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RESEARCH ARTICLE

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Development and validation of an HPLC–MS/MS method to quantify the KRAS inhibitor adagrasib in mouse plasma and tissue-related matrices

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Abstract

We developed and validated an assay utilizing a liquid chromatography–tandem mass spectrometry technique to quantify the KRAS inhibitor adagrasib in mouse plasma and seven tissue-related matrices. The straightforward protein precipitation technique was selected to extract adagrasib and the internal standard salinomycin from the matrices. Gradient elution of acetonitrile and water modified with 0.5% (v/v) ammonium hydroxide and 0.02% (v/v) acetic acid on a C_{18} column at a flow rate of 0.6 ml/min was applied to separate the analytes. Both adagrasib and salinomycin were detected with a triple quadrupole mass spectrometer with positive electrospray ionization in a selected reaction monitoring mode. A linear calibration range of 2–2,000 ng/ml of adagrasib was demonstrated during the validation. In addition, the reported precision values (intra- and inter-day) were between 3.5 and 14.9%, while the accuracy values were 85.5–111.0% for all tested levels in all investigated matrices. Adagrasib in mouse plasma was reported to have good stability at room temperature, while adagrasib in tissue-related matrices was stable on ice for up to 4 h (matrix dependent). Finally, this method was successfully applied to determine the pharmacokinetic profile and tissue distribution of adagrasib in wild-type mice.

KEYWORDS
adagrasib, bioanalysis, KRAS inhibitor, LC–MS/MS, mouse matrices

1 | INTRODUCTION

Kirsten rat sarcoma virus (KRAS) is one of the most frequently mutated oncogenes in human cancer (Bos, 1989; Matikas et al., 2017; Sanchez-Vega et al., 2018). A KRAS mutation is often found in pancreatic, colorectal and lung cancer (Jarvis, 2016). Specifically, non-small cell lung carcinoma patients with a KRAS mutation have been long associated with a poor prognosis (El Osta et al., 2019; Liu et al., 2020). Therefore, targeting the KRAS protein is a promising approach to treating these cancer patients. Despite being a promising pharmacological approach, the development of KRAS inhibitors has come a long way owing to the lack of an allosteric pocket of KRAS protein to provide a binding site for a small molecule inhibitor and the high affinity of KRAS protein in the picomolar range with its natural substrate GTP (Kwan et al., 2022). However, in 2013, some compounds that covalently and irreversibly bind to the cysteine residue of $KRAS^{G12C}$ were

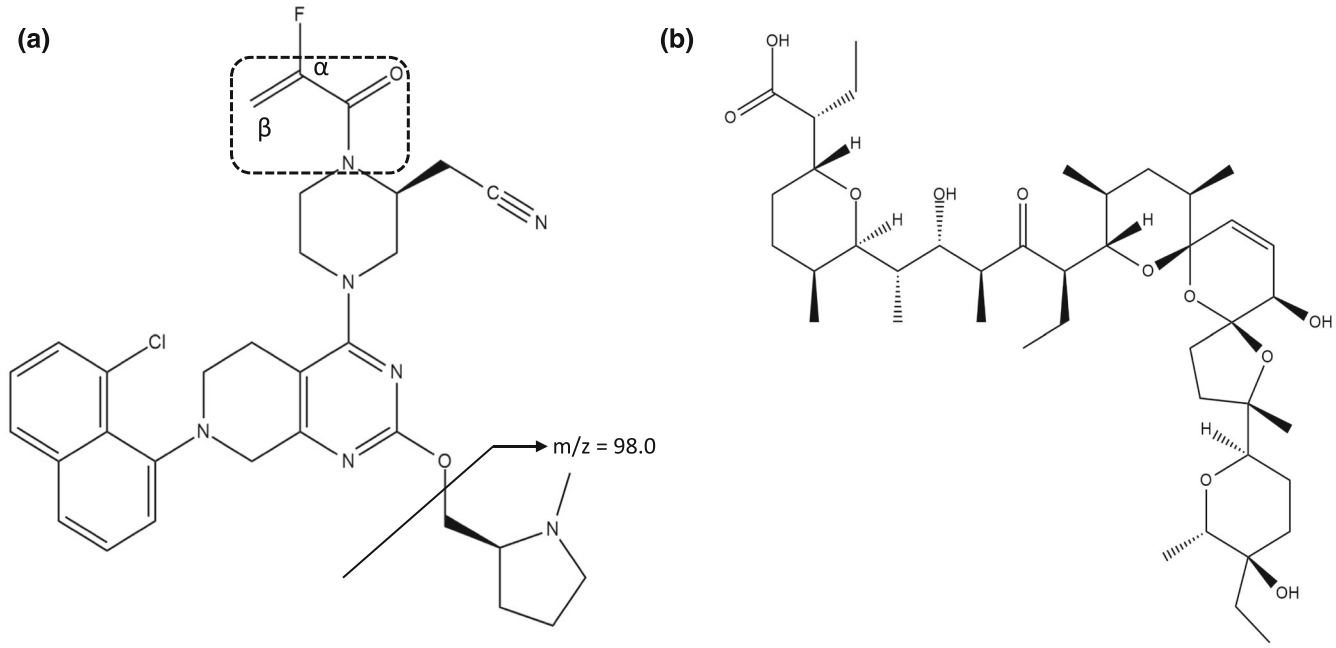

FIGURE 1 (a) The structure of adagrasib and the proposed dissociation pattern used for quantification. Moiety inside the dashed box is the acrylamide group of adagrasib. (b) The structure of salinomycin/internal standard (IS).

successfully developed, embarking the new beginning of the development of KRAS inhibitors (Ostrem et al., 2013). Moreover, using the cysteine residue in $KRAS^{G12C}$ offers the selectivity of this inhibitor to inhibit only the mutant KRAS protein.

Currently, several $KRAS^{G12C}$ inhibitor candidates are being developed and investigated. Among them, adagrasib (MRTX-849) is one of the first $KRAS^{G12C}$ inhibitors to advance to clinical investigation (Hallin et al., 2020). It is shown that the covalent bond between the acrylamide β carbon of adagrasib (Figure 1a) and Cys12 of the $KRAS^{G12C}$ protein and stabilized by some hydrogen bonds and hydrophobic interaction between these two moieties plays a role in the specificity and inhibition activity of adagrasib toward the $KRAS^{G12C}$ protein (Fell et al., 2020). According to the phase I/IB KRYSTAL-1 study, adagrasib 600 mg twice a day shows evidence of clinical activity with a manageable safety profile in patients with an advanced solid tumor harboring the $KRAS^{G12C}$ mutation (Ou et al., 2022). In addition, according to the preliminary data of the phase II study, 41% of evaluable patients with pancreatic and gastrointestinal cancer treated with adagrasib have a partial response, and the remaining 59% have stable disease (Adagrasib Moving Ahead in GI Cancers, 2022). These data show the promising clinical activity of adagrasib.

The application of cancer medication has a well-known risk of a relatively narrow therapeutic window. Moreover, individual dose adjustment depending on each patient's condition is a common practice in hospitals to guarantee the safety and efficacy of the medication (Hendrayana et al., 2017; Lucas & Martin, 2017). Thus, deeper insight into the pharmacokinetic profile at both preclinical and clinical levels is necessary. To obtain such knowledge, a reliable quantification method to determine the adagrasib concentration in biological samples is essential. To our knowledge, only one recent publication describes a validated method to quantify adagrasib in a single biological matrix, that is, rat plasma (Du et al., 2022). The mentioned method utilized protein precipitation (PP) with methanol to treat 50 µl of rat plasma in a vial format.

Therefore, we attempted to develop and to validate a new and straightforward liquid chromatography–tandem mass spectrometry (LC–MS/MS) method to quantitatively measure adagrasib in mouse plasma, six mouse tissue homogenates and small intestinal content homogenates. This method, developed for a low-volume sample of 10 µl in a 96-well plate format, will support further mouse and human studies to gain more information on the pharmacokinetic profile and tissue distribution of this promising drug.

2 | EXPERIMENTAL

2.1 | Chemicals and reagents

The reference material of adagrasib (MRTX849, 99%, MW = 604.13 g/mol) was supplied by MedKoo Bioscience (North Carolina, USA). Salinomycin (as sodium salt pentahemihydrate, MW = 818.039 g/mol) as the internal standard (IS) and ammonium hydroxide solution water (28–30% on NH_3 basis) were both purchased from Sigma-Aldrich (Seelze, Germany). Anhydrous acetic acid was obtained from Merck (Darmstadt, Germany). Acetonitrile (HPLC-S grade), methanol (HPLC-S grade) and water (ULC-MS grade) were all supplied by Biosolve (Valkenswaard, The Netherlands). Both mouse plasma (lithium heparin, pooled gender) and human plasma (lithium heparin, pooled gender) were obtained from BioIVT (West Sussex, UK).

2.2 | Tissue homogenization

The mouse tissue homogenates were prepared by mixing the whole (weighed) organ with 2% (w/v) bovine serum albumin (BSA) in Milli-Q water under iced conditions. Further, the organ and BSA solution were homogenized with a FastPrep-24TM 5G instrument (MP Biomedicals, Santa Ana, USA). The volume of BSA solution used was different for each tissue. For the brain, spleen and lung, 1 ml of BSA solution was used. Two milliliters of BSA solution was added for the kidney and small intestinal content, while 3 ml of BSA solution was used for the liver and small intestines.

2.3 | Analytical instruments

The chromatography system contained a binary ultra-high-performance system from Shimadzu Nexera X2 (Kyoto, Japan) equipped with two LC-30AD pumps, a SIL30-ACmp autosampler, a CTO-20AC column oven and a DGU-20A5R degasser. A triple quadrupole mass spectrometer AB-SCIEX QTRAP 5500 (Ontario, Canada), equipped with a Turbo Ion V™ TurboIonSpray® probe and an inlet valve, were used for the detection. The instrument control and data collection were managed using Analyst 1.6.2 software (SCIEX), while the LC–MS/MS data processing was performed by MultiQuant 3.0.1 software (SCIEX).

2.4 | LC–MS/MS conditions

The gradient elution was performed on an Acquity UPLC® BEH C_{18} column (30 × 2.1 mm, particle size [d_p] = 1.7 µm) guarded by a UPLC® BEH C_{18} Vanguard pre-column (5 × 2.1 mm, d_p = 1.7 µm). Both were provided by Waters (Milford, MA, USA). The mobile phase was a combination of 0.5% ammonium hydroxide and 0.02% acetic acid in water (v/v) as eluent A and acetonitrile as eluent B at a 0.6 ml/min flow rate. The eluent A was freshly prepared every day with a pH range of 10.5–10.7. After a 1 µl injection of the sample, the percentage of acetonitrile was increased from 60 to 75% (v/v) during 1 min, followed by flushing with 100% (v/v) acetonitrile for the next 0.4 min. Ultimately, the column was reconditioned at 60% (v/v) of B for 0.5 min before starting the next injection. The eluent was transferred into the ionization interface between 0.5 and 1.7 min during the elution. The temperature of the column and autosampler were maintained at 40 and 4°C, respectively.

The detection mode utilized the positive selected reaction monitoring (SRM) mode. The individual SRM parameters of adagrasib and salinomycin are listed in Table 1, while the general parameters were curtain gas 10 psi, ion spray voltage 1400 V, temperature 600°C, ion source gas (1) 60 psi and ion source gas (2) 60 psi. These general parameters were optimized by directly introducing 200 ng/ml of adagrasib at 5 µl/min in the mixture of 0.5% (v/v) ammonium hydroxide acidified with 0.01% (v/v) formic acid/methanol (25:75, v/v).

2.5 | Stock and working solutions

A stock solution of 0.5 mg/ml adagrasib was prepared by dissolving adagrasib in methanol. A 50,000 ng/ml working solution of adagrasib was then prepared by diluting the stock solution with 50% methanol (v/v) in water. From this working solution, the calibration samples were prepared. The second set of adagrasib stock and working solutions at the same concentration were prepared to make quality control (QC) samples. A 2.5 mg/ml stock solution of salinomycin was made by dissolving salinomycin sodium in methanol. A working solution of 100,000 ng/ml was prepared by diluting the stock solution with methanol. Further, a solution of 300 ng/ml salinomycin was made via a dilution of the working solution in acetonitrile. This solution was used daily to treat the samples.

2.6 | Calibration and QC samples

The 50,000 ng/ml adagrasib working solution was diluted with the blank lithium heparin mouse plasma to obtain 2,000 ng/ml of the highest calibration sample. Until further use, the highest calibration sample was stored at −30°C. The highest calibration sample was then diluted for the daily calibration samples to 1,000, 200, 100, 20, 10 and 2 ng/ml of adagrasib in blank mouse plasma. Quality control samples were prepared by a sequential dilution from the second set of adagrasib working solutions into 1,600 (high), 80 (medium), 4 (low) and 2 ng/ml (lower limit of quantification, LLOQ) in blank mouse plasma for daily use. Quality control samples at the medium level (80 ng/ml) were also prepared in blank pooled tissue homogenates of the brain, liver, kidney, spleen, lung, small intestine and small intestine contents. Quality controls 4,800 and 480 ng/ml were also prepared from the second adagrasib working solution to investigate the dilution integrity with human plasma.

TABLE 1 The parameter of individual SRM channels of adagrasib and salinomycin.

Compound	*m/z* (Q1)	*m/z* (Q3)	Declustering potential (V)	Collision energy (V)	Cell exit potential (V)
Adagrasib	604.2[a]	98.0	101	63	12
	604.2	70.0	101	129	10
Salinomycin	768.5[a]	733.3	156	27	30
	768.5	715.3	156	30	28
	773.5	431.2	241	69	20
	773.5	531.3	241	63	20

[a]Used for quantification and full validation.

2.7 | Sample preparation

Ten microliters of plasma or tissue homogenates sample were transferred into a polypropylene 96-well microplate with a conical bottom. Into the well, 20 µl of 300 ng/ml salinomycin in acetonitrile (IS) was added. The microplate was then closed with a silicone mat and vortex mixed briefly. Further, the microplate containing the sample was centrifuged for 5 min at 3,500***g***. Fifteen microliters of the supernatant was transferred into a polypropylene 96-deep well plate with a round bottom, followed by the addition of 300 µl of methanol 25% (v/v) to reduce the matrix effect and the elution strength of the sample. Both mouse plasma and all investigated homogenates from mouse tissue-related matrices were prepared in the same manner. The deep well plate was then gently mixed before being put in the autosampler for a chromatographic injection.

2.8 | Analytical method validation

We use guidelines on bioanalytical method validation from the European Medicines Agency (2011) (EMA) and the US Food and Drug Administration (2018) (US FDA) as the bioanalytical framework. According to both guidelines, we conducted a full validation for the mouse plasma and partial validation for the mouse tissue-related matrices with a range of 2–2,000 ng/ml of adagrasib.

2.8.1 | Calibration

All calibration samples together with the additional blank (no analyte) and double blank (no analyte and IS) samples were prepared in duplicate for each daily use ($n = 18$). The calibration curve was determined by the ratio of the analyte (adagrasib) and the IS (salinomycin) peak areas utilizing the least square linear regression with the reversed square of the concentration ($1/x^2$) as the weighting factor.

2.8.2 | Accuracy, precision and dilution integrity

Four different concentrations of adagrasib in pooled mouse plasma—QC-high (1,600 ng/ml), QC-medium (80 ng/ml), QC-low (4 ng/ml) and QC-LLOQ (2 ng/ml)—were used to assess the accuracy and precision of the developed method in mouse plasma. The QC medium was used to define the accuracy and precision of the method in seven tissue homogenates. Accuracy and precision (intra- and inter-day) determination were performed in a sextuplicate analysis for each QC level on three separate days ($n = 18$ per QC level).

In addition, the dilution integrity was investigated by diluting solutions of 4,800 and 480 ng/ml of adagrasib in mouse plasma with 51- and 11-fold factors for the former and a 6-fold factor for the latter with lithium heparin human plasma. Dilution integrity samples were also performed in sextuplicate on three different days ($n = 18$ per dilution factor).

2.8.3 | Selectivity and carryover

Six individual samples of lithium heparin mouse plasma and 28 individual samples of tissue homogenates (four individual samples for each tissue) were processed to determine the selectivity of the developed assay. Each sample was prepared as the LLOQ spiked (2 ng/ml adagrasib) and double blank (no analyte and no IS) samples.

The parameter of carryover was assessed by injecting several blank samples after injection of the highest calibration samples of adagrasib (2,000 ng/ml).

2.8.4 | Recovery and matrix effect

Three types of samples in pooled mouse plasma at three different QC levels—QC-high, QC-medium and QC-low—were prepared in four replications to assess the recovery and matrix effect of this method. The first sample (A) was treated as stated by the sample preparation step. A similar sample to sample A with the analyte added after the extraction step (B) and samples without any matrix constituent (C) were the second and third samples. The recovery was calculated from the ratio of A/B, and the ratio of B/C determined the matrix effect.

In addition, the relative matrix effect was also determined using the same samples to evaluate the selectivity: six individual plasma and 28 individual tissue homogenates samples. These samples were prepared at QC-high and QC-low levels. The relative matrix effect was calculated by comparing their responses with the reference solutions without the presence of any matrix at the same level.

2.8.5 | Stability

The stability of adagrasib in mouse plasma was investigated at the QC-high and QC-low levels, while its stability in tissue homogenates (brain, liver, kidney, spleen, small intestine, small intestine content and lung) was assessed at the QC-medium level. A quadruplicate analysis of plasma samples was performed after exposure to (1) room temperature (~22°C) for 8 h, (2) ice conditions (~10°C on the surface of the ice) for 6 h, (3) storage at −30°C interrupted by three freeze–thaw cycles (thawing at room temperature for at least 1 h, and freezing at least for 20 h), and (4) 6 months' storage at −30°C. The stability of adagrasib in pooled tissue homogenates and pooled small intestines content homogenates was assessed after exposure to ice condition for either 2 or 4 h.

The stability of the stock solutions (in methanol) and working

solutions (in 50% v/v methanol) of adagrasib was also investigated. These solutions were measured after exposure to room temperature with the presence of ambient light for 6 h and after storage at −30°C for 5 months.

A complete validation run with 24 QC samples was reinjected and reanalyzed after 4 and 7 days of storage at 4°C to investigate the stability of the extract.

2.8.6 | Incurred sample reanalysis

Fifty-six samples ($n = 14$ for plasma, $n = 6$ for each investigated tissue-related matrix, 10% of the total study sample) from an initial mouse study were reanalyzed around 1 month after initial analysis as surrogate parameter of the study sample accuracy (European Medicines Agency, 2011; US Food and Drug Administration, 2018; Subramaniam et al., 2015).

2.9 | Pharmacokinetic study in mice

2.9.1 | Mouse treatment

An initial mouse study to obtain more insight into the pharmacokinetic profile of adagrasib was performed in female wild-type mice (>99% FVB genetic background, $n = 6$). The age of the mice was between 8 and 16 weeks. The selected mice fasted for 2–3 h before oral administration of adagrasib at a dose of 30 mg/kg body weight. Subsequently, blood samples were collected from the tail vein of the mouse (~50 µl per sample) at 5, 10, 15, 30 and 60 min after the drug administration. The final blood samples were collected 2 h after the drug administration via cardiac puncture under isoflurane anesthesia. Finally, the mice were sacrificed by cervical dislocation under isoflurane anesthesia, and the tissue-related matrices of interest (brain, liver, kidney, spleen, small intestines, small intestines content and lung) were immediately collected. The housing and handling of the animal followed the institutional guidelines of the Netherlands Cancer Institute and complied with the Dutch and EU legislation.

Plasma samples for quantification were prepared from the collected blood sample via centrifugation at 9,000*g* for 6 min at 4°C. Initial rinsing with a saline buffer was performed for the small intestine and lung tissues prior to homogenization with 2% (w/v) BSA. All samples were stored at −30°C before analysis. The small intestines and small intestines content homogenates were diluted 51 times with human lithium heparinized plasma, while the rest of the matrices except the brain homogenates were diluted six or 11 times before quantitative analysis.

2.9.2 | Pharmacokinetic calculations

The pharmacokinetic parameters were calculated manually with MS Excel software and were reported as the mean ± standard deviation. The maximum plasma concentration (C_{max}) and the time to reach C_{max} (T_{max}) were calculated directly from the highest concentration and corresponding time points for each individual mouse. The elimination rate (K) and half-life ($T_{1/2}$) were calculated from the C_{max} and the last time point whenever possible. The area under the plasma concentration–time curve (AUC) was calculated using the trapezoidal rule.

3 | RESULTS AND DISCUSSION

To the best of our knowledge, this method is the first validated bioanalytical method for adagrasib in mouse plasma and several mouse tissue-related matrices to date. This method is developed with one-step and straightforward protein precipitation (PP) with acetonitrile to treat the sample within less than 3 min of total chromatography run time. Moreover, this method was developed for analyzing a small volume sample (10 µl), enhancing the suitability of the developed method to measure hundreds of low-volume samples from preclinical studies that are often performed in small rodents with a limitation of the blood volume. Our developed method provided merit over the previously published analytical method for adagrasib in rat plasma (Du et al., 2022) because the previous study only refers to plasma analysis while in this new assay we incorporated seven mouse tissue-related matrices in addition to mouse plasma.

3.1 | Development

Electrospray ionization in positive mode was optimized to obtain the highest response of the single protonated adagrasib (m/z 604.2). A product spectrum of the single protonated adagrasib obtained by the optimized triple quadrupole mass spectrometry is depicted in Figure 2a. The optimized product masses were m/z 98.0 and 70.0, but only m/z 98.0 was used for quantifying adagrasib owing to its higher response and lower background noise than the product mass. The fragmentation pathway of the adagrasib quantification transition is shown in Figure 1.

The chromatographic method was optimized empirically based on the MS response, peak shape and retention time. The selected eluent of the developed method was acetonitrile as the organic solvent and water modified with 0.5% (v/v) ammonium hydroxide and 0.02% (v/v) acetic acid. The water phase was prepared daily with a measured pH of around 10.6. The high pH eluent provides a better peak shape of adagrasib than that in a low pH environment. During the development process, we observed an unacceptably broadened and inconsistent peak when adagrasib was eluted in 0.1% (v/v) formic acid in water, a typical acid modifier in LC–MS/MS method. This bad peak shape of adagrasib in the acidic environment may be caused by its pK_a property. Adagrasib has two pK_a values that are feasible for the method development: 3.6 and 8.5 (Data S1). It is known that the retention and peak shape of the analyte may change rapidly in a pH range of ± 1.5 units of the analyte's pK_a. Therefore, the pH eluent will be

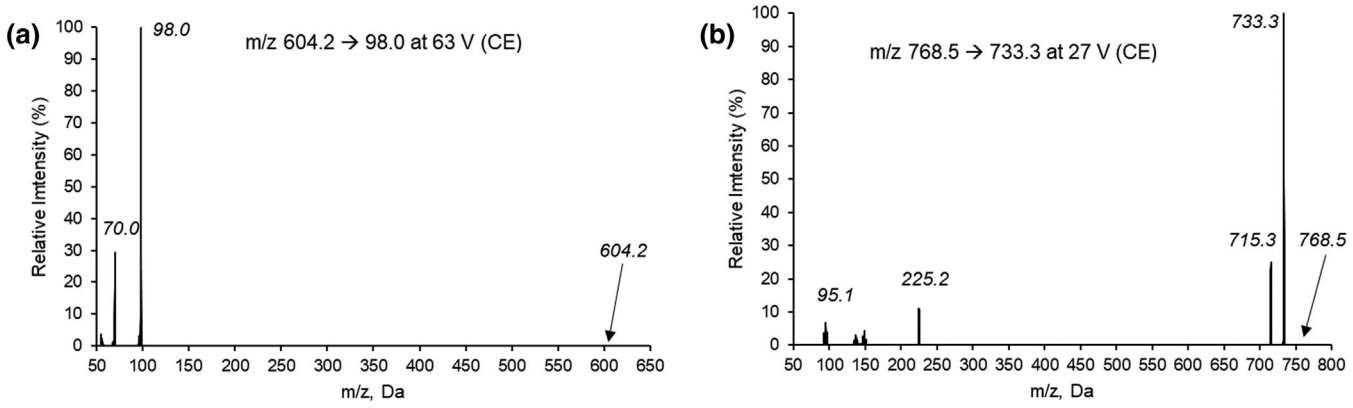

FIGURE 2 Product ion spectra of adagrasib (a) and salinomycin ammonium adduct (b).

adjusted outside that range (Dolan, 2018). This may be one of the reasons for the inconsistent and broadened peak shape of adagrasib in acidic conditions. Since it was challenging to find an MS-friendly and volatile acidic modifier outside the pH range of 2.1–5.1 (1.5 units of adagrasib, pK_a value of 3.6), a basic pH eluent could be a good alternative for this problem. Therefore, we empirically optimized the basic pH eluent. When adagrasib was eluted by the water phase with a fresh pH of 10.6, it showed an acceptable peak shape. Although the selected pH may be considered a relatively high working pH for a reverse chromatography technique, the selected column has a working pH range between 1 and 12. Further, acetonitrile was used to elute adagrasib because it provides an acceptable retention time (∼1.1 min; Figure 3) with a lower percentage of organic solvent than methanol. The representative chromatograms of the processed plasma sample are shown in Figure 3.

Since adagrasib is a relatively new compound and is still under clinical investigation, a stable isotope labeled adagrasib is not yet commercially available. Therefore, we investigated several compounds to be used as the IS for this method. According to our prior investigation, adagrasib is eluted in a high pH environment and a high percentage of acetonitrile. This fact leads to a challenging situation of finding a chromatographically suitable analog IS. An earlier study described the use of MRTX-1257 as the IS for the quantification of sotorasib (Madhyastha et al., 2021). Although MRTX-1257 has a similar molecular structure and produces a similar product ion to adagrasib (*m/z* 98.0), it was reported to be eluted in a low-pH environment, that is, isocratic eluent of 0.2% formic acid and acetonitrile (25:75 v/v) by the same study. This may be incompatible with our preliminary results on the high pH requirement for adagrasib elution. We decided to analyze some anticoccidial medicines such as monensin, salinomycin and narasin for this purpose based on our previous experiments. Although these compounds have a different chemical structure compared with adagrasib, they can be eluted in similar conditions to the adagrasib challenging elution requirement. We empirically investigated them, and salinomycin was chosen owing to its good peak shape at a similar retention time to adagrasib under our specific chromatographic conditions. During optimization, we found that both ammonium and sodium adducts of salinomycin were present in positive mode, while almost no single protonated salinomycin was observed (Sparidans et al., 2007). Although we optimized both the ammonium (*m/z* 768.5/733.3) and sodium (*m/z* 773.5/431.2) adducts of salinomycin, we fully validated this method using the ammonium adduct of salinomycin owing to its higher response during the optimization stage. The monitored transition of ammonium adducts (*m/z* 768.5/733.3) was also reported in previous publications (Dmitrovic & Durden, 2011; Schlüsener et al., 2006). The product ion of *m/z* 733.3 may be the result of the loss of water and ammonium from the parent ion.

However, in the later stage, we found that the ammonium adduct of salinomycin may have an increasing response tendency during a very long analytical run, that is, an analytical run lasting more than 6 h. This led to the relatively lower back-calculated concentration of samples injected in the later order of an analytical sequence. Therefore, we also determined the accuracy and precision of this method using the response of the sodium adduct of salinomycin and summation of both ammonium and sodium adducts of salinomycin as the response of IS.

The chosen sample pretreatment for this quantification method is PP. Although rather crude, PP is time efficient and straightforward. In addition, the short preparation time offered by PP offered an advantage in treating the tissue-related matrices of the preclinical samples owing to a stability issue. The stability issue of adagrasib will be discussed further in Section 3.2.5.

3.2 | Validation

3.2.1 | Calibration

A linear trend has been demonstrated by the relative response of adagrasib over the investigated range of 2–2,000 ng/ml. Therefore, this paper used the simple linear regression equation for the full validation assessment and sample measurement in this paper. The typical equation of $Y = ax + b$ was utilized. Parameter Y is the area ratio of adagrasib to salinomycin as the IS, while x is the concentration of adagrasib (ng/ml). Parameter a denotes the slope of the equation, while b determines the intercept. R defines the correlation coefficient

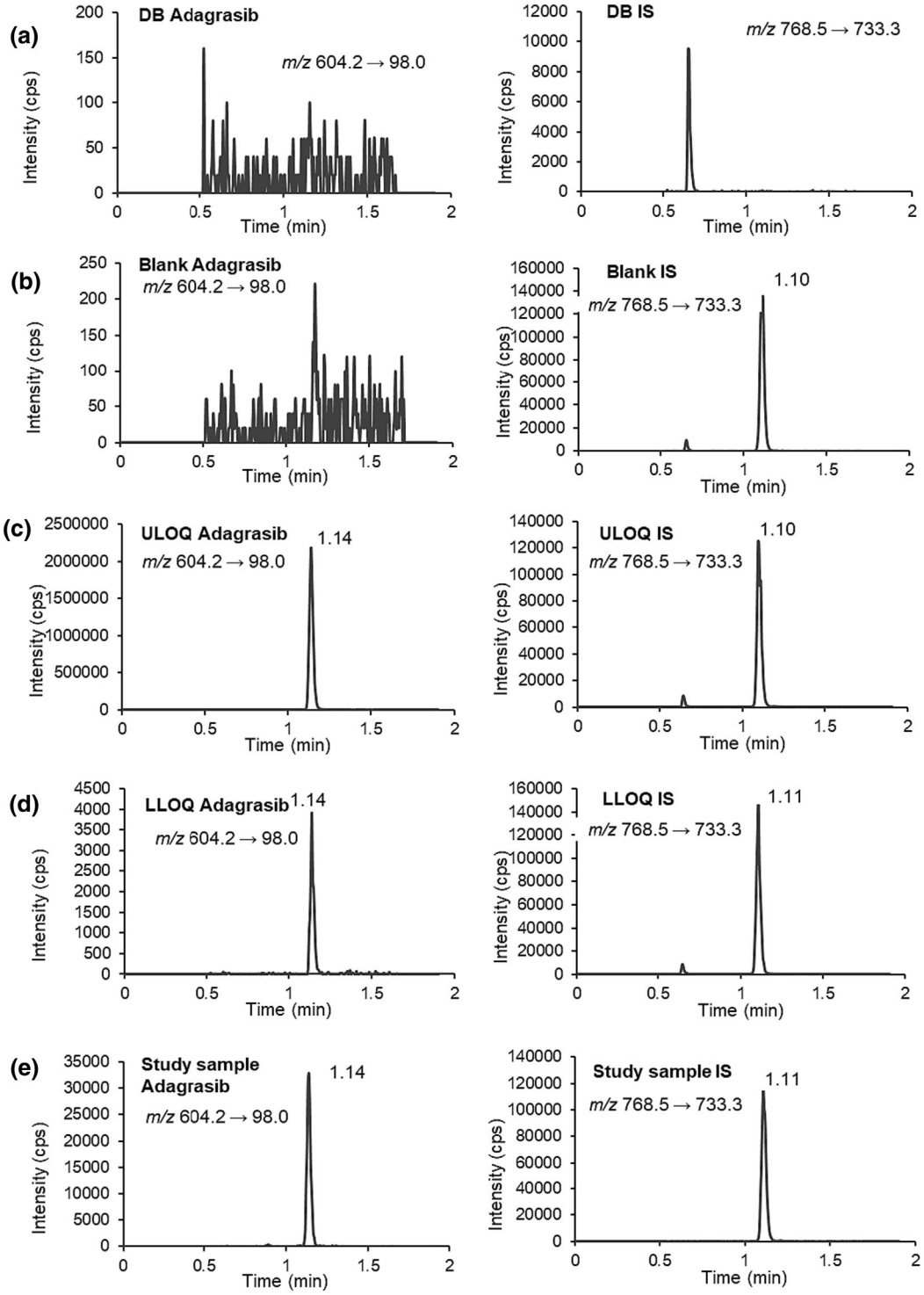

FIGURE 3 Representative multiple reaction monitoring chromatograms of processed mouse plasma samples. (a) Double blank (DB), (b) blank (containing 300 ng/ml, IS), (c) upper limit of quantitation (ULOQ; containing 2,000 ng/ml adagrasib and 300 ng/ml IS), (d) lower limit of quantitation (LLOQ; containing 2 ng/ml adagrasib and 300 ng/ml IS), (e) study sample (containing unknown adagrasib and 300 ng/ml IS).

of the regression. This linear regression utilizes the inverse square of the adagrasib concentration as the weighing factor. We reported the average of 10 calibration runs in different days (mean ± SD) as $Y = 0.0086\ (\pm 0.0020)x + 0.0021\ (\pm 0.0024)$ with $R = 0.9970\ (\pm 0.0011)$. To determine adagrasib concentrations in mouse plasma and homogenates samples from tissue-related matrices, a daily

TABLE 2 The assay performance data ($n = 18$, 3 days) of adagrasib in mouse plasma and dilution integrity with human lithium heparinized plasma.

Quality control level/dilution factor	Concentration (ng/ml)	Accuracy (%)	Intra-day precision (%)	Inter-day precision (%)
High	1600	105.5	3.5	6.5
Medium	80	101.5	6.4	7.8
Low	4	102.2	4.5	8.0
Lower limit of quantification	2	111.0	6.4	9.0
6-Fold dilution	80[a]	103.6	4.6	10.2
11-Fold dilution	436.36[a]	103.2	4.1	10.2
51-Fold dilution	94.12[a]	107.4	3.6	10.8

[a]Concentration after dilution. Accuracy was calculated as the percentage of nominal concentration, while the precision was calculated as the CV of six replications.

TABLE 3 The assay of performance and stability (measured as percentage recovery ± SD after exposure to ice conditions) of 80 ng/ml adagrasib in mouse tissues homogenates.

Tissue	Accuracy (%)	Intra-day precision (%)	Inter-day precision (%)	Stability	Exposure time (h)
Brain	105.3	6.3	5.5	94.1 ± 6.6	2
Liver	109.5	4.1	8.6	89.9 ± 7.4	2
Kidney	85.5	5.4	5.9	94.3 ± 7.6	2
Spleen	91.6	4.5	8.8	89.1 ± 6.0	2
Small intestine[a]	108.2	12.5	14.9	95.1 ± 15.8	4
Small intestine content[a]	87.2	11.8	12.6	101.1 ± 4.8	4
Lung	96.3	5.4	14.5	96.7 ± 3.8	4

[a]Accuracy and precision were performed with 51 times dilution with human plasma (concentration after dilution = 81.69 ng/ml). Accuracy was calculated as the percentage of nominal concentration, while the precision was calculated as the CV of six replications of samples.

calibration equation obtained on the same analytical run as those samples was utilized.

3.2.2 | Accuracy, precision and dilution integrity

The accuracy and precision data of four QC levels for pooled mouse plasma (Table 2) and at QC-medium level for seven mouse tissue-related matrices (Table 3) demonstrated the performance of the developed method. These results were calculated using the area of salinomycin monitored in the channel of its ammonium adduct (m/z 768.5 → 733.3). The accuracy, inter-day precision and intraday precision for QC-high, QC-medium and QC-low levels were within 15% variation, while it was within 20% variation for the QC-LLOQ. These results fulfilled the requirement of our validation framework (European Medicines Agency, 2011; US Food and Drug Administration, 2018). Moreover, the accuracy data and inter-day precision of the LLOQ level confirmed the sensitivity of this method according to US FDA guidelines (US Food and Drug Administration, 2018). Since we observed a tendency of an increasing response of salinomycin (IS) during a long analytical run (>6 h run), we decided to investigate the accuracy and precision parameter using sodium adduct of salinomycin and the summation of both ammonium and sodium adducts of salinomycin. We ran the same sample and simultaneously monitored salinomycin in several channels. Later, the concentration of adagrasib was calculated based on the different responses of salinomycin, that is, (1) using the ammonium adduct of salinomycin, (2) using the sodium adduct of salinomycin, and (3) using the summation of (1) and (2). These results are presented in the Supplementary Information (Data S2). We found that these three calculation methods provide similar performance. This may be caused by the relatively shorter run time of this experiment, that is, ~2.5 h. However, we observed a tendency of a decreasing response of sodium adduct of salinomycin in contrast with the increasing response of ammonium adduct of salinomycin. This tendency is more prominent when the analytical run is longer than 6 h. Therefore, we suggested using the summation of both adducts in a longer analytical run or performing several shorter analytical runs instead of one long analytical run.

The dilution integrity of concentrations above the highest calibration standard (2,000 ng/ml) was performed in three different dilution factors, that is, 6-, 11- and 51-fold with human plasma, since these factors were used for preclinical study sample measurement. Both the accuracy and precision for each tested dilution factor were within

TABLE 4 Stability data (reported as percentage recovery ± SD; $n = 4$) of adagrasib in mouse plasma; calculated as the percentage of the initial concentration.

Condition	Quality control-high (%)	Quality control-low (%)
8 h at room temperature	87.3 ± 5.5	85.1 ± 6.6
6 h at ice condition	94.0 ± 2.5	97.9 ± 4.7
Three freeze–thaw cycles	106.0 ± 1.3	111.3 ± 8.1
6 months at −30°C	102.2 ± 6.8	104.4 ± 11.6

TABLE 5 Stability stock and working solution ($n = 2$) of adagrasib; calculated as the percentage of the initial response (area ratio).

Condition	Percentage recovery ± SD
Working solution after 6 h at room temperature	102.4 ± 0.04
Working solution after 5 months at −30°C	95.4 ± 0.05
Stock solution after 6 h at room temperature	103.4 ± 0.14
Stock solution after 5 months at −30°C	94.0 ± 0.06

15% (Table 2), fulfilling both US FDA and EMA guidelines on bioanalytical method validation (European Medicines Agency, 2011; US Food and Drug Administration, 2018).

3.2.3 | Selectivity and carryover

There was no interfering co-eluting peak more than 3.2% of the LLOQ response at the retention time of adagrasib from six blank individual mouse plasma and all tested blank individual tissue-related matrices. Moreover, the individual spiked sample at LLOQ level has ±20% of the nominal concentration in all individual samples. According to the guidelines (European Medicines Agency, 2011; US Food and Drug Administration, 2018), the developed method is selective enough to measure adagrasib down to 2 ng/ml in mouse plasma and the homogenates of brain, liver, kidney, spleen, small intestines, small intestine content and lungs. For the IS, an additional peak was detected in the SRM channel of the ammonium adduct (768.5 → 733.3) used for the quantification. Since this additional peak was eluted very early (0.7 min) and far from the retention time of salinomycin (~1.1 min; Figure 3b), we are confident that this peak will not interfere with our measurements.

After injection of the highest calibration samples, we found more than 20% of adagrasib traces at the LLOQ level in the blank and/or double blank sample. However, this value decreased significantly to <20% after the second blank injection. We did not observe any salinomycin carryover. Therefore, we always put at least two blank injections after a high concentration sample during the analytical run.

3.2.4 | Recovery and matrix effect

The extraction recoveries with protein precipitation in this method for QC-high, QC-med, QC-low and IS were 96.7 ± 2.3, 84.7 ± 2.3, 88.5 ± 4.3 and 85.8 ± 4.0%, respectively. Since there is no minimum requirement for extraction recovery (European Medicines Agency, 2011; US Food and Drug Administration, 2018), the standard deviation confirmed the reproducibility of the selected pretreatment method in this bioanalytical procedure.

The matrix effects in pooled mouse plasma at three different QC levels were 89.2 ± 2.9, 92.7 ± 3.1 and 89.9 ± 6.5%. The averages of IS-normalized matrix factors (MF) for individual mouse matrices ($n = 34$ for each level) were 0.84 ± 0.07 for QC-low and 0.81 ± 0.06 for QC-high. We also performed IS-normalized MF measurements in six individual human plasma with average values of 0.84 ± 0.09 and 0.79 ± 0.03 for QC-low and QC-high, respectively. According to the EMA guidelines, the variation of the calculated IS-normalized MF was expressed as the coefficient of variation (CV) for each matrix (European Medicines Agency, 2011). At the QC-low level, individual mouse plasma had a CV of 3.4%, individual human plasma showed 11.1% CV, while the CV of individual mouse tissue homogenates ranged from 1.8 to 9.4%. Following the same order of matrices, the CV values obtained at QC-high were 3.4, 4.1 and 1.7–8.3%. The pooled matrix effect data and CV value of the calculated IS-normalized MF in individual matrices confirmed that there is no significant interference of the investigated matrices, both pooled and individual, on the quantification of adagrasib within this newly developed method.

3.2.5 | Stability

The stability of adagrasib at QC-high and QC-low levels in lithium heparin mouse plasma at several exposure conditions is demonstrated in Table 4, while its stability at the QC-medium level in investigated tissue-related homogenates is presented in Table 3.

From these data, adagrasib in mouse plasma was proved stable under all the tested conditions. However, adagrasib in tissue-related matrices showed poor stability when exposed to room temperature for a short period (data not shown). This stability challenge for adagrasib in tissue-related matrices could be improved when these samples were exposed to ice conditions (recorded around 9–11°C), as shown in Table 3. Therefore, we performed sample pretreatment of mouse tissue-related samples under ice conditions. Considering the relatively short-lasting stability of adagrasib in mouse tissue-related matrices, the utilization of the straightforward PP can ensure the integrity of that specific sample during the sample pretreatment.

Reanalysis of the accuracy and precision samples after 3 and 7 days of storage at 4°C resulted in both accuracy and precision

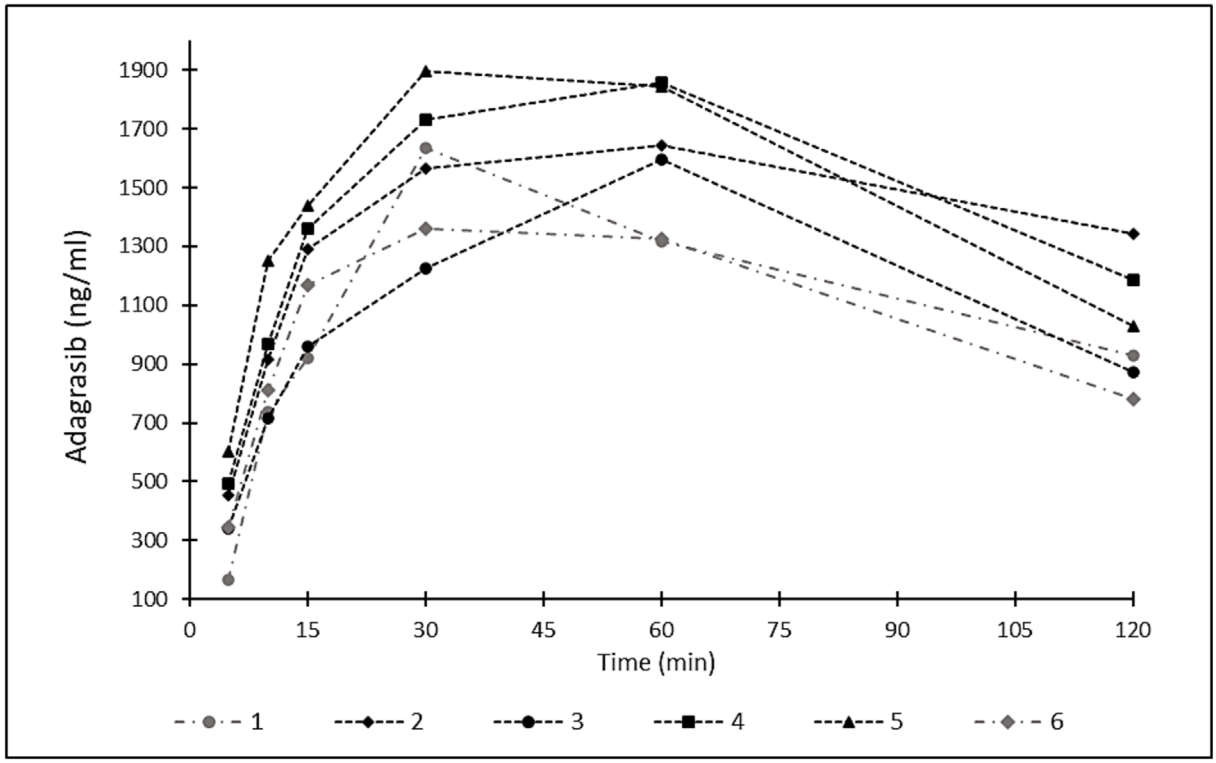

FIGURE 4 The plasma concentration over time curve of six mice (FVB/NRj genetic background) orally administered 30 mg/kg adagrasib.

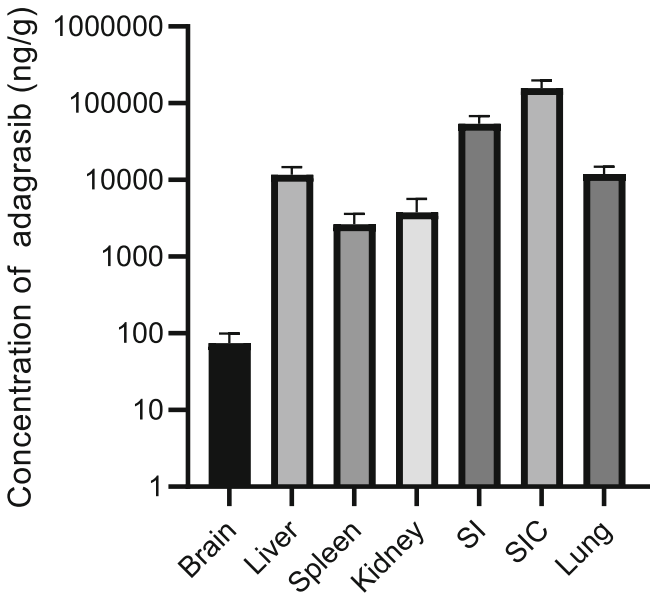

FIGURE 5 Adagrasib concentration in mouse tissue 2 h after a single dose administration of adagrasib (30 mg/kg). SI, small intestines; SIC, small intestines content.

within the 15% requirement for QC-high, QC-medium and QC-low, and within 20% for LLOQ. These data showed that the extract containing adagrasib is stable for up to 7 days when stored at 4°C. In addition, Table 5 demonstrates that adagrasib is stable when stored in academic solutions, in methanol and in a mixture of methanol and water (50:50 v/v) in both short-term and long-term exposures.

3.2.6 | Incurred sample reanalysis

Incurred sample reanalysis (ISR) from a preclinical study of adagrasib 1 month after the initial analysis ($n = 14$ plasma samples, $n = 6$ for each investigated mouse tissue-related matrices) showed that 14 out of 56 samples show a difference between measurements >20%. A 25% deviation of the total sample in this study fulfilled the requirement of the guidelines that allow up to 33% of ISR samples to have more than 20% difference between two measurements (European Medicines Agency, 2011; US Food and Drug Administration, 2018).

3.3 | Pharmacokinetic study in mice

After successful validation, this method was used to measure adagrasib concentration in mouse plasma and homogenized mouse tissues from a mouse pilot study. This pilot study was conducted in female FVB genetic background mice orally administered adagrasib at a dose of 30 mg/kg body weight ($n = 6$). The plasma concentration over the observed time for the six mice is plotted in Figure 4. It shows that three out of six mice have only two points of a decreasing plasma concentration. Therefore, we could not calculate the $T_{1/2}$ and K_e parameters for these animals. For this reason, we only report the parameters

T_{max}, C_{max} and $AUC_{0\rightarrow120}$. According to the data from the six mice, the C_{max} of adagrasib was achieved within 1 h after dose administration, as depicted in Figure 4. The calculated pharmacokinetic parameters were $T_{max} = 45 \pm 16$ min, $C_{max} = 1.67 \pm 0.19$ μg/ml and $AUC_{0\rightarrow120} = 156 \pm 22$ (μg min)/ml.

Figure 5 shows the distribution of adagrasib in mouse tissue and small intestine contents. Adagrasib showed a relatively high concentration in different tissue-related matrices, which reflects its large distribution volume. The concentration of adagrasib in the brain was much lower than those in the other tissues, and the highest adagrasib concentration was observed in small intestine content homogenates. The lower brain concentration compared with the other investigated tissues may be caused by the blood–brain barrier limiting brain accumulation. In contrast, the high concentration of adagrasib in small intestinal contents may be caused by several reasons, for example, a possible affinity of the drug for intestinal efflux transporters or the unabsorbed drug in the gut lumen. Owing to the complex biological processes that happen simultaneously once a drug compound enters the biological system, it will be challenging to pinpoint the exact reason for this high concentration without further experiments.

4 | CONCLUSION

We have successfully developed and validated the first quantification method for the KRAS inhibitor adagrasib in mouse plasma and tissue utilizing salinomycin as IS. The validated method was developed for analyzing a small volume of 10 μl sample with fast and straightforward PP utilizing acetonitrile in a 96-well plate format. The accuracy and precision of the developed method were within 20% for the LLOQ level and within 15% for the remaining tested QC levels in all investigated matrices. This method provides at least 84.7% percentage recovery of both adagrasib and IS with less than 5% standard deviation. Moreover, the IS-normalized MF of this method had a range of 0.79–0.84 with a CV ranging from 1.7 to 11.1% in all investigated mouse matrices and demonstrated that neither significant extraction loss nor matrix effect hampered adagrasib quantification in the investigated matrices. Finally, the method has been used to support a preclinical study investigating the pharmacokinetic properties and tissue distribution of adagrasib in mice and demonstrated its usefulness for pharmacokinetic studies in these laboratory animals.

AUTHOR CONTRIBUTIONS

Irene A. Retmana: Methodology; validation; formal analysis; investigation; visualization; writing—original draft. **Nancy H. C. Loos**: Formal analysis; investigation; visualization; writing—review and editing. **Alfred H. Schinkel**: Conceptualization; resources; writing—review and editing. **Jos H. Beijnen**: Conceptualization; methodology; investigation; resources; writing—review and editing. **Rolf W. Sparidans**: Conceptualization; methodology; investigation; supervision; writing—review and editing.

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