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Validated LC-MS/MS method for simultaneous quantification of KRAS^{G12C} inhibitor sotorasib and its major circulating metabolite (M24) in mouse matrices and its application in a mouse pharmacokinetic study

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ABSTRACT

We have successfully developed and validated a bioanalytical assay using liquid chromatography tandem mass spectrometry to simultaneously quantify the first approved KRAS^{G12C} inhibitor sotorasib and its major circulating metabolite (M24) in various mouse matrices. M24 was synthesized in-house via low-pH hydrolysis. We utilized a fast and efficient protein precipitation method in a 96-well plate format to extract both analytes from biological matrices. Erlotinib was selected as the internal standard in this assay. Gradient elution using methanol and 0.1 % formic acid in water (v/v) was applied on an Acquity UPLC BEH C18 column to separate all analytes. Sotorasib, M24, and erlotinib were detected with a triple quadrupole mass spectrometer in positive electrospray ionization in multiple reaction monitoring mode. During the validation and sample quantification, a linear calibration range was observed for both sotorasib and M24 in a range of 4 – 4000 nM and 1 – 1000 nM, respectively. The % bias and %CV (both intra- and inter-day) for all tested levels in all investigated matrices were lower than 15 % as required by the guidelines. Sotorasib had a rather short room temperature stability in mouse plasma for up to 8 h compared to M24 which was stable up to 16 h at room temperature. This method has been successfully applied to measure sotorasib and M24 in several mouse matrices from three different mouse strains. We can conclude that the plasma exposure of sotorasib in mice is limited via human CYP3A4- and mouse Cyp3a-mediated metabolism of sotorasib into M24.

1. Introduction

Sotorasib, previously known as AMG510, is the first-in-class KRAS^{G12C} inhibitor and has been granted an accelerated market authorization by U.S. FDA (United States Food and Drug Administration) and EMA (European Medicines Agency) to treat non-small cell lung carcinoma (NSCLC) patients harboring the KRAS^{G12C} mutation in 2021 and 2022, respectively [1,2]. Sotorasib showed promising results in phase I/II clinical studies as targeted monotherapy or as a combination with another therapy (Code BreaK 100 study) and is currently under further clinical investigation to compare its efficacy to single-agent docetaxel (Code BreaK 200) [1].

Recently, additional research has been performed in mouse models to provide deeper knowledge of the pharmacokinetics of sotorasib. From the current information, sotorasib is known to be rapidly absorbed in mice, and its plasma exposure is limited by the CYP3A4 enzyme [3].

Abbreviations: ANOVA, one way analysis of variance; AUC, Area Under Curve; BEH, Bridged Ethyl Hybrid; BSA, Bovine Serum Albumin; C_{max}, peak plasma concentration; EMA, European Medicine Agency; EU, European Union; FVB, Friend Virus B-Type; IS, Internal standard; KRAS, Kirsten Rat Sarcoma; LC, Liquid Chromatography; LC-MS/MS, Liquid Chromatography tandem Mass Spectrometry; LLoQ, Lower Limit of Quantification; MF, Matrix Factor; MS, Mass Spectrometry; M_w, molecular weight; NSCCL, Non-small Cell Lung Carcinoma; PP, Protein precipitation; QC, Quality Control; RAS, Rat Sarcoma; TCKI, Targeted Covalent Kinase Inhibitor; T_{max}, time to achieve C_{max}; US FDA, United States Food and Drug Agency.

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It was reported that M10 (cysteine conjugate of sotorasib), M18 (hydroxy-sotorasib), and M24 (des[methylpiperazinylpropenone (MPPO)]-sotorasib dione) were the circulating metabolites in human, rat, and dog [4,5]. Dahal et al. [6] described that M12 (glutathione conjugate), M10, and M24 were major circulating metabolites in rats, whereas in dog the major circulating metabolites were M10 and M24. Vuu and co-workers [7] described that M10 and M24 were observed in human circulation after oral administration of [^{14}C]-sotorasib. Utilizing human liver S9, they showed that M10 was formed via multistep biotransformation involving the non-enzymatic formation of M12 followed by a GGT-mediated reaction resulting in M10. They also demonstrated that M18 and M24 were formed via oxidative metabolism by primarily CYP2C8 and CYP3A enzymes respectively, through pooled human liver microsomes experiments [7]. Among the mentioned metabolites of sotorasib, M10 and M24 are consistently present as the major circulating metabolites across different species.

However, information on the quantitative level of the major circulating metabolite of sotorasib has not been available to date. While there are some validated bioanalytical methods to quantify sotorasib both for preclinical [8,9] and clinical study samples [10], a reliable quantification method for any metabolites has not been available yet. According to our preliminary investigation, only M24 and not M10 showed a significant response in mouse plasma samples and several tissues. Therefore, we wanted to develop and validate a bioanalytical method to provide quantitative data on the major circulating metabolite M24 in several mouse matrices. We also aimed to provide more insight into M24 pharmacokinetics, utilizing samples from a previous preclinical study [3].

2. Material and methods

2.1. Chemical and reagents

Sotorasib (Mw = 560.59 g/mol, >98 %) was purchased from Carbosynth (Compton, Berkshire, UK), while the major metabolite of sotorasib (M24) was synthesized in house at Utrecht University. Erlotinib (Mw = 393.44 g/mol, as hydrochloric acid, > 99 %) used as the internal standard of the analytical method was supplied by Sequoia Research Products (Pangbourne, UK). Analytical grade formic acid (98–100 %) and analytical grade hydrochloric acid fuming (37 %) were both acquired from Merck Darmstadt, Germany. Water (ULC-MS grade), acetonitrile (HPLC grade), and methanol (HPLC grade) were obtained from Biosolve (Valkenswaard, the Netherlands). Pooled CD-1 mouse lithium heparin plasma (mixed gender) and pooled human lithium heparin plasma (mixed gender) were provided by BioIVT (West Sussex, UK).

2.2. M24 formation

The major circulating metabolite of sotorasib, M24, was synthesized on an analytical scale in our lab at Utrecht University. M24 was produced via an incubation of 1 mM sotorasib in a low pH environment. Shortly, a 100 μL of 3.6 mM of sotorasib was added to 260 μL 1 M HCl in water. This solution was then incubated in a shaking water bath for 20 h. The temperature of the shaking water bath was maintained at 40 °C at 50 rpm during the incubation period. The solution was then measured for the residual amount of sotorasib with LC-MS/MS to investigate the yield of the reaction. The identity of the metabolite was additionally ensured with high-resolution mass spectrometry LTQ-Orbitrap XL (Thermo Fisher Scientific, Massachusetts, USA).

2.3. Analytical instruments

The chromatography system used for the separation was a Nexera-X2 UPLC with two LC-30 AD pumps, an inline DGU-250 AR degasser, a Sil30ACmp autosampler, and a CTO-20 AC column oven (Shimadzu, Kyoto, Japan). For the detection a triple quadrupole mass spectrometer QTRAP® 5500 (AB SCIEX, Ontario, Canada), equipped with a Turbo Ion V™ Turbo Ion Spray® and an inlet valve, were utilized. Analyst 1.6.2. software was used for instrument control and data collection, while all LC-MS/MS data were processed with MultiQuant 3.0.1 software. The software used was provided by Sciex. The identification check of synthesized metabolite was performed with a high-resolution linear trap quadrupole-orbitrap mass spectrometer LTQ-Orbitrap XL (Thermo Fisher Scientific, Massachusetts, USA).

2.4. LC-MS/MS conditions

Partial-loop injection (4 μL) was applied on an Acquity UPLC® BEH C18 column (30 × 2.1 mm, d_p = 1.7 μm, Waters, Milford, MA, USA) protected with UPLC® BEH C18 Vanguard pre-column (5 × 2.1 mm, d_p = 1.7 μm, Waters). During the analytical run, the temperature of the column and autosampler were maintained at 40 °C and 4 °C, respectively. The chromatographic eluent consists of 0.1 % formic acid in water (v/v)–methanol (1:1, v/v). To separate all the analytes, a gradient elution program at a flow rate of 0.6 ml/min as follows is used: 0 – 1.50 min, 35 – 70 % B; 1.51 – 1.85 min, 100 % B; 1.86 – 2.30 min, 35 %B. The whole eluate was transferred to the ionization interface between 0.5 and 1.8 min after the injection by switching the MS divert valve. Selected reaction monitoring (SRM) in a positive mode was utilized as the detection mode. The optimized parameters were curtain gas 20 psi, ion spray voltage 1500 V, source temperature 700 °C, ion source gas (1) 60 psi, and ion source gas (2) 75 psi. These parameters were obtained via a direct introduction of 1000 nM of M24 at 5 μL/min in methanol–formic acid 0.1 % (v/v) in water (1:1, v/v) into the mass spectrometer. Individual parameters of sotorasib, M24, and erlotinib as the internal standard are listed in Table 1.

2.5. Stock and working solutions

A stock solution of 2.54 mM IS was prepared by dissolving 500–700 μg of erlotinib with methanol. A working solution of 254 μM erlotinib was obtained by diluting the stock IS with 50 % (v/v) methanol in water. Further, a serial dilution of the working solution was performed to obtain 254 nM and 25.4 nM of erlotinib in acetonitrile. The 25.4 nM of erlotinib in acetonitrile was used as daily IS and precipitating solution.

A solution of 3.6 mM sotorasib (stock A) was prepared by dissolving 1.3 – 1.4 mg of sotorasib in methanol. A 100 μL of stock A was then diluted with aqueous HCl 1 M to obtain 1 mM sotorasib (n = 2) and was further incubated (stock B) to obtain ~ 1 mM M24 A working solution containing both sotorasib (80,000 nM) and its metabolite M24 (20,000 nM) was then prepared from the stock A and stock B (n = 2) with methanol. These two working solutions were used further to prepare calibration and quality control (QC) samples in mouse plasma independently. All solutions were stored in the freezer at −30 °C.

2.6. Standard solutions and quality control

The highest calibration sample was prepared by diluting the first working solution (80,000 nM sotorasib, 20,000 nM M24) to 4000 nM

Table 1
The parameters of the individual SRM settings of sotorasib, M24, and erlotinib.

Compound	Q1 (*m/z*)	Q3 (*m/z*)	DP (V)	CE (V)	CXP (V)
Sotorasib	561.2	134.0*	71	49	8
		317.0	71	57	18
M24	425.1	134.1*	181	43	8
		292.0	181	41	14
Erlotinib	394.1	278.1*	50	47	12

DP: declustering potential, CE: collision energy, CXP: collision cell exit potential.
* Used for quantification

sotorasib and 1000 nM M24 with pooled blank mouse plasma in a polypropylene tube. Until further use, this sample was stored at −30 °C in aliquots. The highest calibration sample was diluted further for daily calibration to 2000, 400, 200, 40, 20, 8, 4 nM of sotorasib and 500, 100, 50, 10, 5, 2, 1 nM of M24 in blank pooled mouse plasma. QC samples were prepared from the second working solution (80,000 nM sotorasib, 20,000 nM M24 via a sequential dilution to QC-high (3200 nM sotorasib, 800 nM M24), QC-high-med (1000 nM sotorasib, 250 nM M24), QC-med (160 nM sotorasib, 40 nM M24), QC-low (10 nM sotorasib, 2.5 nM M24) and QC-lloq (lower limit of quantification, 4 nM sotorasib, 1 nM M24) in pooled blank mouse plasma. QC-med (160 nM sotorasib, 40 nM M24) samples were also prepared in pooled mouse tissue homogenates of brain, liver, kidney, spleen, small intestines, small intestinal content, and lung.

2.7. Sample preparation

A protein precipitation was utilized to treat all preclinical samples. In short, ten μL of plasma sample or tissue homogenate was pipetted into a 200 μL polypropylene 96-well plate with a conical bottom, and 20 μL of IS solution was added to these samples. The 96-well plate was then closed with a silicone mat and briefly shaken with a vortex mixer. Then, the plate was centrifuged (Heraeus Multifuge-3 SR, Kendro Laboratory Products, Hanau, Germany) at 3500 x g and 20 °C for 5 min, and 20 μL of the supernatant was transferred into a 96-deep well polypropylene plate with a 1 ml round bottom. After adding 200 μL of 25 % (v/v) methanol in water, the deep well plate was mixed gently. The prepared plate was placed in the autosampler and was ready for injection. Finally, 4 μL of the final sample was injected into the analytical column for quantification.

2.8. Tissue homogenization

The blank and preclinical sample of mouse tissue homogenates were prepared by mixing the whole harvested organ (weighed) with 2 % (w/v) of bovine serum albumin (BSA) in milli-Q water in an iced condition. For this homogenization, a FastPrep-24™ 5 G instrument (M.P Biomedicals, Santa Ana, USA) was utilized. The volumes of BSA used for every organ were as follows: 1 ml of BSA was used for the brain, spleen, and lung; 2 ml of BSA was added for kidney and small intestinal content; 3 ml of BSA was used for the liver and small intestines.

2.9. Bioanalytical method validation

Full validation was performed on mouse plasma, while partial validation was conducted for all tissue homogenates in the range of 4 – 4000 nM of sotorasib and 1 – 1000 nM of M24. The latest United States Food and Drug Administration (US FDA) and European Medicines Agency (EMA) guidelines on bioanalytical method validation [11,12] were used as the validation framework.

2.9.1. Calibration

Calibration samples, along with an additional blank sample (IS only) and a double blank sample (no analyte, no IS), were prepared in pooled lithium heparin mouse plasma. These samples were prepared for each daily use in duplicate (n = 20). A linear regression with the reversed square of the concentration ($1/x^2$) as the weighting factor was utilized to define the calibration curve using the area ratio of the analyte/IS against analyte concentration.

2.9.2. Precision and accuracy

The assay performance, defined as accuracy and precision, was assessed at five different concentrations (QC-high, QC-high-med, QC-med, QC-low, QC-lloq) for mouse plasma and only at one concentration (QC-med) for mouse tissue homogenates. Precision and accuracy were performed in sextuple analysis in three independent runs (n = 18 per QC level). In addition, dilution integrity was investigated by diluting 10,000 nM sotorasib and 2500 nM M24 in mouse plasma with dilution factors of 5-fold and 11-fold in human plasma (n = 6 for each dilution factor on three different days).

2.9.3. Selectivity and carry-over

Six individual mouse plasma and 28 individual tissue homogenates (4 individual samples for each tissue) were processed to determine the selectivity of the assay. Each sample was investigated at LLoQ level (individual matrices spiked with 4 nM sotorasib and 1 nM M24) and double blank (no analytes, no IS). The carry-over was investigated by injecting the blank samples after the injection of the highest level of the calibration samples.

2.9.4. Recovery and matrix effect

Three different samples at three different QC levels, QC-high, QC-med, and QC-low (n = 4 for each QC level), were prepared to investigate the recovery and matrix effect. The first sample was a normal plasma sample (A) treated as stated in the sample preparation step (Section 2.7). A similar sample as A in which the analyte was added after the extraction step (B) and samples without any matrix constituents (C) were the second and the third samples, respectively. Recovery was calculated from the ratio A/B, while the matrix effect was the ratio of B/C. The relative matrix effect was evaluated using the same matrix used to determine the selectivity: 6 individual mouse plasma and 28 individual tissue homogenates. These samples were prepared at QC-high and QC-low levels. The relative matrix effect was calculated by comparing their response to the reference solution (without the presence of any matrix) at the same level.

2.9.5. Stability

The stability of sotorasib and M24 in mouse plasma was investigated in both QC-high and QC-low levels (n = 4 for each QC level). Quadruplicate analysis of these samples was performed following several exposure conditions, namely room temperature (22 °C) for 6 h, at −30 °C for two months, and at −30 °C interrupted by three freeze-thaw cycles (thawing at room temperature for ± 1 h and freezing again at least for 20 h). Additional room temperature stability of sotorasib and its major metabolite for both QC levels were investigated after 2, 4, 6, 8, and 16 h after exposure to ambient temperature. The stability of sotorasib and M24 in several tissue homogenates was investigated in QC-med level exposure to room temperature for 6 h.

The stability of the working solution (containing both sotorasib and M24) in methanol was assessed after the exposure to room temperature with the presence of the light for 6 h and after 3 months of storage at −30 °C. The response of these solutions was compared to the response of freshly prepared working solutions at the same level.

An analytical run was reinjected and reanalyzed after 7 days of storage at 4 °C to assess the stability of the extract.

2.9.6. Incurred samples reanalysis

A reanalysis of twenty-seven samples from the mouse study (n = 6 for plasma, n = 3 for each investigated tissue homogenate) was performed three weeks after the first measurement. In between the two analysis, these samples were stored in the freezer at - 80 °C.

2.10. Preclinical studies

2.10.1. Mouse treatment

The preclinical study used female mice with ages between 8 and 16 weeks. We utilized some sets of samples from a previously published preclinical study of sotorasib [3]. The specific sample set chosen for this investigation was wild-type (FVB/NRj), Cyp3a knockout ($Cyp3a^{-/-}$), and transgenic overexpression of human CYP3A4 in liver and small intestine (Cyp3aXAV) mice. Since M24 has been reported as CYP3A-mediated metabolite in vitro, these specific mouse strains were selected to

further investigate the effect of both mouse Cyp3a and human CYP3A4 enzymes on the pharmacokinetic profile of both sotorasib and M24. In short, the mice were orally dosed with 20 mg/kg body weight of sotorasib after they were fasted for 2 – 3 h. Their blood was then withdrawn from the tail vein at 0.125, 0.25, 0.5, 1, and 2 h after drug administration. Four hours after sotorasib administration, the mice were anesthetized with isoflurane to collect the final blood samples by cardiac puncture. Finally, the mice were sacrificed by cervical dislocation, and the tissues of interest (brain, liver, spleen, kidney, small intestines, and lungs) were immediately harvested. Small intestinal content was also collected during organ harvesting. The housing and handling of the mice followed the institutional guidelines of the Netherlands Cancer Institute and complied with the Dutch and EU legislation. Plasma samples were obtained from withdrawn blood via centrifugation at 9000 *g* for 6 min at 4 °C. All the harvested organs were prepared according to Section 2.8. Prior to homogenization with 2 % (w/v) BSA, small intestines and lungs were first rinsed with saline. All samples were stored at −30 °C before further quantification. All the plasma samples were diluted 5 times, while small intestines and small intestinal contents homogenates were diluted 11 times with human lithium heparin plasma before quantitative analysis. The remaining biological matrices were directly prepared according to Section 2.7.

2.10.2. Data processing

Since we have reported the pharmacokinetic profile of sotorasib in our previous publication [3], these data were not recalculated. As for M24, only the area under the curve (AUC), the peak plasma concentration (C_{max}), and the time of peak plasma concentration (T_{max}) parameter is reported here. The AUC was calculated according to the linear trapezoidal rule without extrapolation to infinity using Microsoft Excel, while the C_{max} and T_{max} were assessed from the raw data. The comparison of AUC and peak plasma concentration for both sotorasib and M24 among three different mouse strains were also performed. One-way analysis of variance (ANOVA) was used to compare those parameters. Sidak's post hoc correction was applied to account for multiple comparisons. All the statistical analyses and graphs were performed and made with GraphPad Prism 9 (GraphPad Software, La Jolla, CA). A significant difference was reported when $P < 0.05$.

3. Results and discussion

There are several quantification methods to determine sotorasib in different biological matrices [8–10]. However, to the best of our knowledge, this is the first validated bioanalytical method for the simultaneous quantification of sotorasib and its major metabolite, M24, in several mouse matrices. This quantitative assay can analyze a small volume of sample with less than 3 min total of analytical run time, emphasizing the suitability of this method to support further preclinical studies.

3.1. Method development

Prior to the method development, we screened some samples from a mouse study (plasma, liver, small intestines, and small intestinal content, n = 2 for each matrix) for the presence of possible metabolites. Our results showed there are several metabolites detected in mouse, in which M24 has the highest response (more than 10 % of sotorasib response) in all investigated matrices (Fig S1.1). In contrast to previous studies in different species, we did not observe a considerable response of M10 (less than or equal to 1 % of sotorasib's response) either in mouse plasma or other tissue homogenates in our preclinical samples (Fig S1.1) [4,6,7]. Owing to this fact, we decided to only quantify M24 further in preclinical samples and provide the pharmacokinetic profile of this metabolite. In addition, we found that there are three peaks detected in the mass transition of hydroxy-sotorasib in mouse metabolizing organs (Fig S1.1). The presence of three peaks of hydroxy-sotorasib was possibly caused by the hydroxylation or oxidation that occurs in three different regions of sotorasib, resulting in three different metabolites with a different lipophilicity. However, further investigation of hydroxy-sotorasib was not conducted due to their quite low responses (Fig S1.1). Furthermore, it has been reported that the pharmacodynamic activity of one of the identified hydroxy-sotorasib entities (M18) is considerably lower than that of sotorasib [4]. While M24 has been reported not to show any of the pharmacologic effect of sotorasib [4], the high metabolism rate of sotorasib into M24 will decrease the plasma exposure of sotorasib. Therefore, it can decrease the effectiveness of the drug.

Since the M24 metabolite is not commercially available to date, we decided to self-synthesize this compound via hydrolysis at low pH. During a pilot experiment, we found that sotorasib can be chemically hydrolyzed utilizing extreme pH conditions (pH lower than 2 and pH higher than 12). We also observed that after 8, 12, and 24 h of the incubation period of sotorasib in HCl 1 M, the response of M24 remained stable (data not shown). A twenty-hour incubation of sotorasib was selected for practical reasons (overnight incubation). After the incubation, only around 1 % of sotorasib remained, indicating virtual completion of the hydrolysis reaction. In addition, other forms of degradation were not observed with LC-UV identification at $\lambda = 254$ nm (data not shown). This information obtained related to the non-enzymatic hydrolysis of sotorasib in an extreme pH environment may affect the fate of this drug inside the stomach.

An identity check of M24 was performed with LTQ-Orbitrap mass spectrometry. The mass error of the observed protonated M24 was 1.88 ppm, suggesting that the synthesized compound has the expected mass of M24 (Table S2.1).

The parameters of electrospray ionization in positive mode were optimized to obtain the highest response of the single protonated M24 (*m/z* 425.1), while the electrospray ionization condition for the single protonated sotorasib (*m/z* 561.2) has been published in our prior publication [9]. Fig. 1 shows the product spectrum of both single protonated sotorasib (A) and M24 (B) obtained by triple quadrupole mass spectrometry. In addition, a high-resolution product spectrum of a single protonated M24 and the proposed dissociation pathway are provided as supplementary data (Fig S2.3, Table S2.2). Two product masses of M24 (*m/z* 134.1 and 292.0) and two product masses of sotorasib (*m/z* 134.0 and 317.0) were optimized during the development stage. However, only product masses *m/z* 134.1 and 134.0 were used for the quantification of M24 and sotorasib, respectively.

The chromatographic separation on the analytical C18 column was developed and optimized empirically based on MS response, peak shape, and its retention time. The use of water acidified with 0.1 % formic acid and methanol in the chromatographic eluent has been proven reliable for sotorasib quantitative determination [9] and it provided a good peak shape and separation for both sotorasib and M24. Further, erlotinib was used as the internal standard and was monitored at *m/z* 394.1 → 278.1 following the previous publication [9,13]. One analytical study used a stable isotope labeled sotorasib (self-synthesized) as an internal standard [10]. However, the proven reliability of erlotinib as an internal standard offers the opportunity to develop a quantification method utilizing a commonly available compound instead of using a rather expensive stable isotope labeled compound. The final proportion of 0.1 % (v/v) formic acid in water and methanol as the eluent provides a satisfactory chromatographical separation of the analytes and IS with a total run time of 2.3 min. This proportion of mobile phase eluted all the compounds on the selected column within 1.5 min, as illustrated by Fig. 2.

A fast and straightforward protein precipitation technique with acetonitrile as the organic solvent was selected to extract both analytes from the mouse matrices. This technique has been proven capable and reliable in extracting sotorasib in many preclinical study samples before. In the current study, we showed the suitability of this technique to extract both sotorasib and its M24 in mouse matrices.

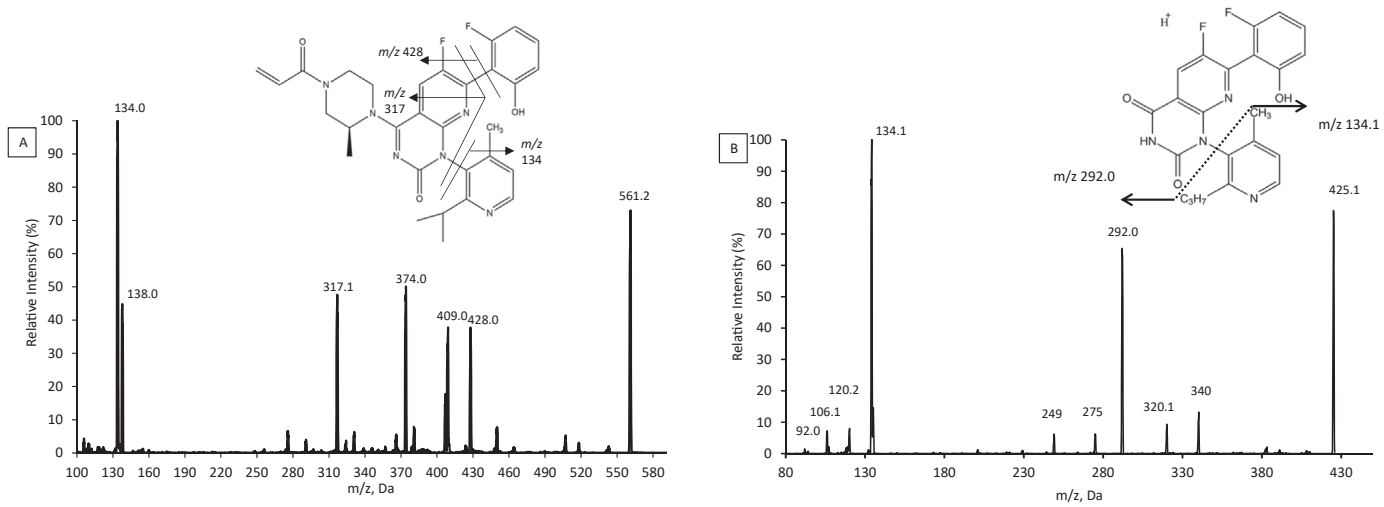

Fig. 1. Chemical structures of sotorasib (A) and M24 (B) and the product ion mass spectra formed by collision-induced dissociation of protonated sotorasib (A, *m/z* 561.2 @ 49 V) and M24 (B, *m/z* 425.1 @ 40 V) with their expected fragments.

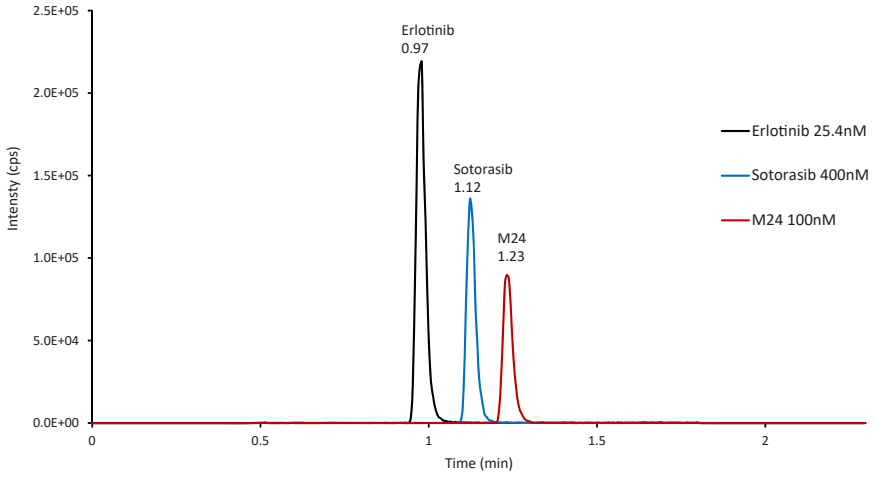

Fig. 2. Representative chromatogram of a mouse plasma sample containing erlotinib (*394.1 → 278.1*) 10 ng/ml, sotorasib (*561.2 → 134.0*) 400 nM, and M24 (*425.1 → 134.0*) 100 nM.

The calibration range of 4 – 4000 nM of sotorasib was chosen because this range has proven sufficient for previous preclinical studies [9], while the 1 – 1000 nM range of M24 was selected because of the expected lower concentration of M24 compared to the parent compound in preclinical samples.

3.2. Method validation

3.2.1. Calibration

A linear trend has been demonstrated by the relative response of both sotorasib M24 over the selected range. Therefore, the linear regression equation with a typical equation of $Y = a \cdot X + b$ was used for the quantification. Y denotes the area ratio of analyte to erlotinib as IS, X defines the concentration of the analyte in nM, a is the slope, and b denotes the intercept. The regression coefficient was determined by the R value. The inverse square of the analyte's concentration is used as the weighting factor. We calculated the average calibration equation of eight consecutive analytical runs as $Y = 0.00164 (\pm 0.00013) X + 0.00071 (\pm 0.00023)$ with $R = 0.99846 (\pm 0.00091)$ for sotorasib and $Y = 0.00475 (\pm 0.00032) X + 0.00111 (\pm 0.00072)$ with $R = 0.99838 (\pm 0.00069)$ for its metabolite M24.

3.2.2. Precision and accuracy

The detailed data of method performance on mouse plasma at five different QC levels is demonstrated in Table 2, in which %CV determines the precision while %bias defines the accuracy of the method. All calculated %CV and %bias in all investigated QC levels in all three independent runs for both sotorasib and M24 had a value of less than 15 %. These results confirm the reliability of this method to produce accurate and precise data when used to measure both sotorasib and M24 in mouse plasma matrices.

The performance of the method was also investigated in different mouse tissue matrices at QC-medium level as part of the partial validation of this method. In addition, we assessed the performance of the method when sample dilution is needed for future measurement. Dilution factors of 5-fold and 11-fold from mouse plasma samples with a concentration above the highest calibration level were selected and assessed for three independent runs. The results of these investigations are reported in Table 3 as %bias and %CV (both intra- and inter-day). The data shows that all tested tissue-related matrices and selected dilution factors have less than 7 % of %CV (both intra- and inter-day) and less than 13 % of %bias for both analytes. These data confirmed that the performance of this method is fulfilling the requirements of both US FDA and EMA guidelines [11,12].

Table 2
Detailed data on method performance in mouse plasma.

Day	Statistic	Sotorasib					M24				
		LLoQ 4 nM	QC-low 10 nM	QC-medium 160 nM	QC-med high 1000 nM	QC high 3200 nM	LLoQ 1 nM	QC-low 2.5 nM	QC-medium 40 nM	QC-med high 250 nM	QC high 800 nM
1	Intraday mean (n = 6)	3.90	9.35	157.5	921.7	3131	1.04	2.42	40.26	233.2	800.3
	%CV	9.6 %	6.1 %	2.1 %	5.7 %	3.2 %	10.4 %	4.1 %	1.6 %	6.8 %	2.5 %
	%Bias	-2.5 %	-6.5 %	-1.6 %	-7.8 %	-2.2 %	4.1 %	-3.0 %	0.7 %	-6.7 %	0.0 %
2	Intraday mean (n = 6)	3.62	9.50	159.5	939.6	3082	1.02	2.39	40.98	237.8	801.0
	%CV	8.5 %	4.8 %	3.1 %	3.4 %	3.5 %	11.4 %	5.7 %	2.7 %	2.8 %	2.5 %
	%Bias	-9.5 %	-5.0 %	-0.3 %	-6.0 %	-3.7 %	1.5 %	-4.3 %	2.5 %	-4.9 %	0.1 %
3	Intraday mean (n = 6)	3.61	9.22	154.6	893.3	2982	0.86	2.28	38.19	222.7	755.0
	%CV	6.1 %	4.0 %	4.1 %	1.2 %	2.1 %	3.3 %	4.5 %	0.028	1.0 %	2.2 %
	%Bias	-9.7 %	-7.8 %	-3.4 %	-10.7 %	-6.8 %	-14.4 %	-8.8 %	-4.5 %	-10.9 %	-5.6 %
1–3	Interday mean (n = 18)	3.71	9.36	157.2	918.2	3065	0.97	2.37	39.81	231.2	785.4
	%CV	8.6 %	4.9 %	3.3 %	4.3 %	3.5 %	12.5 %	5.3 %	3.8 %	4.9 %	3.6 %
	%Bias	-7.2 %	-6.4 %	-1.8 %	-8.2 %	-4.2 %	-2.9 %	-5.4 %	-0.5 %	-7.5 %	-1.8 %

LLoQ = lower limit of quantification, QC = quality control, CV = coefficient of variation.

Table 3
The method performance on mouse tissue-related matrices at QC-medium level and dilution integrity in human plasma at 10,000 nM of sotorasib and 2500 nM of M24.

Matrix	Sotorasib				M24			
	Conc. (nM)	%Bias	%CV (intraday)	%CV (interday)	Conc. (nM)	%Bias	%CV (intraday)	%CV (interday)
Brain homogenates	160	6.5	6.0	6.5	40	8.6	5.8	6.4
Lung homogenates	160	0.9	2.8	4.9	40	3.5	2.9	5.1
Liver homogenates	160	6.9	5.9	6.6	40	8.5	6.1	6.7
Spleen homogenates	160	5.8	3.5	4.9	40	12.1	2.9	3.0
Kidney homogenates	160	8.6	3.3	6.3	40	10.4	3.5	4.6
SI homogenates	160	6.7	4.7	4.6	40	10.4	4.5	5.4
SIC homogenates	160	3.8	4.0	4.3	40	4.2	4.9	5.1
5-fold dilution with human plasma	2000	-0.2	2.2	5.4	500	2.1	2.3	4.3
11-fold dilution with human plasma	909	0.8	3.2	5.2	227	2.9	3.1	5.2

Conc. = concentration, SI = small intestines, SIC = small intestinal content

3.2.3. Selectivity and carry-over

There was no interfering co-eluting peak that was higher than 20 % of LLoQ response observed in all individual blank matrices at the retention time of both sotorasib and M24, with neither a co-eluting peak of erlotinib exceeding 5 % of its normal response. These results fulfilled the required guidelines [11,12]. Moreover, all the spiked samples at the LLoQ level in all individual matrices fulfilled the guidelines requirement [11,12] of 80 – 120 % accuracy, as depicted in Table 4, indicating that this method is capable of quantifying sotorasib and M24 down to 4 nM and 1 nM, respectively. These results also show that the developed method is selective enough to quantify both analytes without any interferences from the matrix variability. We found that the response of M24 was slightly over 20 % of LLoQ response when blank samples were injected after the highest calibration sample, while we did not observe such a result for sotorasib. However, the slightly over-requirement response of M24 decreased below 20 % after the second blank injection. Therefore, at least two blank samples were injected after an injection of known high-concentration samples.

3.2.4. Recovery and matrix effect

The extraction recovery for sotorasib and M24 was investigated in QC-high, -med, and -low plasma samples. The obtained recovery (mean ± SD) was 100.7 ± 3.1 %, 94.5 ± 3.5 %, and 100.8 ± 2.5 %, respectively, for sotorasib. The observed recovery for M24 in the same order was 102.0 ± 2.3 %, 86.7 ± 3.6 %, and 100.6 ± 4.5 %, while the recovery for the IS was 97.8 ± 4.0 %. Since there is no minimum requirement for extraction recovery, its standard deviation confirmed the reproducibility of the selected protein precipitation method as the sample pretreatment in this procedure [11,12].

Further, the matrix effect of pooled mouse plasma matrix tested at QC-high, -med, and -low was 95.4 ± 5.0 %, 101.8 ± 5.8 %, and 100.0 ± 2.8 %, respectively, for sotorasib, and 96.1 ± 4.4 %, 110.0 ± 6.1 %, and 102.3 ± 5.5 %, respectively for M24. To elaborate more on the matrix effect investigation, individual matrices were investigated at QC-high and -low levels. The IS-normalized matrix factor (MF) was calculated to assess the relative matrix effect. The data are presented in Table 5. According to Table 5, the MF of all investigated matrices ranged from 0.9 to 1.1, showing that there is no significant matrix effect;

Table 4
Selectivity data of all individual matrices at LLoQ level for sotorasib (4 nM) and M24 (1 nM).

Matrix	Sotorasib		M24		n
	mean measured concentration (nM)	Accuracy (%)	mean measured concentration (nM)	Accuracy (%)	
Plasma	4.089	102.2 %	0.934	93.4 %	6
Brain homogenates	3.829	95.7 %	1.030	103.0 %	4
Liver homogenates	3.952	98.8 %	1.099	109.9 %	4
Spleen homogenates	3.718	92.9 %	1.114	111.4 %	4
Kidney homogenates	4.028	100.7 %	1.109	110.9 %	4
Small intestines homogenates	4.416	110.4 %	1.143	114.3 %	4
Small intestinal content homogenates	3.913	97.8 %	0.974	97.4 %	4
Lung homogenates	3.820	95.5 %	1.030	103.0 %	4

Table 5
The relative matrix effect of individual mouse matrices.

Matrix	Sotorasib			M24			n
	Conc. (nM)	mean IS-normalized matrix factor (MF)	% CV	Conc. (nM)	mean IS-normalized matrix factor (MF)	% CV	
Plasma	3200	1.013	0.3 %	800	0.984	1.6 %	6
	10	1.086	5.6 %	2.5	1.063	3.4 %	6
Brain homogenates	3200	1.010	1.4 %	800	0.995	2.4 %	4
	10	1.066	5.5 %	2.5	1.036	4.6 %	4
Liver homogenates	3200	1.016	2.5 %	800	1.013	2.4 %	4
	10	1.067	5.0 %	2.5	1.054	7.2 %	4
Spleen homogenates	3200	1.018	1.5 %	800	1.005	2.3 %	4
	10	1.016	1.7 %	2.5	1.050	5.5 %	4
Kidney homogenates	3200	1.006	2.0 %	800	0.988	1.3 %	4
	10	1.073	1.8 %	2.5	1.037	3.7 %	4
Small intestines homogenates	3200	1.049	2.2 %	800	1.045	1.9 %	4
	10	1.054	4.5 %	2.5	1.109	0.8 %	4
Small intestinal content homogenates	3200	1.067	1.5 %	800	1.073	1.7 %	4
	10	1.106	3.2 %	2.5	1.056	1.8 %	4
Lung homogenates	3200	0.995	1.1 %	800	0.979	1.8 %	4
	10	1.055	5.6 %	2.5	1.025	5.4 %	4

Conc. = concentration, CV = coefficient of variation

therefore, neither matrix enhancement nor matrix suppression will interfere with the measurement of either sotorasib or M24 in investigated matrices.

3.2.5. Stability

The stability of sotorasib and its major circulating metabolite M24 in plasma and investigated mouse tissue homogenates is illustrated in Table 6. Sotorasib and its metabolite were proven stable under all tested conditions. We have shown in our previous publication that sotorasib has a rather short stability in mouse plasma under room temperature conditions [9]. Therefore, we exposed plasma samples containing both sotorasib and its metabolite to room temperature for several different exposure periods. The result of this investigation is illustrated in Fig. 3. This figure shows that sotorasib is stable for up to 6 h during room temperature exposure, however, recovery was slightly lower than 85 % when the sample was exposed to room temperature for 8 h. We obtained similar stability data as in our previous publication [9]. On the other hand, M24 was more stable than sotorasib. It was stable up to 16 h when the plasma sample was exposed to room temperature. These stability differences may be caused by the presence of the acrylamide moiety in sotorasib. Previously, we hypothesized that the reactive acrylamide group may affect the short stability of sotorasib at room temperature [9]. In contrast to sotorasib, M24 does not have the reactive acrylamide moiety in its structure (Fig. 1). Therefore, the longer stability data of M24 compared to sotorasib at room temperature in this study are in line with our previous hypothesis.

3.2.6. Incurred samples reanalysis

We reanalyzed 27 samples out of 234 total preclinical samples three weeks after the initial measurement. The Bald-Altman graph in Fig. 4 illustrates the obtained data. This figure shows that six out of 27 samples (22 %) had more than 20 % differences from the average concentration of sotorasib, and only two out of 27 samples (7 %) exceeded 20 % differences from the average concentration of M24. These numbers are lower than the permitted number by the guidelines (33 % of the total sample is allowed to have 20 % deviation from the average concentration) [11,12]. The better performance of the metabolite may be attributed to its better stability in plasma. In addition, samples that showed deviation from the average concentration of M24 during the reanalysis were not the same samples yielding deviation from the average concentration of sotorasib, suggesting that the deviated result was not caused by back metabolism during the storage.

3.3. Preclinical study results

The pharmacokinetic parameters, including the plasma concentration versus time curve, C_{max}, and AUC for both sotorasib and M24, are presented in Fig. 5. The T_{max} of sotorasib was between 15 and 30 min, while for M24 it was between 1 and 2 h in all strains. The plasma concentration of sotorasib was the highest in the $Cyp3a^{-/-}$ mouse strain, while the plasma concentration of M24 in this mouse strain was the lowest (Fig. 5A, 5B, 5D & 5E). Compared to wild-type mice, the plasma exposure of sotorasib in $Cyp3a^{-/-}$ mouse was significantly increased (2.54-fold, $P < 0.001$), while the plasma exposure of M24 was

Table 6
Stability of sotorasib and M24 in different mouse matrices (reported as the mean of the percentage of the recovered concentration ± SD).

Matrix	Exposure condition	QC level	Sotorasib		M24	
			Conc. (nM)	Stability (%)	Conc. (nM)	Stability (%)
Mouse plasma	6 h at RT	QC-low	10	92.8 ± 7.9	2.5	99.8 ± 7.8
		QC-high	3200	89.0 ± 3.2	800	101.0 ± 2.3
	three freeze thaw cycle	QC-low	10	101.5 ± 10.3	2.5	111.2 ± 6.2
		QC-high	3200	93.4 ± 2.5	800	98.7 ± 3.5
	2 months at −30 °C	QC-low	10	114.9 ± 0.7	2.5	103.8 ± 2.7
		QC-high	3200	90.4 ± 2.3	800	103.0 ± 1.9
Brain homogenates	6 h at RT	QC-med	160	102.0 ± 3.5	40	107.5 ± 3.4
Lung homogenates	6 h at RT	QC-med	160	101.4 ± 3.2	40	109.6 ± 3.9
Liver homogenates	6 h at RT	QC-med	160	109.7 ± 1.8	40	113.3 ± 1.4
Kidney homogenates	6 h at RT	QC-med	160	106.1 ± 3.2	40	111.5 ± 4.9
Spleen homogenates	6 h at RT	QC-med	160	102.1 ± 5.2	40	107.7 ± 3.5
Small Intestines homogenates	6 h at RT	QC-med	160	104.4 ± 3.6	40	106.9 ± 2.9
Small Intestinal content homogenates	6 h at RT	QC-med	160	109.0 ± 4.1	40	112.4 ± 6.7

Conc. = concentration, h = hour, RT = room temperature

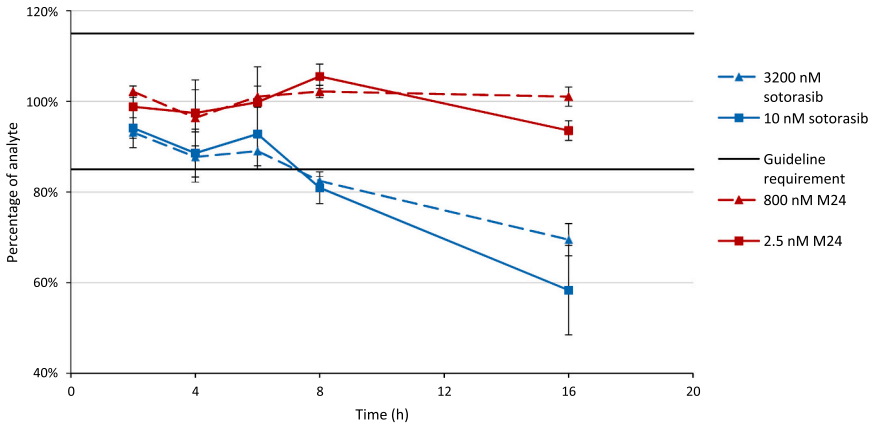

Fig. 3. Time dependent room temperature stability of both sotorasib and its major circulating metabolite (M24) in mouse plasma (reported as the average of % recovery, n = 4).

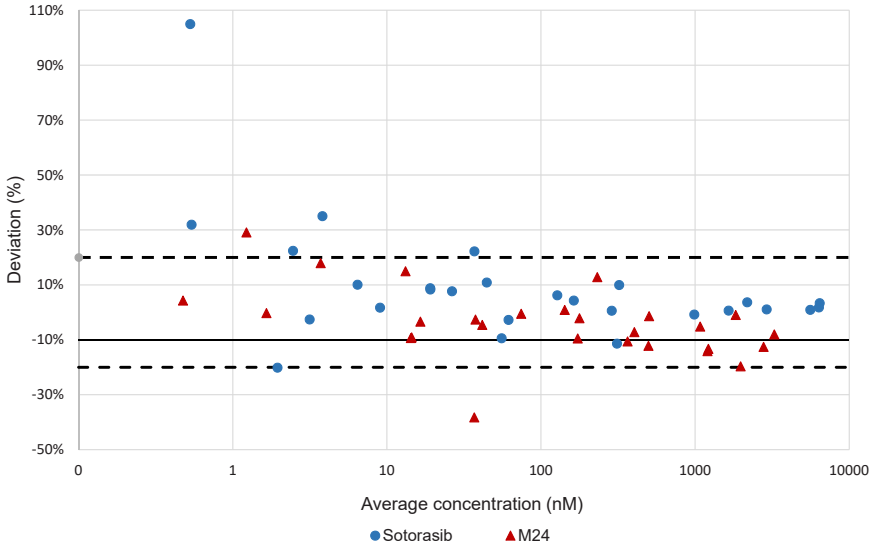

Fig. 4. The Bald-Altmann graphs illustrating the reanalysis of 27 mouse samples across eight different mouse matrices.

significantly decreased (0.01-fold, $P < 0.0001$). Conversely, the plasma exposure of sotorasib in Cyp3aXAV mouse was significantly decreased (0.26-fold, $P < 0.001$), whereas the plasma exposure of M24 in the same mouse strain was significantly increased (152-fold, $P < 0.0001$) compared to Cyp3a$^{-/-}$ mice (Fig. 5C, 5F, Table S3.1). These data clearly indicate that both mouse Cyp3a and human CYP3A4 enzymes limit the plasma exposure of sotorasib by metabolizing sotorasib into M24. These data also suggest that almost all metabolism from sotorasib to M24 is mediated by Cyp3a and CYP3A4.

A higher tissue concentration of sotorasib and lower concentration of M24 in Cyp3a$^{-/-}$ compared to the other two strains was observed (Fig. 6). These data reconfirm that M24 was formed almost exclusively via oxidative metabolism both by Cyp3a and CYP3A4 enzymes. In general, the tissue-to-plasma ratios of both sotorasib and M24 showed less pronounced differences than the tissue concentration among the three strains (Figs S4.1 – S4.7), suggesting that plasma exposure has a strong influence on the tissue concentration of sotorasib and M24. The M24-to-sotorasib ratio in all investigated tissues in different strains shows a consistently similar trend to the area under curve ($AUC_{0\rightarrow 4\ h}$) M24-to-sotasib ratio (Figs S3.1, Fig S4.1 – S4.7) These observations confirm the strong influence of plasma exposure to the tissue concentration of sotorasib and M24.

4. Conclusion

A bioanalytical method for simultaneous quantification of both sotorasib and its major circulating metabolite (M24) in mice has been developed and validated. This method has good performance in quantifying both analytes without any significant extraction loss and matrix interferences. The applicability of this method has been successfully proven for preclinical samples to support further investigation of sotorasib and M24. Lastly, we showed that sotorasib plasma and tissue exposure after an oral administration was markedly limited by both human CYP3A4 and mouse Cyp3a enzymes via its metabolism into its major circulating metabolite (M24). Inter- and intra-individual variation in CYP3A activity in patients might therefore potentially have a marked impact on the effective exposure to sotorasib, and hence its therapeutic efficacy.

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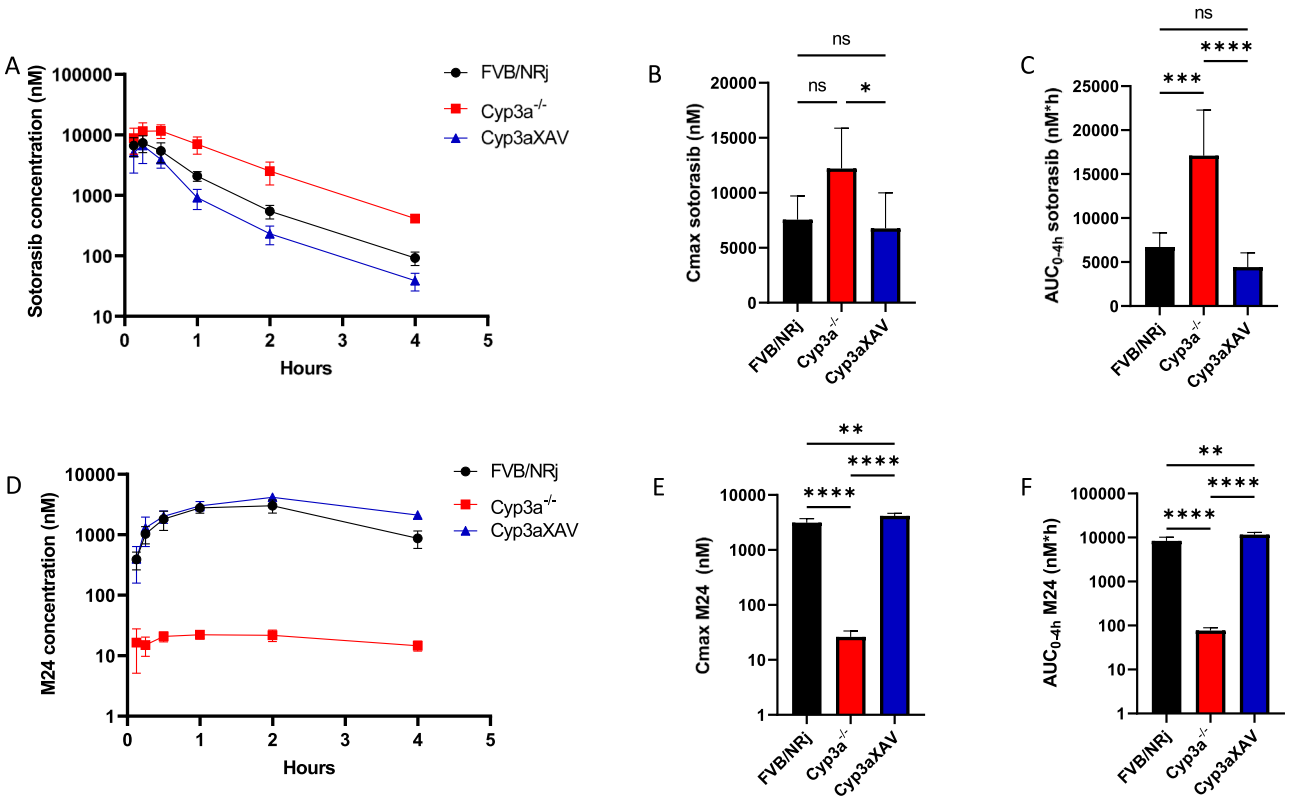

Fig. 5. Pharmacokinetic parameters of sotorasib and M24 in female wild-type (FVB/NRj), Cyp3a$^{-/-}$, and Cyp3aXAV (transgenic overexpression of human CYP3A4 in liver and small intestines) mice over 4 h after dosing with 20 mg/kg body weight sotorasib (n = 6). A = plasma concentration vs. time curve of sotorasib. B = maximum concentration of sotorasib in mouse plasma. C = AUC $_{(0-4\,h)}$, area under the plasma concentration vs. time curve of sotorasib from 0 to 4 h. D = plasma concentration vs. time curve of M24. E = maximum concentration of M24 in mouse plasma. F = AUC $_{(0-4\,h)}$, area under the plasma concentration vs. time curve of M24 from 0 to 4 h. Data are presented as mean ± SD. ns = not significant; *= P < 0,05; ** = P < 0,01; ***= P < 0001; **** = P < 0,0001.

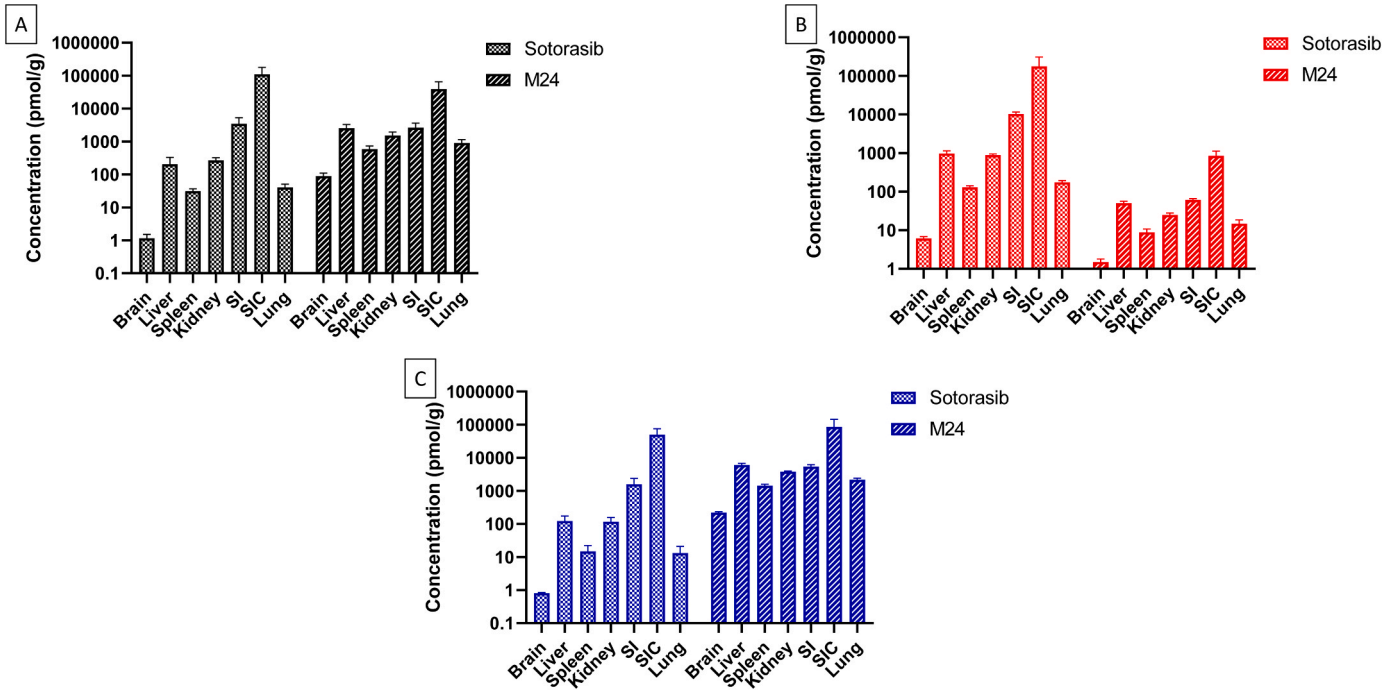

Fig. 6. Tissue distribution of sotorasib and M24 in three different mouse strains 4 h after a single oral administration of 20 mg/kg body weight sotorasib (n = 6 per strains). The concentration was plotted in logarithmic scale. A = female wild-type (FVB/NRj) mouse. B = female Cyp3a$^{-/-}$ mouse. C = female Cyp3aXAV (transgenic overexpression of human CYP3A4 in liver and small intestines) mouse. SI = small intestines, SIC = small intestines content. Data are presented as mean ± SD.

CRediT authorship contribution statement

Irene A. Retmana: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Visualization, Writing – original draft; **Nancy Loos:** Formal analysis, Investigation, Visualization, Writing – review & editing; **Alfred H. Schinkel:** Conceptualization, Resources, Writing – review & editing, Supervision; **Rolf W. Sparidans:** Conceptualization, Methodology, Investigation, Resources, Writing – review & editing, Supervision; **Jos H. Beijnen**: Conceptualization, Resources, Writing – review & editing, Supervision.

Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2023.115612.

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