-injection needle wash with different solvent mixtures was also tested, but could only reduce the carry-over to a minor extent.

Plasma protein precipitation was utilized for the sample preparation due to its fast, simple and cost-effective nature [24]. Acetonitrile was selected as the precipitant due to its high efficacy in removing plasma protein at low precipitant-to-plasma ratios [25]. For sample preparation, the IS as well as the working standards were generally added at low volumes (4 μL) in methanol to avoid any precipitation during the addition of analyte or IS and to allow their equilibration with plasma proteins. After equilibration for 20 s, the extraction of analyte and IS from rat plasma was achieved by a 1:2 ratio of plasma to acetonitrile. Higher acetonitrile to plasma ratios were tested but did not yield improved results.

3.2. Method validation

3.2.1. Linearity and range

The bioanalytical methods for ARV-110 and ARV-471 exhibited good linearity within the range of 0.5 – 2000 ng/mL with correlation coefficients (r^2) greater than 0.995. Representative linear regression equations were $y = 0.0292x + 0.0000012$ ($r^2 > 0.996$) and $y = 1.4492x + 0.000361$ ($r^2 > 0.995$) for ARV-110 and ARV-471 for one of the three respective calibration curves. The representative calibration curves are shown in Fig. 4. None of the eight standards deviated more than 8.6 % from the fitted curves. The LLOQ was set to 0.5 ng/mL for the assay as this was the lowest expected concentration in plasma samples after oral administration of the drugs. Moreover, with high signal-to-noise ratios of 26:1 (ARV-110) and 126:1 (ARV-471) the LLOQ could have been set lower but was ultimately limited by the carry-over.

3.2.2. Precision and accuracy

Precision and accuracy were assessed by computing intra- and inter-day variation and accuracy at four different QC concentration levels (0.5, 1.5, 150, and 1500 ng/mL) from three analytical batches with five replicates each. The intra-day accuracy, expressed as %RE, ranged between −8.7 % and 9.5 % for the two compounds while the inter-day accuracy was found to be between −6.8 % and 9.7 % at the four concentration levels. The intra-day coefficient of variance, i.e., the precision was in the range of 11.0 and 11.5 % for the LLOQs and in the range of 2.1 and 6.3 % for the other QC concentration levels. Inter-day precision values were in the range of 9.4 and 11.2 % for the LLOQs and in the range of 2.8 and 6.5 % for LQC, MQC, and HQC. The detailed overview of the precision and accuracy of the bioanalytical methods can be seen in Table 3. All measured values were within the acceptable limits with most of the values showing less than 5 % relative error or coefficient of variance, indicating a high reliability of the analytical method for ARV-110 and ARV-471 in rat plasma.

3.2.3. Selectivity and matrix effects

The chromatograms of blank plasma samples, zero-calibrator, calibrator at LLOQ, and a plasma sample taken 40 min after IV injection of the respective drug are shown in Figs. 5 and 6. Symmetrical peaks with retention times of 1.83 minutes and 2.17 minutes for ARV-110 and warfarin as well as 2.02 and 2.07 for ARV-471 and verapamil were obtained. All chromatograms showed a clear separation of the analyte from endogenous components of the plasma matrix with constant retention times for QC and calibration samples and plasma samples taken in the pharmacokinetic studies.

As shown in Table 4 very low interference from endogenous substances in six different sources of the matrix was observed for the analytes and the IS. All integrated peak areas at the analyte retention times were below 5.0 % of the LLOQ signal, while the integrated peak areas at the IS retention time were below 1.3 % of the IS signal, thereby fulfilling the criteria.

The determination of accuracy and precision of LQC and HQC samples prepared as triplicates in six different rat plasma sources revealed acceptable matrix effects within the limits (15 % RE and CV) set by the regulatory agencies. The matrix effect was evaluated according to the newly described procedure in the ICH M10 guideline on bioanalytical method validation [20]. This suggests that the simple protein precipitation method is able to reliably and efficiently remove interfering matrix components from the plasma matrix. Table 5 provides an

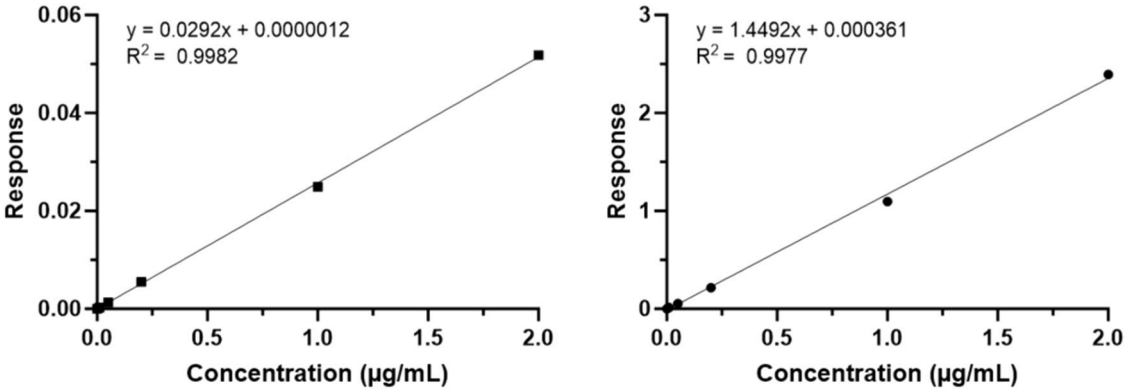


Fig. 4. Representative calibration curves with eight calibration standards in the range of 0.0005 μg/mL to 2.0 μg/mL for ARV-110 (left) and ARV-471 (right).

Table 3
Intra- and inter-day accuracy and precision of the LC-MS/MS assay at four concentration levels.

Nominal Concentration (ng/mL)	Intra-day (n = 5)			Inter-day (n=15)		
	Measured Concentration ± SD (ng/mL)	Accuracy (% RE)	Precision (% CV)	Measured Concentration ± SD (ng/mL)	Accuracy (% RE)	Precision (% CV)
ARV-110						
0.50	0.50 ± 0.06	-0.20	11.0	0.51 ± 0.05	0.93	9.4
1.50	1.50 ± 0.05	0.13	4.9	1.51 ± 0.07	0.31	4.6
150	145 ± 5	-3.2	3.1	147 ± 6	-1.7	3.8
1500	1370 ± 66	-8.7	4.7	1400 ± 74	-6.8	5.3
ARV-471						
0.50	0.47 ± 0.05	-5.6	11.5	0.48 ± 0.05	-3.7	11.2
1.50	1.64 ± 0.04	9.5	2.1	1.65 ± 0.05	9.7	2.8
150	149 ± 9	-0.89	6.3	152 ± 10	1.5	6.5
1500	1490 ± 64	-0.68	4.3	1520 ± 84	1.6	5.5

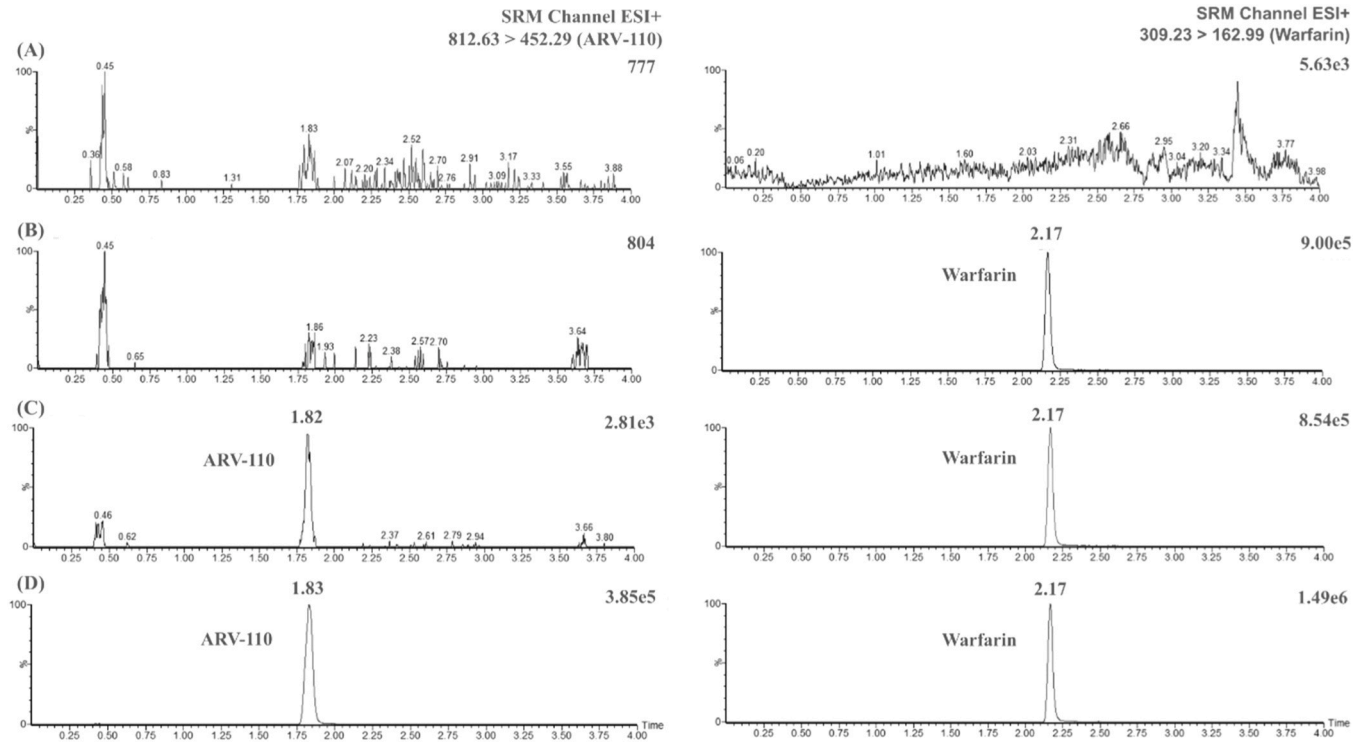


Fig. 5. Representative SRM chromatograms of the ARV-110 (left) and warfarin (IS) channel (right) in rat plasma samples. (A) Blank plasma sample; (B) Zero-calibrator sample; (C) Calibrator at the LLOQ concentration level; (D) Rat plasma sample taken 40 minutes after intravenous bolus administration at a dose of 1 mg/kg.

overview of the matrix effects at both concentration levels.

3.2.4. Carry-over

The mean carry-over (n = 3) found in a zero-calibrator sample after injection of the highest calibrator was 16.55 % of the mean signal from three injected LLOQ samples for ARV-471. These results indicate an acceptable carry-over effect, manifesting the reliability of the bio-analytical assay. For ARV-110 the mean carry-over was found to be 27.85 %. To investigate the propagation of the carry over, two further zero-calibrator samples were injected after the first one. It could be shown that the carry-over diminishes gradually with each further injection, being at 12.24 % already in the second zero-calibrator injection and thereby below the limit. For the quantification of ARV-110 these findings imply that an additional blank sample should be injected when a sample with an anticipated low concentration is injected after a highly concentrated sample to minimize the influence on accuracy and precision of the assay. In the quantification of samples from pharmacokinetic studies these implications were considered and practically applied in the analysis.

3.2.5. Stability studies

Table 6 displays the stability test data of the analytes in plasma samples as well as in processed samples and stock solutions. With accuracy within the acceptance criteria, neither of the PROTACs showed signs of instability in rat plasma during short-term (20 min) storage when covered from light at room temperature as well as during long-term storage for 7 weeks at −20°C or three freeze-thaw cycles. Furthermore, samples from stock solution in MeOH were stable over 8 weeks at −20°C, and processed samples did not degrade during storage in the autosampler at 6°C for 24 h.

Plasma samples stored for 20 min directly exposed to light revealed slight instabilities to a similar degree for both PROTACs which however still lied within the acceptance criteria for accuracy. A more pronounced degradation was apparent in ARV-110 plasma samples after 60 min storage at room temperature, covered from light. Despite formally fulfilling the regulatory criteria, a tendency to degradation of ARV-471 can

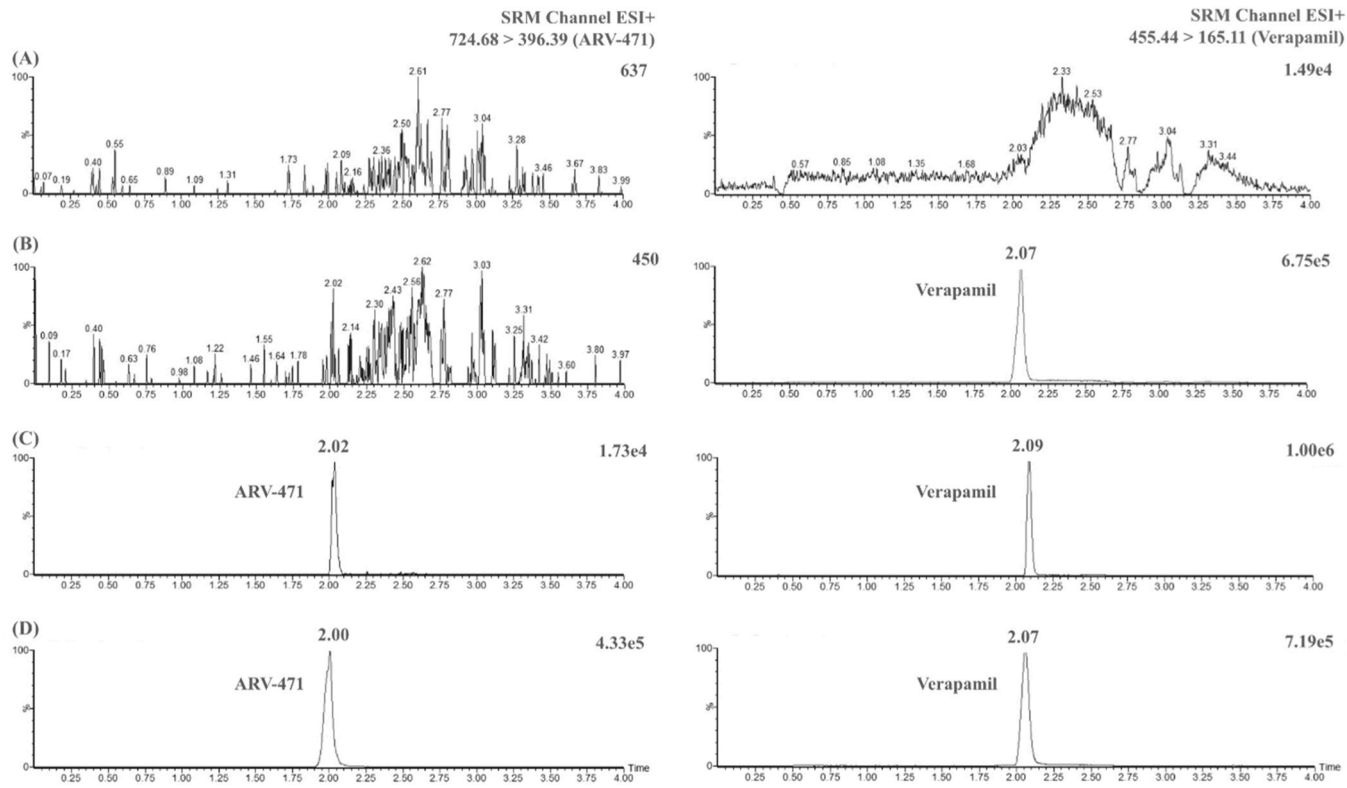


Fig. 6. Representative SRM chromatograms of the ARV-471 (left) and warfarin (IS) channel (right) in rat plasma samples. (A) Blank plasma sample; (B) Zero-calibrator sample; (C) Calibrator at the LLOQ concentration level; (D) Rat plasma sample taken 40 minutes after intravenous bolus administration at a dose of 1 mg/kg.

Table 4
Overview of the selectivity of the LC-MS/MS method for the analytes and their corresponding internal standards in rat plasma. The responses attributable to interfering components are expressed as the percentage of the analyte response at the LLOQ concentration level and the percentage of the IS response in the LLOQ sample.

Blank Plasma Source	ARV-110		ARV-471	
	Peak area at the retention time of the analyte [%]	Peak area at the retention time of the IS [%]	Peak area at the retention time of the analyte [%]	Peak area at the retention time of the IS [%]
1	2.8	0.7	3.6	1.3
2	1.7	1.1	4.1	0.9
3	2.2	1.1	3.2	1.1
4	3.8	1.3	0.4	0.8
5	2.6	1.0	5.0	0.9
6	2.0	0.8	2.6	0.7

Table 5
Matrix effects, expressed as the accuracy and precision of plasma samples from six different plasma sources in triplicates at the LQC and HQC concentration levels respectively.

Nominal Concentration [ng/mL]	Measured Concentration [ng/mL]	Accuracy [% RE]	Precision [% CV]
ARV-110			
1.50	1.50 ± 0.09	-0.33	6.3
1500	1420 ± 74	-8.5	5.2
ARV-471			
1.50	1.59 ± 0.21	6.2	13.2
1500	1620 ± 87	4.7	5.3

Table 6
Stability of ARV-110 and ARV-471 in rat plasma, processed samples and stock solution under various storage conditions. The stability is calculated as an accuracy by dividing the mean concentration of the samples by the nominal concentration and multiplying the ratio by 100 % to highlight the ratio of unchanged drug after storage.

Storage Conditions	Nominal Concentration [ng/mL]	Stability ± RSD [%]	
		ARV-110	ARV-471
Short term (25°C, 20 min) - covered	1.5	99.33 ± 7.64	98.62 ± 7.92
	1500	100.26 ± 2.91	98.70 ± 2.63
Short term (25°C, 20 min) – light exposed	1.5	85.66 ± 11.01	90.46 ± 3.89
	1500	93.24 ± 5.89	90.10 ± 6.84
Intermediate (25°C, 60 min) - covered	1.5	78.35 ± 2.43	86.67 ± 8.42
	1500	82.84 ± 2.42	91.67 ± 5.78
Autosampler (6 °C, 24 h)	1.5	100.77 ± 8.18	100.99 ± 6.64
	1500	98.75 ± 3.36	100.08 ± 3.47
Freeze-thaw (3 cycles)	1.5	102.60 ± 4.12	97.09 ± 5.53
	1500	101.90 ± 3.15	99.86 ± 1.65
Long term (-20 °C, 7 weeks)	1.5	100.00 ± 4.35	98.26 ± 6.47
	1500	101.66 ± 4.62	99.80 ± 3.35
Working solution (-20°C, 8 weeks)	1.5	99.32 ± 2.70	98.06 ± 2.84
	1500	99.79 ± 2.83	99.83 ± 3.13

be seen after 60 min storage at room temperature with a lower accuracy compared to the samples analyzed to determine precision and accuracy.

The tendency to degradation of the two PROTACs to a similar extent during longer storage is presumably associated with spontaneous hydrolysis that might be assisted by hydrolases in the plasma. ARV-471 and ARV-110 both employ a glutarimide functionality at their E3 ligase-binding moiety. In two former studies on thalidomide, these thalidomide-derived moieties which are also used in CRBN-based PROTACs have already been shown to be prone to hydrolysis in plasma samples [26,27]. In addition, ARV-110 has a phthalimido ring which has been hypothesized to further activate hydrolysis of the glutarimide ring and can itself be cleaved to form an α–(carboxybenzamido) glutarimide [27,28]. These mechanisms are likely to cause the degradation at room temperature of both PROTACs with more pronounced degradation for ARV-110 due to the additional electron-withdrawing phthalimide moiety. This instability can however be prevented by storing the plasma samples at −20°C for up to 7 weeks and avoiding storage of the samples for more than 20 min at room temperature prior to sample preparation, as shown by the data presented in Table 6.

3.3. *In vivo pharmacokinetic study*

By applying the developed LC-MS/MS methods, plasma concentration-time profiles over a 6 h study period of ARV-110 and ARV-471 (Fig. 7) following single IV and intraduodenal bolus doses at 1 mg/kg in rats could be obtained.

Plasma concentrations of ARV-110 in a range between 0.5 and 228 ng/mL could reliably be quantified at a dose of 1 mg/kg. The range of observed plasma concentrations for ARV-471 was 4.9–707 ng/mL. Assuming dose-linearity in absorption and bioavailability of ARV-471, a successful quantification of the drug in plasma at doses as low as 0.1 mg/kg in rats can be expected using the bioanalytical method. The plasma concentration-time curves underlined that the developed LC-MS/MS assays are capable of reliably quantifying the drug over a wide range of concentrations. This further enables the execution of in-depth pharmacokinetic studies as well as comprehensive studies of the biopharmaceutical *in vivo* properties of PROTACs.

The corresponding $AUC_{0-6\ h}$ and bioavailability values are depicted in Table 7. The bioavailability (0–6 h) following intraduodenal bolus administration was low for both PROTACs, indicating limited intestinal absorption and/or high first-pass extraction of ARV-110 and ARV-471. A full mechanistic rat *in vivo* evaluation of the rate and extent of intestinal absorption and first-pass extraction of the two drugs will be evaluated in a separately published study. This includes the impact of dose and formulation, metabolic enzyme and transporter protein contributions, as well as luminal stability, bile excretion and food effects.

Table 7
Selected pharmacokinetic parameters of ARV-110 and ARV-471 following intravenous and intraduodenal bolus administration in rats (n = 4).

Parameter	ARV-110		ARV-471	
	Intravenous	Intraduodenal	Intravenous	Intraduodenal
$AUC_{0-6\ h}$ (ng•h/mL)	374.4 ± 17.7	27.7 ± 3.4	585.4 ± 43.82	73.3 ± 3.8
F (%)	-	7.4 ± 0.9	-	12.5 ± 0.6

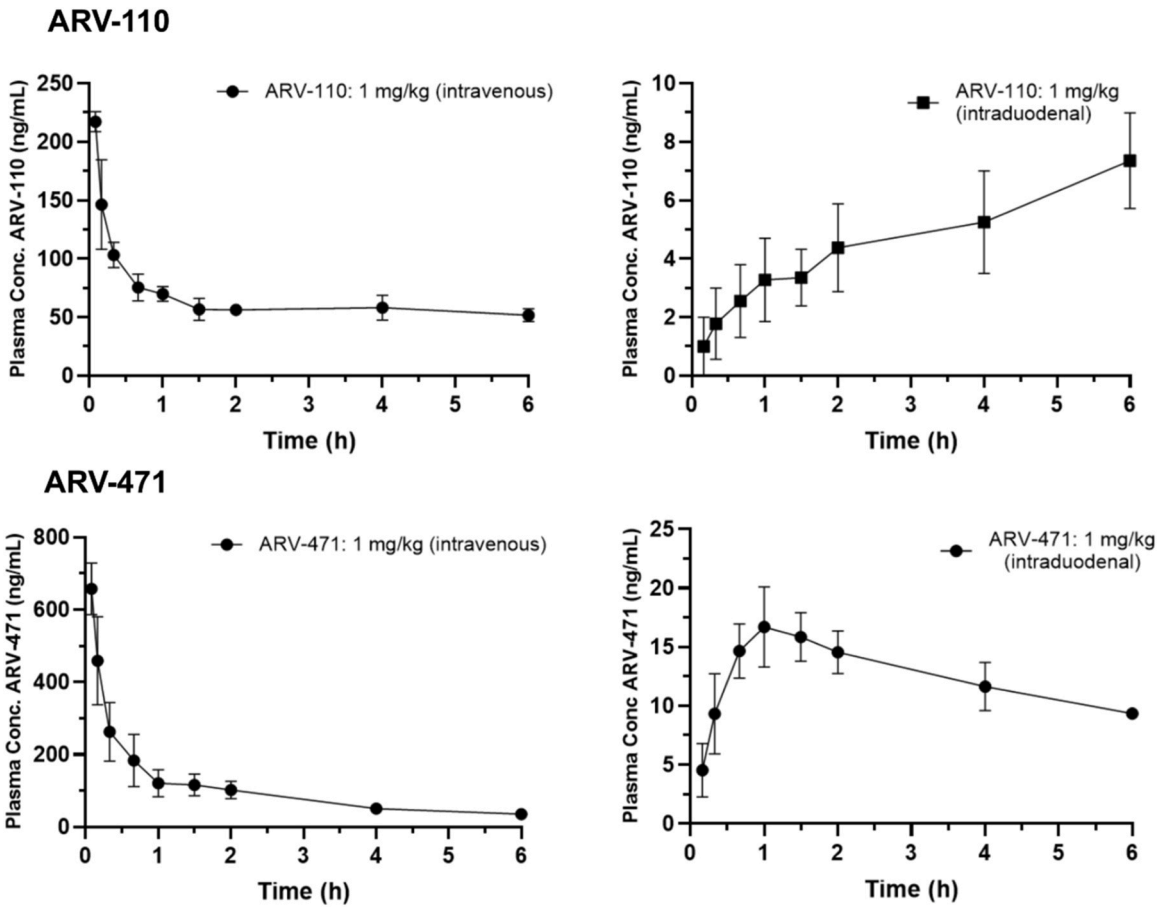


Fig. 7. Plasma concentration-time profiles of ARV-110 (top) and ARV-471 (bottom) after single-dose intravenous (left) and intra-duodenal (right) administration at a dose of 1 mg/kg to rats. Data are presented as mean ± SD (n = 4).

4. Conclusion

This work describes the development and validation of two LC-MS/MS methods with high reliability for the quantification of ARV-110 and ARV-471 in rat plasma passing all bioanalytical validation criteria defined in the most recent guidelines of EMA and FDA. Both PROTACs were shown to be stable in rat plasma for 7 weeks at −20 °C, three freeze-thaw cycles as well as for 20 min when stored protected from light at room temperature (25 °C). The methods were successfully applied in pharmacokinetic studies measuring drug plasma concentrations in rats after single IV and intraduodenal doses at 1 mg/kg, revealing a low oral bioavailability for both PROTACs. To the best of our knowledge, this is the first publication of data on the plasma pharmacokinetics including oral bioavailability of ARV-471 after single IV and intraduodenal administration in rats. These findings can serve as a pivotal precursor to further investigate and address the issues associated with limited intestinal absorption of PROTACs, ultimately expediting the advancement of these promising drug candidates toward clinical application.

Ethical approval and animal welfare

The animal study was approved by the local ethics committee for animal research (5.8.18–00250/2022) in Uppsala, Sweden. The animal studies were carried out in compliance with the EU regulations for animal welfare. Animals were evaluated for general welfare twice daily by the Animal Department staff.

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CRediT authorship contribution statement

Mirko Koziolek: Writing – review & editing, Supervision, Resources. **Hans Lennernäs:** Writing – review & editing, Supervision, Funding acquisition. **David Dahlgren:** Writing – review & editing, Supervision, Funding acquisition. **Jenny M. Nilsson:** Methodology. **Karsten Peters:** Investigation. **Anura Indulkar:** Writing – review & editing, Supervision, Resources. **Thomas Borchardt:** Writing – review & editing, Supervision, Resources. **Mikael Hedeland:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Janis Niessen:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: David Dahlgren reports financial support was provided by AbbVie Inc. AI, TB and MK are employees of AbbVie and may own AbbVie stock. The other 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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