



# Quantitative bioanalytical assay for the selective RET inhibitors selpercatinib and pralsetinib in mouse plasma and tissue homogenates using liquid chromatography-tandem mass spectrometry



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## ABSTRACT

Selpercatinib and pralsetinib are potent and selective tyrosine kinase inhibitors targeting the rearranged during transfection (RET) receptor in various types of cancer. In this study, a bioanalytical assay was developed and fully validated for selpercatinib and pralsetinib in mouse plasma and partially in eight mouse tissue homogenates using liquid chromatography-tandem mass spectrometry. Samples were pre-treated by protein precipitation with acetonitrile using erlotinib as internal standard. Separation of the analytes was performed on an ethylene bridged octadecyl silica C18 column by gradient elution using ammonium hydroxide (in water) and methanol. Analytes were detected by positive electrospray ionization in selected reaction monitoring mode. A linear concentration range of 2–2000 ng/ml was used for the validation of the assay for both inhibitors. The precision values (within-day and between-day) ranged between 3.4 and 10.2% for selpercatinib and 3.1–14.6% for pralsetinib in all matrices. Furthermore, data obtained for accuracy were between 91.7 and 109.3% and 85.1–114.1% for selpercatinib and pralsetinib, respectively. No significant matrix effects or extraction losses were observed and both analytes were stable under all investigated conditions. Finally, a pilot study for selpercatinib in mice was conducted employing this method, followed by a successful incurred sample reanalysis.

## 1. Introduction

Cancer is still the leading global cause of death and is characterized by the abnormal division of cells, potentially spreading to different parts of the body. This disease is caused by damage to genes that are responsible for maintaining cellular functions. Such damage leads to altered cellular functions and could allow a cell to become malignant [1]. An example of a genetic alteration that is currently treated with different kinds of specific small molecules is rearranged during transfection (RET). RET is identified in different types of cancer, such as non-small cell lung cancer (NSCLC), papillary thyroid cancer, medullary thyroid cancer, colon cancer and other solid tumors [2]. The growth of tumors in NSCLC strongly depends on RET activity. Specific mutations can lead to continuous autophosphorylation and activation of the

tyrosine kinase signal transduction pathway. This mutation causes a change in the specificity of the tyrosine kinase, which leads to tumor formation. Several drugs have been developed to attack this process and ensure that tyrosine kinases (TKIs) are inhibited [3]. The first selective and potent oral RET TKIs selpercatinib (LOXO-292, Fig. 1A) and pralsetinib (BLU-667, Fig. 1B) are currently in phase 1/2 studies for patients with advanced RET fusion-positive solid tumors [3–5]. Both inhibitors show improved results for patients with RET fusion-positive cancer due to increased potency and minimal side effects.

For further development of both RET inhibitors availability of a bioanalytical assay for these drugs is indispensable. As far as we know, such an assay has not been reported hitherto for both, selpercatinib and pralsetinib. An quantitative bioanalytical method was therefore developed and validated for both drugs in mouse plasma as well as eight

**Abbreviations:** BSA, bovine serum albumin; IS, internal standard; PPT, protein precipitation; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LLOQ, lower limit of quantification; NSCLC, non-small cell lung cancer; QC, quality control; RET, rearranged during transfection; R<sup>2</sup>, coefficient of determination; SD, standard deviation; SI, small intestine; SRM, selected reaction monitoring; TKI, tyrosine kinase

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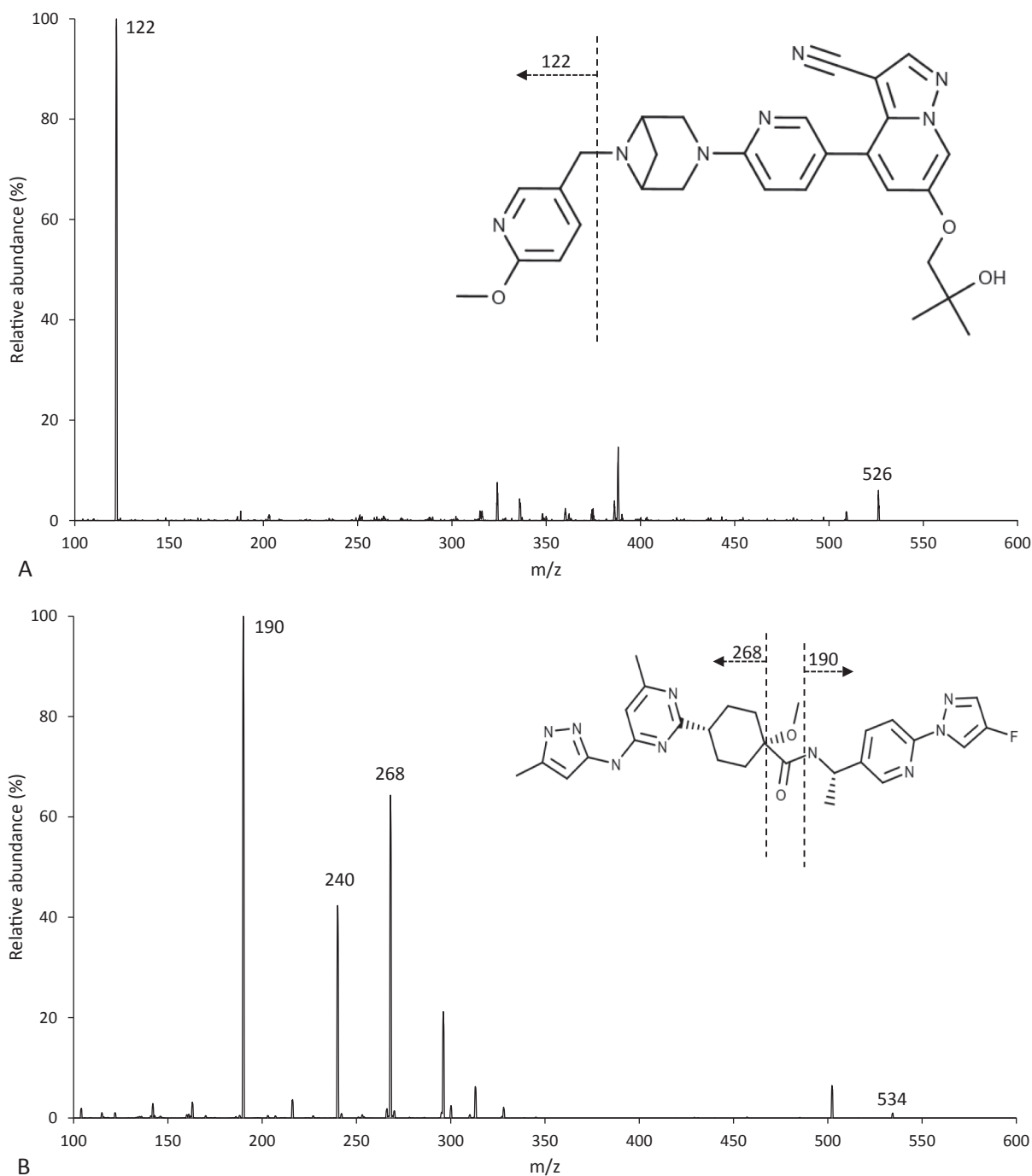


Fig. 1. Product spectra of (A) selpercatinib,  $m/z$  526.6 @  $-33$  V and (B) pralsetinib, 534.3 @  $-35$  V.

tissue homogenates (brain, lung, spleen, liver, kidney, testis, small intestine (SI) and SI contents) using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Although SI content is not a real “tissue”, it is a matrix that can give pivotal information of the non-absorbed fraction of the drug and it may also reflect the hepatic-biliary circulation. It was therefore also included in the present bioanalytical method without making further distinction with real tissues. The method was successfully applied in a pre-clinical study of selpercatinib in mice studying plasma pharmacokinetics and tissue distribution.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Selpercatinib (LOXO-292; > 99%) and Pralsetinib (BLU-667; > 99%) were obtained from Chemgood (Glen Allen, VA, USA), internal standard (IS) erlotinib (> 99%, as hydrochloric acid) was supplied by Carbosynth (Compton, Berkshire, UK). Acetonitrile (HPLS-S), methanol (HPLC) and water (ULC-MS) were purchased from Biosolve (Valkenswaard, The Netherlands) and analytical grade ammonium hydroxide was obtained from Sigma Aldrich (Steinheim, Germany). Blank human lithium-heparin plasma and lithium-heparin plasma from female mice were provided by Sera Laboratories (Haywards Heath, UK). Blank homogenized mouse organs were

prepared in ice-cold 2% (w/v) bovine serum albumin (BSA) in water using the FastPrep-24™ 5G instrument (M.P. Biomedicals, Santa Ana, CA, USA) for 1 min. Different volumes of BSA solution were used for different organs: 3 ml (liver and SI), 2 ml (two kidneys and SI contents), and 1 ml (brain, spleen, two lungs and two testes), respectively. All samples were stored at a temperature of  $-30\text{ }^{\circ}\text{C}$ .

## 2.2. Analytical instruments

An Accela LC system (quaternary pump and autosampler), coupled to a TSQ Quantum Ultra triple quadrupole mass spectrometer with heated electrospray ionization (ESI) was used for this study. Equipment and software for controlling, data recording and processing (Xcalibur version 2.07) were supplied by Thermo Fischer Scientific (San Jose, CA, USA).

## 2.3. LC-MS/MS conditions

An Acquity UPLC® BEH C18 column ( $30 \times 2.1\text{ mm}$ ,  $d_p = 1.7\text{ }\mu\text{m}$ , Waters, Milford, USA) and the corresponding VanGuard pre-column ( $5 \times 2.1\text{ mm}$ , Waters) were used for chromatographic separation at  $40\text{ }^{\circ}\text{C}$ . The samples, in a 96-well plate placed at  $4\text{ }^{\circ}\text{C}$ , were transferred to the column with an injection volume of  $10\text{ }\mu\text{l}$ . The separation of the analytes was performed using gradient elution at a  $600\text{ }\mu\text{l}/\text{min}$  flow rate. Solvents for gradient elution were (A) 0.2% (v/v) ammonium hydroxide in water and (B) methanol, where after (C) acetonitrile was used shortly after each run to flush the column. The method consisted of a linear increase for 1.5 min from 55% to 60% methanol supplemented with solvent A, followed by a flush with 100% acetonitrile for 0.2 min. To equilibrate, the initial composition of 0.2% ammonium hydroxide and methanol (45:55) (v/v) was set for 0.3 min before starting the next injection procedure of ca. 0.6 min. In order to optimize the electrospray and SRM conditions,  $5\text{ }\mu\text{g}/\text{ml}$  selpercatinib, pralsetinib and erlotinib were infused separately at  $5\text{ }\mu\text{l}/\text{ml}$  and mixed with  $600\text{ }\mu\text{l}/\text{min}$  of 50% methanol/49.9% water/0.1% formic acid (v/v/v). The final positive electrospray conditions for the method were: 3500 V spray voltage; 50, 8, and 40 (arbitrary units) nitrogen sheath, ion sweep, and aux gasses; 164 and  $345\text{ }^{\circ}\text{C}$  vaporizer and capillary temperatures; no skimmer offset, and a 1.7 mTorr argon collision pressure. Optimized SRM parameters were a 121 V tube lens offset with  $m/z\ 526.6 \rightarrow 122.0$  @  $-33\text{ V}$  collision energy for selpercatinib and a 109 V tube lens offset with  $m/z\ 534.3 \rightarrow 190.0$ ; 240.1; 268.1@  $-35$ ;  $-38$ ;  $-32\text{ V}$  collision energies for pralsetinib. For quantification, signals of all three pralsetinib transitions were added up. The parameters of the IS erlotinib were a 100 V tube lens offset with  $m/z\ 394.1 \rightarrow 278.1$  @  $-31\text{ V}$  collision energy. Transitions were monitored at a 100 ms dwell time for selpercatinib and at 50 ms for the others, all with unit mass resolutions (0.7 FWHH).

## 2.4. Stock and working solutions

Stock solutions were prepared at 0.5 and 0.2 mg/ml for selpercatinib and 0.2 mg/ml for pralsetinib in methanol. Working solutions were prepared as a mixture of both compounds with concentrations of  $50\text{ }\mu\text{g}/\text{ml}$  in 50% (v/v) methanol/water. The erlotinib (IS) stock solution had a concentration of 0.5 mg/ml. This solution was diluted 5000-fold in acetonitrile in order to obtain the precipitating agent.

## 2.5. Sample pre-treatment

Preparation of tissue homogenates was reported in Section 2.1. Protein precipitation was carried out by transferring  $10\text{ }\mu\text{l}$  of a sample into a conical 200- $\mu\text{l}$  well of a polypropylene 96-well plate. Subsequently,  $30\text{ }\mu\text{l}$  of the protein precipitant containing 100 ng/ml IS in acetonitrile was added, which was followed by vortex mixing the closed plate for approximately 15 s. Afterwards, the plate was placed in the

centrifuge at  $2643 \times g$  for 5 min. Thirty  $\mu\text{l}$  of supernatant was diluted with  $300\text{ }\mu\text{l}$  of 25% (v/v) methanol (in water) in a 1-ml round-bottom well of a polypropylene 96-deep well plate. Finally, the wells were sealed with a silicone mat, gently shaken and placed in the autosampler for analysis.

## 2.6. Bioanalytical method validation

The validation was performed according to the EMA [6] and FDA [7] guidelines for plasma and tissue homogenates. Full validation was performed for plasma samples and partial validation for the tissue homogenates. The partial validation consisted of one level for the determination of the accuracy and precision, four individual blank homogenates to assess selectivity and relative matrix effect, stability assessment at short term (bench-top) conditions only and incurred samples reanalysis, analogously to our recently published validation procedures for other kinase inhibitors [8,9].

### 2.6.1. Calibration

Calibration samples were prepared by diluting a 50- $\mu\text{g}/\text{ml}$  working solution in 4 plasma working solutions at 2000, 200, 20 and 4 ng/ml to obtain standards at 2000, 1000, 200, 100, 20, 10, 4 and 2 ng/ml in mouse plasma. Plasma calibration samples were also used for quantification in tissue homogenates and were freshly prepared in duplicate for every measurement. Duplicate blank (no analytes) and double blank (no analytes and no IS) samples were also prepared.

### 2.6.2. Accuracy and precision

The accuracy and precision in all matrices were assessed by analyzing quality control (QC) samples together with the calibration curve. The QC samples were prepared at 1500 (high), 150 (medium), 5 (low) and 2 (LLOQ) ng/ml in mouse plasma. The accuracy and precision were assessed in three different runs of 6 replicates for each QC sample. For the inter-assay precision squared deviations were calculated using the overall average, for intra-day precision squared deviations were calculated using the within-day averages. Further, QC samples at 150 ng/ml in all eight tissue homogenates were prepared to be processed in three times 6 replicates as well. Dilution integrity of the assay was assessed by preparing 20-fold dilutions. A mouse plasma sample containing 10  $\mu\text{g}/\text{ml}$  of both analytes was diluted to 0.5 ng/ml with human plasma in 5 replicates.

### 2.6.3. Selectivity

The selectivity of the assay was determined by employing plasma samples of 6 different mice. Tissue homogenates from four different mice were used for brain, lung, spleen, liver, kidney, testis, SI and SI contents. Each matrix was analyzed as double blank (no analytes, no IS) and at LLOQ (2 ng/ml).

### 2.6.4. Recovery and matrix effect

Three types of samples (A, B and C), were used to determine the extraction recovery and matrix effect from mouse plasma at three QC levels (high, medium and low) using four replicates for each level. Recovery experiments were carried out in duplicate. Concentrations of the reference QC samples for recovery and matrix effect were corrected for the 25% sample loss during the original sample pre-treatment procedure.

(A) The original method (Section 2.5) was used for plasma QC samples. (B) reference QC samples were prepared in 50% (v/v) methanol (in water) with concentrations of 1125 (high), 112.5 (medium), and 3.75 ng/ml (low). A volume of  $30\text{ }\mu\text{l}$  blank extract containing IS was added to  $10\text{ }\mu\text{l}$  methanolic QC sample, followed by dilution with  $290\text{ }\mu\text{l}$  of 25% (v/v) methanol. (C) The same methanolic reference QC samples of method B were used. Following the transfer of  $10\text{ }\mu\text{l}$  of each sample,  $20\text{ }\mu\text{l}$  of acetonitrile and  $300\text{ }\mu\text{l}$  of 25% (v/v) methanol were added.

The extraction recoveries and matrix effects of the drugs were calculated from absolute responses for ratios A/B and B/C, respectively.

In addition, relative matrix effects were assessed in individual plasma samples ( $n = 6$ ) and tissue homogenates ( $n = 4$ , for lung and testis  $n = 3$ ) at high and low levels, by comparing samples containing extract to samples without extract.

### 2.6.5. Stability

The stability of the analytes was investigated in mouse plasma at high and low QC levels. Ten- $\mu$ l portions of each level were stored under different conditions in 200- $\mu$ l well plates. The investigated conditions were 6 and 24 h at room temperature, three additional freeze-thaw cycles (1.5 h thawing at room temperature), and 2 months at  $-30\text{ }^{\circ}\text{C}$ . Tissue homogenates at medium QC levels were tested for stability at room temperature for 6 h. In addition, calibration samples were stored at  $4\text{ }^{\circ}\text{C}$  and reanalyzed five days later with freshly prepared QC samples to assess stability in diluted extracts under autosampler conditions.

### 2.6.6. Incurred samples reanalysis

Incurred samples reanalysis was performed by remeasuring 6 study samples taken at the end of the 4-h experiment reported in the next section of each 9 mouse matrices ( $n = 54$ ) stored at  $-30\text{ }^{\circ}\text{C}$ .

## 2.7. Pharmacokinetics and tissue distribution of selpercatinib in mice

A pilot study in male mice ( $n = 6$ ; 28.0–41.2 g; work protocol 2.3.9450 of the animal laboratory facility, The Netherlands Cancer Institute, Amsterdam, The Netherlands) with a FVB/NRj genetic background was performed. In brief, selpercatinib was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 20 mg/ml, and further diluted with polysorbate 20:ethanol (1:1 (v/v)) and 5% (w/v) glucose water [DMSO:polysorbate 20:Ethanol:glucose water = 5%:15%:15%:65% (v/v/v/v)] to yield a drug working solution of 1 mg/ml to be dosed in 280–412  $\mu$ l volumes. Mice were housed and handled according to institutional guidelines complying with Dutch legislation and treated similar to earlier reported protocols [10]. All the mice were 9 to 16 weeks of age and housed in a temperature-controlled environment with a 12-h light/12-h dark cycle. Animals received a standard diet and acidified water *ad libitum* and were fasted for 3 h before selpercatinib (10 mg/kg) was administered by oral gavage into the stomach, using a blunt-ended needle. Serial blood samples (50  $\mu$ l) were collected at 7.5 min, 15 min, 30 min, 1 h and 2 h following oral administration of the drug selpercatinib in mice by tail cutting. Mice were anesthetized with isoflurane after 4 h and a final blood sample was acquired by cardiac puncture. Plasma was obtained by centrifugation at  $9000 \times g$  for 6 min at  $4\text{ }^{\circ}\text{C}$  and stored at  $-30\text{ }^{\circ}\text{C}$  until analysis. Organs (brain, lung, spleen, liver, kidney, testis, SI and SI contents) were quickly removed after sacrificing the animal at 4 h, weighed, and homogenized as described in Section 2.1. All plasma, liver, kidney, SI and SI contents samples were diluted 10 times with human plasma before analysis. Human plasma was chosen instead of mouse plasma to be used for dilution because of the limited availability of plasma from this small animal.

Non-compartmental pharmacokinetic parameters were all calculated as mean  $\pm$  standard deviation (SD). The maximum plasma concentration ( $C_{\max}$ ) and time to reach the maximum plasma concentration ( $T_{\max}$ ) were calculated directly from the highest levels. The area under the plasma concentration-time curve until 4 h ( $AUC_{0-4h}$ ) was calculated using the trapezoidal rule.

## 3. Results and discussion

### 3.1. Method development

A novel LC-MS/MS method was developed and validated for selpercatinib and pralsetinib with high selectivity, sensitivity, and reproducibility. Moreover, different LC parameters were tested to achieve

the optimal response in terms of peak area, shape and resolution. Further, a fast and effective protein precipitation method was developed. The short Acquity UPLC BEH C18 column was chosen for fast analysis as it provides sufficient resolution in a short run time of ca. 2.6 min (injection time in between runs included). Among several mobile phase combinations of 0.1% (v/v) aqueous formic acid, 0.2% (v/v) ammonium hydroxide with acetonitrile and methanol as organic modifiers, ammonium hydroxide showed the highest response for ESI-MS. Moreover, better peak shapes were formed and therefore 0.2% (v/v) ammonium hydroxide (in water) was selected as a solvent.

The optimization of the mass spectrometric conditions was carried out to obtain optimally stable and sensitive responses for the compounds. Mass transitions  $m/z$  526.6  $\rightarrow$  122.0 for selpercatinib and 534.3  $\rightarrow$  190.0; 240.1; 268.1 for pralsetinib were used for quantification. Product spectra of selpercatinib and pralsetinib are shown in Fig. 1. The  $m/z$  240 fragment of pralsetinib could be explained by  $C_2H_4$  loss of the  $m/z$  268 fragment, other fragments of both analytes used for quantification are suggested in Fig. 1. A spectrum of erlotinib was reported previously [11]. For maximal response and sensitivity, signals of all three pralsetinib transitions were added up for quantification. This quantification mode can be advantageous when similar abundancies for these transitions are observed and was used previously for kinase inhibitors [12,13].

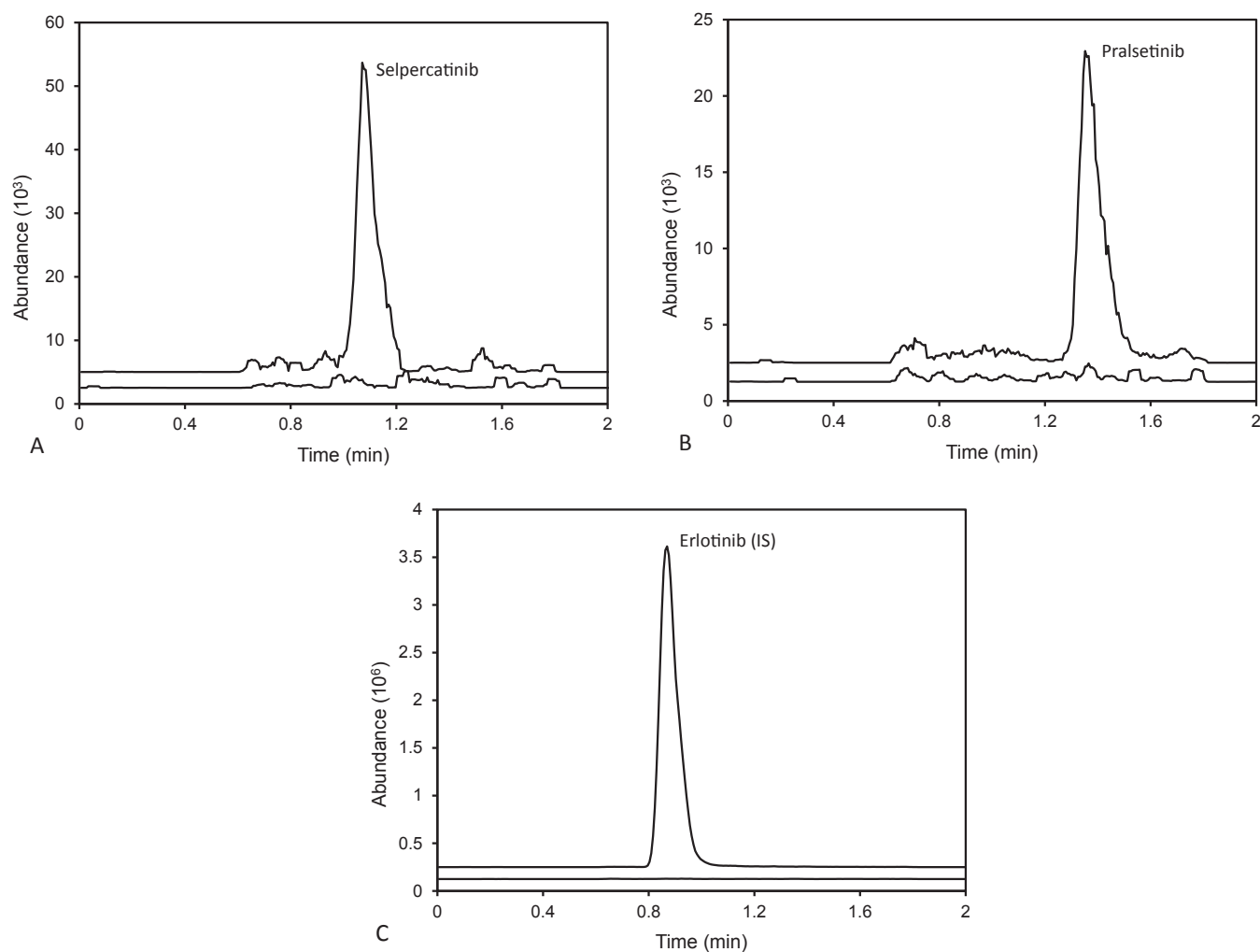
A one-step protein precipitation (PPT) procedure was preferred over other sample pre-treatment techniques since it is inexpensive, easily accessible without any additional equipment and less time consuming. In addition, PPT was successfully used for the extraction of other kinase inhibitors from plasma and tissue homogenates in previously reported studies [10,11,14,15]. Since no stable isotopically labelled internal standards were available, various compounds with similar chromatographic properties as selpercatinib and pralsetinib were screened. Finally, erlotinib was selected as most suitable IS for this method.

### 3.2. Validation

EMA guidelines [6] were followed to carry out a complete validation for the plasma and a partial validation for the tissue homogenates. The 2 to 2000 ng/ml calibration range was selected based on experience with earlier TKI studies in mice since no bioanalytical data of both drugs were available yet. Since the sex of the mice for future study samples was not known at the time of starting the validation, female plasma was chosen because of its larger supplies in our laboratory. The requirements for an acceptable value for the standards and quality control samples were as follows: calculated concentrations of calibration standards and QC samples should be  $\pm 15\%$  of the nominal value, with exception of the LLOQ, which should be  $\pm 20\%$  [6,7]. Representative chromatograms of the analytes in plasma are shown in Fig. 2.

#### 3.2.1. Calibration

Since there was no significant difference in deviations of the calibration standards at different concentrations, least squares weighted ( $1/x^2$ ) linear regression of the analyte/IS peak ratios was employed to determine the calibration curve ( $y = A + B \cdot x$ ), with the parameters: A as intercept, B as slope, y as peak area relative to IS and x as the concentration (ng/ml) of the analyte. Linear regression parameters (mean ( $\pm$  SD);  $n = 11$ ; Supplemental Table 1) were:  $y = 0.018 (\pm 0.021) + 0.066 (\pm 0.094) \cdot x$  with  $R^2$  (coefficient of determination) = 0.989 ( $\pm 0.007$ ) for selpercatinib. Linear regression parameters (mean ( $\pm$  SD);  $n = 11$ ) were:  $y = 0.020 (\pm 0.027) + 0.0360 (\pm 0.0059) \cdot x$  with  $R^2$  (coefficient of determination) = 0.991 ( $\pm 0.004$ ) for pralsetinib. The acceptability of the chosen method was confirmed by average deviations from the target values (coefficients of variation were  $\leq 1.2\%$  and  $\leq 1.3\%$  for selpercatinib and pralsetinib, respectively).



**Fig. 2.** Representative chromatograms of a blank and LLOQ spiked plasma sample showing (A) selpercatinib, (B) pralsetinib and (C) erlotinib. Signals were given an artificial offset.

### 3.2.2. Accuracy and precision

The assay performance for plasma samples was studied at four levels (Table 1) and for each of the eight tissue homogenates at one level (Table 2). The precision values in plasma ranged between 3.4 and 10.2% for selpercatinib and from 4.3 to 14.6% for pralsetinib. Furthermore, accuracy data were all between 91.7 and 109.3% and 90.8–102.9% for selpercatinib and pralsetinib, respectively, which indicated that the method was accurate and precise enough to perform quantification of study samples for both drugs. The precision values in

**Table 1**

Accuracy and precision data for selpercatinib and pralsetinib in mouse plasma (n = 18).

Analytes	Level (ng/ml)	Intra-day precision (%)	Inter-day precision (%)	Accuracy (%)
<b>Selpercatinib</b>				
QC high	1500	4.4	4.6	109.3
QC medium	150	3.4	4.4	101.0
QC low	5	5.9	6.0	98.8
LLOQ	2	9.9	10.2	91.7
<b>Pralsetinib</b>				
QC high	1500	6.5	6.1	102.9
QC medium	150	4.3	5.3	93.5
QC low	5	6.4	8.5	95.9
LLOQ	2	8.1	14.6	90.8

**Table 2**

Accuracy, precision, and stability (recovery after 6 h at ambient temperature) data for selpercatinib and pralsetinib in tissue homogenates at 150 ng/ml (QC medium) (n = 18).

	Intra-day precision (%)	Inter-day precision (%)	Accuracy (%)	Recovery (%)
<b>Selpercatinib</b>				
Brain	6.8	7.9	103.6	97.7 ± 4.0
Lung	5.3	8.2	104.2	103.1 ± 19.1
Spleen	4.6	7.0	104.1	95.4 ± 15.2
Liver	10.0	9.9	106.1	104.3 ± 4.1
Kidney	5.4	6.4	101.1	104.8 ± 4.3
Testis	4.7	6.6	98.7	101.3 ± 11.6
SI	5.4	6.4	101.4	110.5 ± 10.5
SI contents	4.2	7.7	102.1	102.9 ± 8.1
<b>Pralsetinib</b>				
Brain	6.4	7.0	100.7	103.4 ± 8.5
Lung	4.6	6.5	100.0	109.3 ± 40.6
Spleen	4.8	5.0	101.2	85.1 ± 22.0
Liver	8.6	10.1	99.2	104.1 ± 7.4
Kidney	8.6	9.2	100.6	96.7 ± 8.0
Testis	3.9	5.0	98.3	96.2 ± 12.9
SI	6.8	8.4	97.9	114.1 ± 12.5
SI contents	3.1	4.8	101.7	89.5 ± 8.0

all eight tissue homogenates were  $\leq 10.0\%$  for selpercatinib and  $\leq 10.1\%$  for pralsetinib. Accuracy values ranged from 95.4% to 110.5% and 85.1%–114.1% for selpercatinib and pralsetinib, respectively. All QC levels in different matrices met the  $\pm 15\%$  requirements and the  $\pm 20\%$  for the LLOQ samples [6,7].

Moreover, a 20-fold dilution ( $n = 5$ ) was performed to assess the dilution integrity at 10  $\mu\text{g/ml}$ , which resulted in 2.0 and 3.2% precisions and 102.2 and 99.4% accuracies for selpercatinib and pralsetinib, respectively. This result suggested that high level study samples will produce precise and accurate data too.

### 3.2.3. Selectivity

In order to conclude that interfering compounds are absent in the matrices, the responses of the analytes and IS in the blank samples should be lower than 20% of LLOQ and 5% of the normal IS response respectively [6,7]. No responses of individual plasma ( $n = 6$ ) or tissue homogenate ( $n = 32$ ) samples exceeded these requirements (Supplemental Table 2) and no interferences or co-eluting peaks were found in all these 38 blank samples. Further, levels observed in all individual LLOQ spiked samples ( $n = 38$ ; Supplemental Table 2) never exceeded  $\pm 20\%$  of the LLOQ value for more than half of the samples of each matrix. Thus, the LLOQ of 2  $\text{ng/ml}$  is suitable for the use in all different matrices with this assay.

### 3.2.4. Recovery and matrix effect

The recovery and absolute matrix effect were determined for plasma, to assess reproducibility of the extraction method. The data (Table 3) for recovery (93.3–124.2%) and matrix effect (97.3–107.6%) revealed that any significant extraction loss or matrix effect could be excluded at all QC levels for both compounds.

The overall relative matrix effects in all matrices (Supplemental Table 3) were  $105.6 \pm 4.3\%$  and  $94.1 \pm 4.2\%$  at the high level and  $108.5 \pm 7.1\%$  and  $108.8 \pm 7.8\%$  at the low level for selpercatinib and pralsetinib, respectively. All values of this experiment showed that the method was reproducible and matrix effects could be considered negligible.

### 3.2.5. Stability

Results of the stability studies for selpercatinib and pralsetinib in plasma and tissue homogenates are summarized in Table 4. QC-high and QC-low samples of the analytes in plasma were tested for stability under the following conditions: room temperature for 6 and 24 h, three additional freeze-thaw cycles, 2 months at  $-30^\circ\text{C}$ . All recoveries were between 85.0 and 106.7% for selpercatinib and between 88.0 and 105.5% for pralsetinib. Furthermore, recoveries for QC-medium in all tissue homogenates after 6 h at ambient temperature were between 95.4 and 110.5% for selpercatinib and between 85.1 and 114.1% for pralsetinib (Table 2). Reanalysis of calibration samples to demonstrate stability in diluted extracts was performed after 5 days at  $4^\circ\text{C}$  and resulted in successful processing of freshly prepared QC samples ( $n = 6$ ) with only one sample exceeding 115% accuracy for selpercatinib. So overall, sufficient stability of both drugs in all biological matrices and diluted extract was guaranteed.

### 3.2.6. Incurred samples reanalysis

Incurred samples reanalysis ( $n = 54$ ) resulted in only 2 samples

**Table 3**  
Data of recovery and matrix effect in plasma samples ( $n = 4$ ).

Level	Recovery (%)		Matrix effect (%)	
	Selpercatinib	Pralsetinib	Selpercatinib	Pralsetinib
QC high	$115.6 \pm 3.0$	$107.6 \pm 3.4$	$106.3 \pm 4.8$	$107.6 \pm 3.4$
QC medium	$124.2 \pm 3.4$	$115.7 \pm 3.7$	$97.3 \pm 11.5$	$99.7 \pm 8.9$
QC low	$94.5 \pm 10.3$	$93.3 \pm 4.2$	$101.2 \pm 7.2$	$105.0 \pm 10.0$

**Table 4**

Recovery for selpercatinib and pralsetinib in plasma ( $n = 4$ ) after exposure to different storage conditions.

Storage conditions	6 h at room temperature	24 h at room temperature	3 freeze-thaw cycles	2 months at $-30^\circ\text{C}$
<b>Selpercatinib</b>				
QC high	$106.7 \pm 2.7$	$91.6 \pm 9.8$	$98.7 \pm 3.7$	$101.3 \pm 4.1$
QC low	$104.8 \pm 11.9$	$97.0 \pm 5.4$	$85.0 \pm 4.0$	$106.3 \pm 11.1$
<b>Pralsetinib</b>				
QC high	$105.5 \pm 4.8$	$88.0 \pm 2.4$	$90.2 \pm 10.1$	$102.4 \pm 3.6$
QC low	$99.5 \pm 12.2$	$85.9 \pm 1.3$	$99.6 \pm 11.7$	$102.8 \pm 10.1$

(spleen +24.3%; brain –22.3%) exceeding the  $\pm 20\%$  requirement as shown in Fig. 3. This is far below the allowance of exceeding replicates (33%) [6,7].

### 3.3. Pharmacokinetics and tissue distribution of selpercatinib in mice

The new method was used for a pre-clinical study of selpercatinib, chromatograms of plasma and brain samples of one mouse are shown in Fig. 4. A pharmacokinetic plot for selpercatinib in mouse plasma is shown for 6 wild type (FVB/NRj) mice in Fig. 5. Pharmacokinetic parameters were:  $T_{\text{max}} = 1.8 \pm 1.2$  h,  $C_{\text{max}} = 7862 \pm 1814$   $\text{ng/ml}$ , and  $\text{AUC} = 26649 \pm 6360$   $\text{ng}\cdot\text{h}\cdot\text{ml}^{-1}$ . The drug was not rapidly absorbed, taking at least one hour to reach the maximum concentration in the plasma. After  $T_{\text{max}}$  the plasma level decreased only slightly and in one mouse  $T_{\text{max}}$  was even 4 h. Therefore, elimination rates and  $T_{1/2}$  (4–10 h in 3 out of 6 mice) could not be calculated for all mice in this 4-h pilot experiment. It is recommended to observe the plasma levels of the drug for a longer time period in order to estimate the pharmacokinetic profile completely.

Tissue levels decreased in the order SI contents > liver > kidney > SI > lung > spleen > testis > brain (Fig. 6) under the investigated conditions at 4 h. Drug levels in brain were found to be relatively low, most likely due to the presence of the blood-brain barrier. In various studies it has been shown that the ATP-binding cassette (ABC) drug efflux transporters, such as P-glycoprotein (P-gp; ABCB1) and breast cancer resistance protein (BCRP; ABCG2), affect the oral absorption, tissue distribution and toxicity of anti-cancer drugs [16]. Brain (and testis) results suggested that high expression of these ABC transporters presumably restricted selpercatinib penetration and distribution in comparison to other tissues. Furthermore, the drug is especially metabolized by the liver, followed by hepatobiliary excretion. All concentrations were above the LLOQ, but all plasma samples, liver, kidney, SI, and SI contents homogenates exceeded the upper limit of quantification. Those samples were diluted 10 times, which was acceptable due to the successful dilution integrity test.

## 4. Conclusions

The first bioanalytical assay for novel RET inhibitors selpercatinib and pralsetinib has successfully been developed and validated using LC-MS/MS. The assay includes an easy and efficient sample pre-treatment using only 10- $\mu\text{l}$  sample volumes. Results showed that accuracy, precision and stability values at all levels and matrices were within the requirements of current guidelines [6,7]. Furthermore, no significant matrix effects or extraction losses were observed, and the drugs were selectively detected in all studied matrices. The LC-MS/MS method was successfully applied for a pilot study in mice and will be used in further mouse studies.

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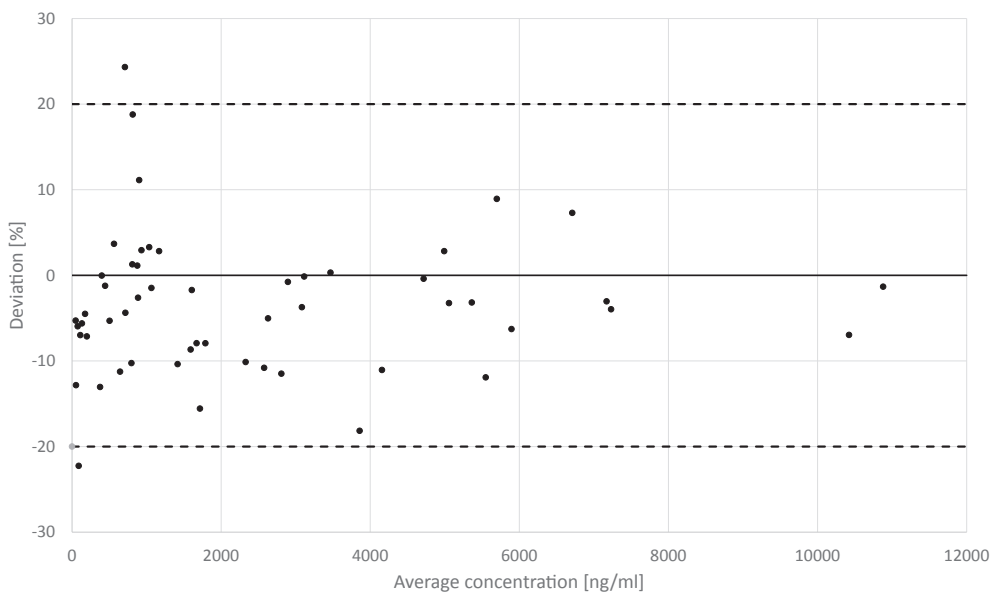


Fig. 3. Bland-Altman plot representing reanalysis of 54 mouse samples of all 9 different matrices.

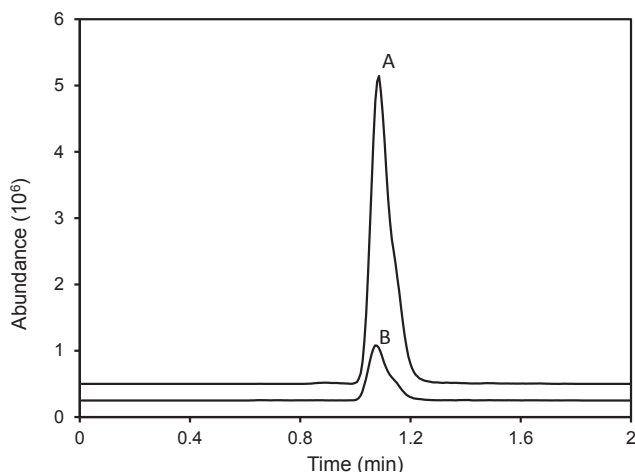


Fig. 4. SRM Chromatograms of selpercatinib in (A) plasma, 2437 ng/ml, after 10-fold dilution and (B) brain, 48.0 ng/ml, of a male mouse 4 h after oral administration of 10 mg/kg selpercatinib. Signals were given an artificial offset.

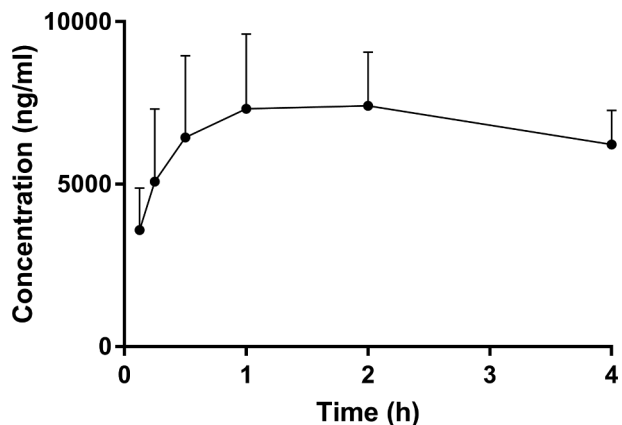


Fig. 5. Plasma concentration-time curve of selpercatinib in male FVB/NRj mice during 4 h after oral administration of 10 mg/kg selpercatinib (n = 6).

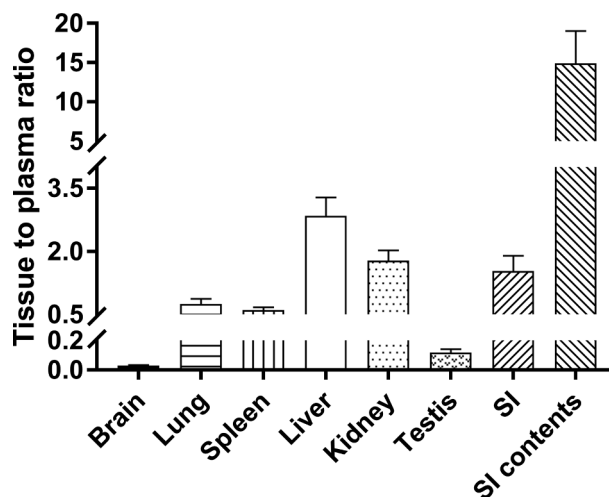


Fig. 6. Tissue concentration-to-plasma ratios (ng/g to ng/ml) of selpercatinib in male FVB/NRj mice (n = 6) for 8 tissues, 4 h after oral administration of 10 mg/kg selpercatinib.

**CRedit authorship contribution statement**

**Rahime Şentürk:** Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Visualization, Project administration. **Yaogeng Wang:** Formal analysis, Investigation, Writing - review & editing, Visualization. **Alfred H. Schinkel:** Conceptualization, Resources, Writing - review & editing, Supervision. **Jos H. Beijnen:** Conceptualization, Resources, Writing - review & editing, Supervision. **Rolf W. Sparidans:** Conceptualization, Methodology, Validation, Investigation, Resources, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration.

**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.

## Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jchromb.2020.122131>.

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