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Short communication

Bioanalytical assay for the novel TRK inhibitor selitrectinib in mouse plasma and tissue homogenates using liquid chromatography-tandem mass spectrometry

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ABSTRACT

Selitrectinib is a next generation tropomyosin receptor kinase (TRK) inhibitor developed to overcome acquired resistance to first generation TRK inhibitors. The drug is a cyclic analogue of larotrectinib. An existing bioanalytical assay for larotrectinib was therefore redesigned for selitrectinib. The assay used liquid chromatography-electrospray tandem mass spectrometry in positive selected reaction monitoring mode. Mouse plasma and tissue homogenates of brain, heart, kidney, liver, lung, small intestine, spleen, and testis were pretreated using acetonitrile protein precipitation with larotrectinib added as internal standard. Successful validation using current guidelines was obtained in the range 0.5–1000 ng/ml. Precision was within 5–12% and accuracy within 91–108% for all matrices investigated. The drug was stable in all matrices under the relevant storage conditions. Pharmacokinetics and tissue distribution of selitrectinib were monitored in a pilot study in mice demonstrating the applicability of the presented assay.

1. Introduction

In several adult and pediatric tumor types neurotrophin tropomyosin receptor kinase (*NTRK*) gene fusions can be involved as oncogenic drivers. The *NTRK1*, *NTRK2* and *NTRK3* genes encode the neurotrophin receptors TRKA, TRKB and TRKC, respectively [1]. Histology-agnostic efficacy was shown by larotrectinib, a selective TRK tyrosine kinase inhibitor (TKI) in patients with TRK fusion–positive cancers [2]. Although high response rates by first generation TRK inhibitors were obtained, acquired resistance to these drugs, larotrectinib and entrectinib, eventually leads to therapeutic failures [1,2]. Selitrectinib (LOXO-195, Fig. 1), a novel macrocyclic compound analogous to larotrectinib, was specifically designed through structural modeling to overcome the solvent front substitutions that promote acquired resistance to larotrectinib and other TRK inhibitors. Selitrectinib showed promising activity against these mutations in enzyme and cell-based assays and also in *in vivo* tumor models [2]. Moreover, two patients with TRK fusion-positive cancers who developed solvent front substitution-mediated acquired resistance to larotrectinib were treated with selitrectinib. Both patients got rapid tumor responses and the overall duration of disease control was extended.

A bioanalytical assay for selitrectinib was used previously by Drilon et al. [2] but any details of the method were not reported. Larotrectinib is the linear analogue of selitrectinib. Therefore, we decided to redesign our existing assay for larotrectinib [3] in the development of a bioanalytical method for selitrectinib. Mouse plasma as well as eight tissue homogenates (brain, heart, kidney, liver, lung, small intestine, spleen, and testis) was subjected to validation of the assay using protein precipitation and liquid chromatography – tandem mass spectrometry (LC-MS/MS). The applicability of the assay has been demonstrated in a pharmacokinetic experiment with selitrectinib in mice.

Abbreviations: LC-MS/MS, liquid chromatography-tandem mass spectrometry; LLOQ, lower limit of quantification; NTRK, neurotrophic tropomyosin receptor kinase; QC, quality control; R^2, coefficient of determination; SD, standard deviation; SRM, selected reaction monitoring

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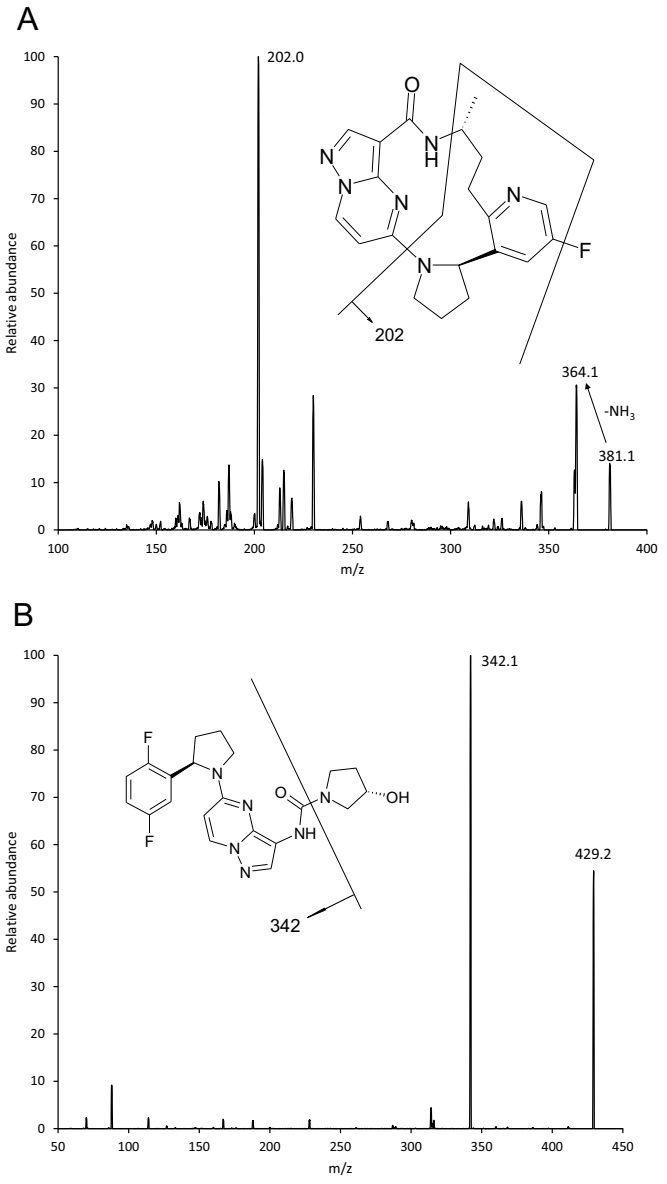

Fig. 1. Chemical structures and product spectra formed by collision induced dissociation of the protonated molecules of (A) selitrectinib (*m/z* 381.1 @ −30 V) and (B) larotrectinib (*m/z* 429.2 @ −24 V). Identities of the monitored products are indicated.

2. Materials and methods

2.1. Chemicals and reagents

Selitrectinib (LOXO-195; > 99.5%) was obtained from ChemieTek (Indianapolis, IN, USA) and larotrectinib sulphate (> 99%) from Carbosynth (Compton, Berkshire, UK). Acetonitrile (HPLS-S), methanol (HPLC), and water (ULC-MS) were supplied by Biosolve (Valkenswaard, The Netherlands). Plasma was provided by Sera Laboratories (Haywards Heath, West Sussex, UK) [3]. Blank homogenized mouse organs were prepared in ice-cold 4% (w/v) bovine serum albumin in water using the FastPrep-24™ 5G instrument (M.P. Biomedicals, Santa Ana, CA, USA) for 1 min. The volumes of albumin solution used were 3 ml for a liver and small intestine, 2 ml for two kidneys, and 1 ml for brain, hart, two lungs, two testes and spleen [3].

2.2. Analytical instruments

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

2.3. LC-MS/MS conditions

An Acquity UPLC® BEH C18 column (30 × 2.1 mm, d_p = 1.7 µm, Waters, Milford, USA) and the corresponding VanGuard pre-column (5 × 2.1 mm, Waters) were used for chromatographic separation at 40 °C. From the samples in the 96-well plate stored at 4 °C in the sample rack, 10-µl partial-loop injections were transferred to the column. Details for the linear 1-min gradient from 40 to 60% methanol in water (containing 0.1% formic acid) and for the optimization of the positive electrospray and SRM conditions have been reported previously [3]. ESI settings for the new assay were: 5000 V spray voltage; 50, 4, and 55 (arbitrary units) nitrogen sheath, ion sweep, and aux gasses; 400 and 300 °C vaporizer and capillary temperatures; no skimmer off set, and a 1.5 mTorr argon collision pressure. SRM parameters were a 110 V tube lens off set with *m/z* 381.1 → 202.0; 364.1 @ −30; −21 V collision energies for selitrectinib and a 105 V tube lens off set with *m/z* 429.2 → 342.1 @ −24 V collision energy for the internal standard (IS) larotrectinib. Dwell times all were 100 ms and mass resolutions of both separating quadrupoles were 0.7 FWHH (unit resolution).

2.4. Stock and working solutions

Selitrectinib stock solutions were prepared at 0.5 and 1 mg/ml in methanol, for larotrectinib (as free base) the concentrations were 0.2 and 0.5 mg/ml. Stock solutions of selitrectinib were diluted to 20 µg/ml working solutions in 50% (v/v) methanol in water while larotrectinib was diluted to 50 ng/ml in acetonitrile and to be used as the precipitation solution.

2.5. Sample pre-treatment

Ten-µl samples (plasma or tissue homogenate) were precipitated with 20 µl of the precipitation solution (50 ng/ml IS in acetonitrile), other details were as reported previously for larotrectinib sample pretreatment [3].

2.6. Bioanalytical method validation

The assay was submitted to full validation for mouse lithium heparin plasma and to partial validation for eight mouse tissue homogenates (brain, heart, kidney, liver, lung, small intestine, spleen, and testis) according to current FDA [4] and EMA guidelines [5].

2.6.1. Calibration

One selitrectinib working solution was diluted to 1000 ng/ml in mouse plasma (pooled from females) and stored in small portions at −30 °C. Lower calibration levels (500; 100; 50; 10; 5; 1; 0.5 ng/ml) were obtained by daily dilution of the highest level and all calibration samples were processed in duplicate together with an additional blank sample and zero calibrator. Least squared regression in a second degree polynomial (quadratic) was used for calibration using the ratio of the peak areas (selitrectinib/IS) as function of the concentration.

2.6.2. Precision, accuracy and selectivity

The second selitrectinib working solution was diluted to five QC-samples at 2000 (dilution), 800 (high), 40 (medium), 1.2 (low) and 0.5

(LLOQ) ng/ml in pooled mouse plasma. The QC-dilution sample was diluted 10-fold (10 µl sample with 90 µl blank pooled human lithium heparin plasma) prior to further treatment for each individual sample. Tissue homogenate QC samples from the eight different organs were prepared at the medium level (40 ng/ml) for each organ homogenate. Intra- and inter-day precision and accuracy were assessed for all QC samples in three separate runs containing 6 replicates of each sample.

Mouse plasma (n = 6) and tissue homogenate samples (n = 20; 3 kidney, liver, lung, small intestine, and lung; 2 brain and spleen; 1 heart) from different animals were analyzed twice to assess selectivity; once as a blank (no selitrectinib, no IS) and once as a LLOQ spiked sample (with IS).

2.6.3. Recovery and matrix effect

Recoveries and matrix effects of selitrectinib in mouse plasma were assessed using the previously reported procedures [3] using pre- and post-treatment spiked QC samples and corresponding academic solutions. Relative matrix effects at high and low QC levels were determined for the same plasma and tissue homogenate samples used for the selectivity experiments.

2.6.4. Stability and incurred sample reanalysis

Stability of high and low QC levels of selitrectinib (each n = 4) in mouse plasma were investigated under different conditions: (A) bench top (24 h ambient); (B) freeze-thaw (3 additional cycles); (C) long term (2 months at −30 °C). Stability in tissue homogenates was assessed for bench top conditions (6 h ambient) for all eight organs (each n = 4). In addition, extracts of QC samples (n = 4 for high, medium and low levels) and plasma extracts from study samples (n = 24) were reinjected after 48 h storage at 4 °C together with freshly prepared calibration samples to test autosampler stability.

Pilot study samples of plasma and six tissue homogenates (brain, kidney, liver, small intestine, spleen, and testis) were reanalyzed (n = 6 for each matrix) within a few days to assess incurred sample reanalysis.

2.7. Pharmacokinetics and tissue distribution in mice

2.7.1. Mouse treatment

A pilot study with selitrectinib was conducted in wild-type male mice (FVB/NRj genetic background). Selitrectinib was administered orally at 10 mg/kg orally (n = 6). The drug was dissolved in dimethyl sulfoxide (DMSO) at concentration 50 mg/ml and diluted with 10 mM hydrochloric acid to yield a concentration of 1 mg/ml selitrectinib and 2% (v/v) DMSO. Mice were housed and handled according to institutional guidelines complying with Dutch legislation and treated similar to earlier reported protocols [6]. Shortly, mice were 11 to 13 weeks of age and housed in a temperature-controlled environment with a 12-h light/12-h dark cycle. Animals were administered a standard diet and acidified water *ad libitum* and they were fasted for 2–3 h before treatment with the drug. Selitrectinib was administered by gavage into the stomach, using a blunt-ended needle. At 7.5, 15, 30 min, 1, and 2 h after administration, blood was collected from the tail vein in heparinized capillary tubes (Sarstedt, Germany). After 4 h, isoflurane was used to narcotize the mice and a final blood sample was obtained with cardiac puncture. In addition, organs (brain, kidney, liver, small intestinal tissue, spleen, testis) were quickly removed after sacrificing the animals at 4 h, weighed, and homogenized as described in Section 2.1. Plasma was acquired by centrifugation at 9000 ×*g* for 6 min at 4 °C and was stored at −30 °C. Mouse plasma samples were diluted 1:10 using human lithium heparin plasma before further sample treatment and analysis.

2.7.2. Pharmacokinetic calculations

Maximal plasma concentrations (C_{max}) as well as times to reach the maxima (t_{max}) were calculated from the highest concentrations of each animal, the half-life ($t_{1/2}$) was calculated from $C_{0.125}$ to C_1. The area under the plasma concentration-time curve until 4 h ($AUC_{0\rightarrow4}$) was calculated using the trapezoidal rule.

3. Results and discussion

3.1. Method development

Because a sensitive bioanalytical assay for the linear analogue of selitrectinib, larotrectinib, was developed recently [3], properties of this assay were transferred to the new assay as far as possible. The method used small 10-µl samples in a 96-well format. Chromatographic conditions and column were identical while ESI conditions and SRM setting were optimized for selitrectinib. The linear analogue larotrectinib was used as internal standard because a stable isotopically labeled analogue was not available, and further sample treatment in a high-throughput 96-well format was identical to the existing assay. Product spectra of selitrectinib and larotrectinib are shown in Fig. 1.

3.2. Validation

Based on supplemental data [2], the peak concentrations of selitrectinib in two patients were in the range of 143–800 ng/ml at a dose from 20 to 100 mg BID. Thus, the validated concentration range (0.5–1000 ng/ml) is pharmacologically relevant. To date, there is not a lot of publicly available information about the pharmacokinetics of selitrectinib in mice. Oral bioavailability was investigated in mice using 10 mg/kg selitrectinib [2]. Based on this information and experience in our group, we also adopted 10 mg/kg in the pilot pharmacokinetic study in mice.

To facilitate quantification of drug levels in all sample types the assay was validated at the lowest concentrations and in the largest range possible (0.5–1000 ng/ml) on the present instrument using current international guidelines [4,5] with full validation for mouse plasma and partial validation for tissue homogenates of this species. LLOQ levels on mass basis (ng/g), for tissues prior to homogenization, are reported in Table 1 and representative chromatograms are shown in Fig. 2.

3.2.1. Calibration

Deviation from linearity was only observed at the highest concentrations, therefore a second degree polynomial function ($y = A + B{\cdot}x + C{\cdot}x^2$) was used for calibration with parameters A (intercept), B (slope) and C (quadratic factor), and *y* being selitrectinib peak area relative to IS and *x* the concentration in ng/ml. Least squares regression parameters (mean (± SD); n = 7) were: $y = 0.62(\pm 0.76) + 0.0292(\pm 0.0030){\cdot}x - 5.7(\pm 1.9){\cdot}10^{-6}{\cdot}x^2$ with R^2 (coefficient of determination) = 0.995(± 0.003). Average deviations from de target values were all ≤2.4% supporting the applicability of the selected model.

Table 1
Assay performance data (n = 18, 3 days), stability (n = 4), expressed as recovery after 6 h at ambient temperature, of selitrectinib in tissue homogenates at 40 ng/ml, and the quantification limit (approximately) in tissue before homogenization.

Tissue	Intra-day precision (%)	Inter-day precision (%)	Accuracy (%)	Recovery (%)	LLOQ (ng/g)
Brain	4.8	5.5	101.4	108.3 ± 2.5	1.5
Heart	5.4	5.9	93.7	101.0 ± 9.1	5
Kidney	7.8	7.9	106.4	90.6 ± 5.3	3
Liver	5.1	5.4	93.3	103.5 ± 2.9	1.5
Lung	5.7	7.3	103.1	93.1 ± 2.4	4
Small intestine	5.0	6.3	107.3	93.1 ± 2.4	2
Spleen	6.7	6.8	95.0	98.8 ± 4.1	5
Testis	10.7	11.4	94.5	106.0 ± 1.0	3

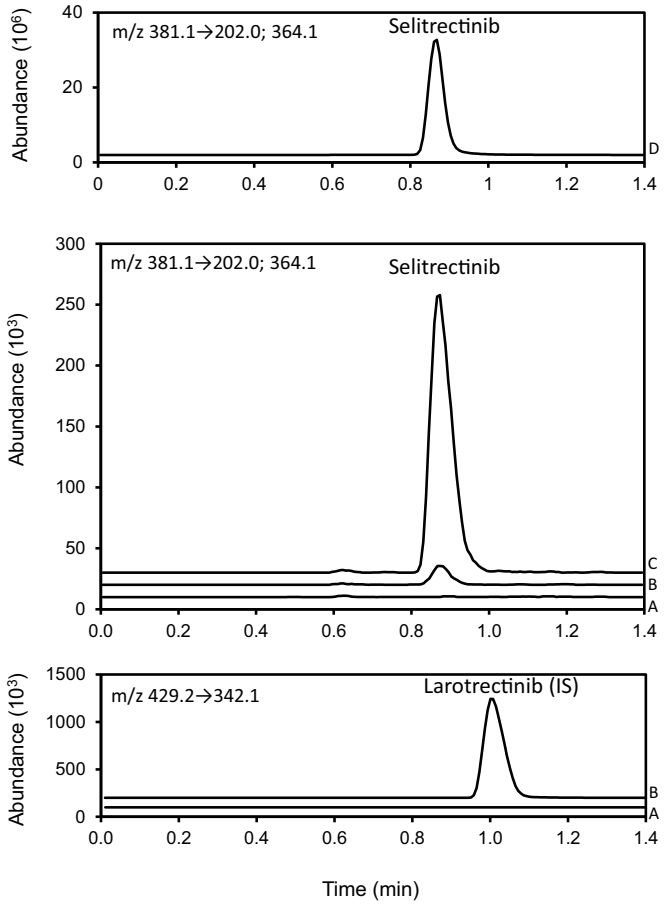

Fig. 2. SRM chromatograms of selitrectinib and larotrectinib in mouse matrix. (A) Blank plasma sample; (B) LLOQ plasma sample; (C) brain homogenate sample 4 h after administration of 10 mg/kg selitrectinib to a wild type mouse (6.9 ng/ml) and (D) the corresponding 4-h plasma sample (916 ng/ml).

3.2.2. Precision, accuracy and selectivity

Parameters for four concentrations in mouse plasma and one concentration in diluted plasma and eight tissue homogenates are reported in Tables 1 and 2, all precision and accuracy values were within ± 15% deviation as required by the guidelines [4,5].

Responses of selitrectinib in blank samples (n = 26) were ≤5% of the LLOQ level (0.5 ng/ml), while IS responses were ≤1% of the normal response. For LLOQ spiked samples back calculated concentrations were 0.532 ± 0.040 ng/ml in mouse plasma (n = 6) and 0.489 ± 0.071 ng/ml in tissue homogenates (n = 20), demonstrating sufficient selectivity of the present assay for all nine matrices.

3.2.3. Recovery and matrix effects

Extraction losses from plasma could not be observed (calculated average recoveries ≥100%), as well as any significant matrix effects (85–98%), similar to the previous larotrectinib assay [3]. Relative matrix effects at high (107.1 ± 4.4%; 99.9 ± 5.6%) and low (109.6 ± 6.3%; 101.9 ± 9.4%) QC levels in plasma (n = 6) and tissue homogenates (n = 20), respectively, were absent as well. These results also facilitate the accurate assessment of the selitrectinib levels in all matrices by using plasma calibration only.

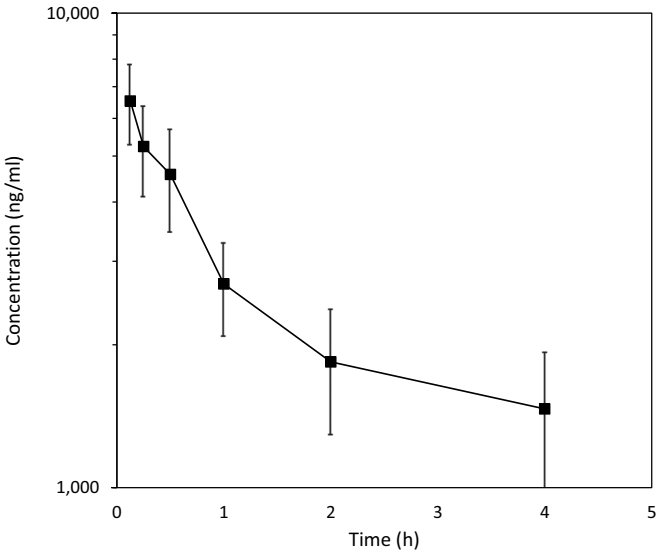

Fig. 3. Semi-log plot of plasma pharmacokinetics of selitrectinib in wild-type male mice (n = 6; mean ± SD) after oral administration of 10 mg/kg of the drug.

3.2.4. Stability and incurred sample reanalysis

Drug recoveries from biological matrices after using different storage conditions and in different matrices are reported in Tables 1 and 2. No significant losses could be observed with average recoveries in the range 90–109%. Reinjection of diluted extracts after 48 h at 4 °C using fresh calibration samples resulted in relative differences of 8.0 ± 8.5% for QC samples (n = 12) and 1.9 ± 10.0% for plasma study samples (n = 24). So, any stability issues for selitrectinib in this assay seem to be absent.

Reanalysis of study samples for seven matrices (n = 6 each) resulted in only three differences exceeding 20% for the individual samples, one for liver, spleen and testis homogenate samples each. Overall, the failure rate (3 out of 42) was far below 33% as required [5].

3.3. Selitrectinib in mice

Pharmacokinetic parameters were calculated for the drug after oral administration in wild-type mice (t_{max} = 0.17 ± 0.06 h, C_{max} = 6623 ± 1153 ng/ml, $t_{1/2}$ (0.125–1 h) = 0.82 ± 0.36 h, $AUC_{0\rightarrow4}$ = 9754 ± 1521 ng·h·ml^{-1}). The drug was absorbed very fast, seemed moderately distributed, with drug levels a decade higher compared to larotrectinib [3], and showed fast initial elimination.

Table 2
Assay performance data (n = 18, 3 days) and stability under different conditions at high and low levels only for selitrectinib in mouse lithium heparin plasma.

Level	Intra-day precision	Inter-day precision	Accuracy	Stability (expressed as recovery (%))		
(ng/ml)	(%)	(%)	(%)	3 freeze-thaw cycles	24 h at ambient temperature	2 months at −30 °C
2000[a]	12.3	13.1	99.6			
800	2.9	3.6	101.4	92.7 ± 3.1	96.9 ± 9.3	101.6 ± 2.0
40	2.7	2.9	98.3			
1.2	7.2	8.0	93.1	104.0 ± 10.8	109.0 ± 3.3	98.3 ± 9.3
0.5	10.4	11.1	87.4			

[a] 5-Fold diluted with human plasma.

However, the kinetic curve shown in Fig. 3 demonstrates slower elimination after 1 h. Therefore, one-compartment modeling was not possible and only non-compartmental data were reported.

Tissue distribution (tissue-plasma concentration ratios) of selitrectinib was assessed. Drug distribution decreases in the order small intestine (0.56 ± 0.20) > liver (0.51 ± 0.06) > kidney (0.34 ± 0.05) > spleen (0.082 ± 0.017) > testis (0.053 ± 0.007) > brain (0.021 ± 0.004). These data suggest that selitrectinib shows very poor penetration into tissues, especially for brain and testes with efficient tissue-to-blood barriers. The multidrug efflux transporters, such as P-glycoprotein (MDR1; ABCB1) and breast cancer resistance protein (BCRP; ABCG2), in these barriers may be actively pumping selitrectinib out of tissues, leading to low tissue distributions. Although the brain-to-plasma ratios were relatively low, due to markedly higher selitrectinib plasma concentration, the absolute concentrations of the drug in brain still were much higher compared to larotrectinib [3], which might be due to the macrocyclic structure of selitrectinib.

4. Conclusions

This assay for selitrectinib is the first validated bioanalytical method published for this new TRK inhibiting drug. Validated parameters all met current international guidelines [4,5]. The method can be used for 9 mouse matrices, using only 10-µl sample volumes and will be applied for further studies in this species.

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