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A validated UHPLC-MS/MS method for rapid determination of senicapoc in plasma samples

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ABSTRACT

The clinically tested KCa3.1 channel blocker, senicapoc, has been proven to have excellent pharmacological properties and prior clinical trials found it to be safe for use in patients with sickle cell anaemia. Currently, several preclinical projects are aiming to repurpose senicapoc for other indications, but well-described analytical methods in the literature are lacking. Our aim was to develop a sensitive, rapid and accurate ultra-high-performance liquid chromatography-tandem mass spectrometry method using pneumatically assisted electrospray ionisation (UHPLC-ESI-MS/MS) suitable for the determination of senicapoc in plasma samples. Unfortunately, direct analysis of senicapoc in crude acetonitrile extracts of human plasma samples by UHPLC-ESI-MS/MS was subjected to significant and variable ion suppression from coeluting phospholipids (PLs). The interferences were mainly caused by the presence of phosphatidylcholine and phosphatidylethanolamine classes of PLs, including their lyso-products. However, the PLs were easily removed from crude extracts by filtration through a sorbent with Lewis acid properties which decreased the total ion suppression effect to approximately 5%. Based on this technique, a simple high-throughput UHPLC-MS/MS method was developed and validated for the determination of senicapoc in 100-μL plasma samples. The lower limit of quantification was 0.1 ng/mL. The mean true extraction recovery was close to 100 %. The relative intra-laboratory reproducibility standard deviations of the measured concentrations were 8% and 4% at concentrations of 0.1 ng/mL and 250 ng/mL, respectively. The trueness expressed as the relative bias of the test results was within ± 2% at concentrations of 1 ng/mL or higher.

1. Introduction

Senicapoc (2,2-bis(4-fluorophenyl)-2-phenylacetamide) (Fig. 1), also known as ICA-17043, is a blocker of KCa3.1, a calcium-activated potassium with intermediate conductance. It was initially developed for the treatment of sickle cell anaemia [1,2]. Despite a positive outcome in a phase-2 clinical trial [3], senicapoc failed to achieve its primary clinical endpoint in phase-3 trials [4]. However, senicapoc was demonstrated to be safe and well tolerated in humans, thereby allowing easier repurposing of the drug in other preclinical and clinical studies. Examples include neuroinflammation [5–8], cardiovascular and respiratory disorders (fibrosis or lung oedema) [9–12], other diseases with red cell dehydration [13,14] and, latest, a clinical trial using senicapoc to treat COVID-19 patients in intensive care (COVIPOC, EudraCT 2020-001420-34).

The increasing interest in senicapoc furthermore sparks a call for improved methods to assess pharmacokinetic parameters. Few sparsely described analytical methods for the determination of senicapoc in plasma have been presented in the scientific literature. Ataga et al. [15] used a liquid chromatography tandem mass spectrometry (LC–MS/MS) method with a lower limit of quantification (LLOQ) of 1 μg/L in their study on the pharmacokinetics of senicapoc. The clean-up was based on turbulent flow technology and ICA-18756 (2,2-bis-(4-fluoro-phenyl)-2-(2-fluoro-phenyl)-acetamide), which is not commercially available, was used as an internal standard (IS). Jin et al. [5] used ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) after clean-up by solid phase extraction (SPE) on a C18 sorbent in their study on using senicapoc in relation to Alzheimer's disease. The use of IS was not described and the concentration of the lowest calibrant was 50 nM (11 ng/mL). To

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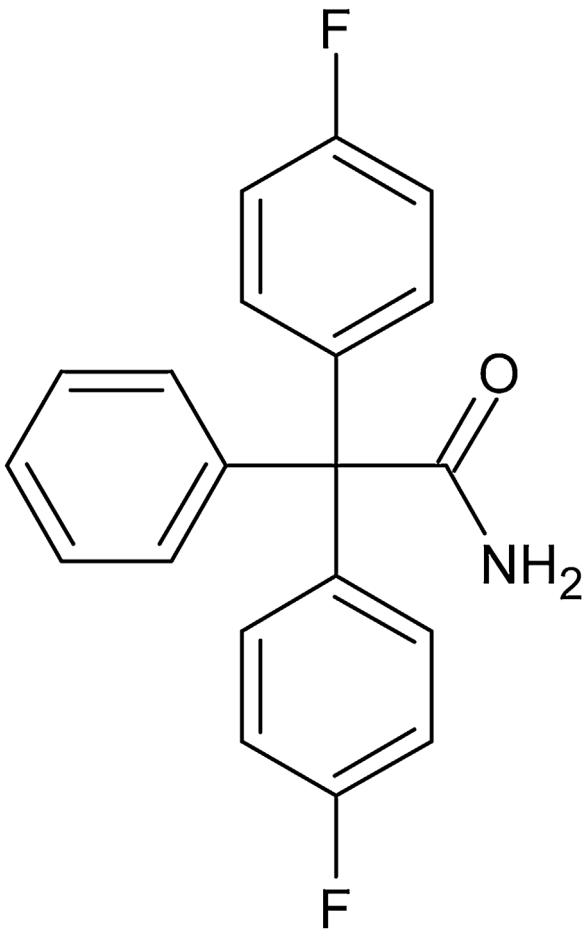

Fig. 1. Molecular structure of senicapoc.

our knowledge there are no fully described and validated MS/MS methods that are accessible in the scientific literature. Because of the lipophilic nature of senicapoc, plasma phospholipids (PLs) represent one primary source of matrix interference. PLs cannot be removed from the crude extract by reversed-phase SPE without simultaneous loss of the analyte. However, PLs can easily be removed from the crude extract by simple filtration through a sorbent with Lewis acid properties leaving a clean extract ready for direct LC–MS/MS analysis.

Here we present a UHPLC-MS/MS method that was developed and fully validated for high-throughput and sensitive determination of senicapoc in plasma samples using commercially available chemicals and materials. Interfering PLs were rapidly removed by filtration through a sorbent with bonded zirconia.

2. Materials and methods

2.1. Standards and reagents

Senicapoc was obtained from Sigma-Aldrich (Schnelldorf, Germany). Diazepam-D_5 and 11-hydroxy-Δ^9-tetrahydrocannabinol-D_3 (THC-OH-D_3) were purchased from Cerilliant (Round Rock, Texas). Methanol (MeOH), acetonitrile (MeCN), dimethyl sulphoxide (DMSO) and formic acid (FA) were obtained from Sigma-Aldrich. Water was purified using a Direct-Q 3 apparatus (Millipore, Bedford, MA).

Stock solutions of senicapoc (1 mg/mL) were prepared in DMSO. Separate stock solutions (0.1 or 1 mg/mL) of the deuterated substances were obtained in MeOH. Standard solutions to spike the samples and prepare the calibrants were prepared by diluting the stock solutions with MeOH. An IS solution containing 0.05 μg/mL diazepam-D_5 and 0.5 μg/mL THC-OH-D_3 was prepared in MeOH. Mobile phases A and B consisted of 0.1 % FA and 0.1 % FA in MeCN, respectively.

2.2. Materials

Different batches of blank human plasma were obtained from the blood banks at the University Hospitals of Aalborg and Aarhus (Denmark). The samples were stored in Vacuette tubes (no. 454297) containing a mixture of sodium fluoride and potassium oxalate (FX mixture) and in Vacuette tubes (no. 454513) containing a mixture of sodium fluoride, sodium EDTA, citric acid and sodium citrate (FC mixture) (Greiner Bio-one GmbH, Kremsmünster, Austria). The plasma samples were stored at -20 ± 2 °C. Blank porcine EDTA plasma and porcine EDTA plasma containing senicapoc from oral drug administration were provided by the Department of Biomedicine at Aarhus University. Human EDTA plasma containing senicapoc from drug administration were provided by the University hospital of Aarhus. The porcine and human plasma samples containing senicapoc were stored at a maximum of −70 °C. Sample treatments were performed in 2 mL 96-well plates from Eppendorf (Hamburg, Germany). HybridSPE 96-well plates containing 50 mg of a stationary phase with bonded zirconia (Supelco, Bellefonte, Pennsylvania) were used to clean-up the extracts. Amber 1-CRV(A) glass vials for the insertion into multi-well plates were obtained from Chromacol (Welwyn Garden City, UK). Pierceable heat-sealing foil was obtained from Waters (Milford, MA).

2.3. Animal experiments

Animal experiments were approved by the National Committee on Animal Research Ethics (2019-15-0201-00208). Female cross-bred Landrace/Yorkshire/Duroc pigs (40–42 kg) were prepared as previously described [16]. Fifty milligrams of senicapoc dissolved in 60 mL of saline was administered orally at baseline. The gastric tube was flushed with a second bolus of 60 mL of saline. Arterial blood samples were collected in EDTA tubes hourly during an 8 -h observation period. Plasma was isolated and stored at −80 °C.

2.4. Patient study

Four patients included in the trial "Senicapoc in COVID-19 Patients with Severe Respiratory Insufficiency – A Randomized, Open-Label, Phase II Trial (COVIPOC, EudraCT 2020-001420-34)" were randomised to Senicapoc. They were orally administered 50 mg of senicapoc at baseline and after 24 h. Forty-eight hours after the first dose of senicapoc 4 mL of blood was collected from an arterial line and placed in an EDTA tube. The samples were immediately centrifuged at 3000 rpm at 4 °C and stored at −80 °C

2.5. Equipment

The liquid chromatography system was an Exion UHPLC system that consisted of two Exion AD pumps, an Exion AD multiplate autosampler set at 10 ± 2 °C and an Exion AC column oven set at 40 ± 2 °C (Sciex, Ontario, Canada). Separation was performed using a Kinetex Biphenyl UHPLC column (1.7 μm, 2.1 mm I.D. × 100 mm) (Phenomenex, Torrance, CA). The mass spectrometer was a Sciex QTRAP 6500+ with a TurboIonSpray probe for electrospray ionisation (ESI). Other equipment included a vacuum manifold for 96-well plates (Supelco), a MixMate plate shaker (Eppendorf), a plate heat sealer (Eppendorf) and single- and 8-channel eLine pipettes (Biohit, Helsinki, Finland).

2.6. Extraction and clean-up

Plasma samples (100 μL) were transferred to a 96-well plate. Then, 100 μL of MeOH, 100 μL of IS solution and 200 μL of MeCN were added in succession to each well. The plate was shaken at 1650 rpm for 30 s after the addition of each reagent. The sample suspension was transferred to a HybridSPE plate using wide bore tips. The plate had been previously washed with 500 μL of MeOH and dried for at least 10 min under full vacuum (−70 kPa). Vacuum was applied to the plate, first at −30 kPa for 4 min, and then vacuum at −60 kPa was applied for an additional 2 min. Approximately 200 μL of the filtrate was transferred to a glass-lined multi-well plate. Finally, the plate was sealed with pierceable foil.

2.7. Calibration

Calibrants based on blank plasma were used for the construction of 8-point calibration curves. The calibrants were treated according to the above procedure, except that 100 μL of MeOH was replaced by 100 μL of the standard solution containing the analyte. Calibrants were prepared at concentrations of 0.1, 1, 50, 100, 150, 200, 250 and 300 ng/mL senicapoc in human plasma. In addition, a blank plasma sample processed without any added analyte and a blank plasma sample spiked with the IS mixture were included to verify the absence of detectable concentrations of the analytes due to processing or the IS mixture. The calibration curves were created by weighted (1/x) regression analysis of the IS-normalised peak areas (analyte area/IS area).

2.8. LC–MS/MS conditions

UHPLC: A 5-μL volume was injected onto the column running 60 % mobile phase A. The column flow rate was 400 μL/min, and the column temperature was maintained at 40 ± 2 °C. The mobile phase was changed through a linear gradient to 20 % A (80 % B) over 3 min. Then, the mobile phase was changed to 100 % B over 0.1 min. Five minutes after the initial injection, the gradient was returned to 60 % A (40 % B) over 0.1 min, and the column was equilibrated for 1.9 min before the next injection, resulting in a total run time of 7 min. The eluent was diverted to waste during the time intervals of 0–1 min and 4–7 min after injection using a post-column switch.

MS-MS: The probe temperature (TEM) was set to 500 °C. The curtain gas (CUR), ion source gas 1 (GS1), ion source gas 2 (GS2) and collision gas (CAD) were set at 20, 60, 60 and 9 psi, respectively. The mass spectrometer was operated in positive ion mode at unit mass resolution. The ion spray voltages were set at 4.0 kV. At least 12 data points were obtained across each peak. The applied MRM conditions are listed in Table 1. Nitrogen was used as the CAD gas. Data acquisition and processing were performed using Analyst 1.7 and MultiQuant 3.0.3 (Sciex), respectively.

2.9. Method validation

2.9.1. Selectivity

The selectivity of the method against endogenous interference was investigated by the analysis of 10 different blank samples each of human plasma and porcine plasma. Human plasma samples were also spiked with licit and illicit drugs and their metabolites frequently detected in blood from subjects suspected of driving under the influence of drugs at concentrations of 0.8–1.6 μg/mL, unless otherwise specified: alfentanil, alprazolam, 7-aminoclonazepam, 7-aminoflunitrazepam, amphetamine, amitriptyline, amlodipine (0.2 μg/mL), atomoxetine, atropine (0.2 μg/mL), baclofen, barbital (40 μg/mL), benzoylecgonine, bromazepam, buprenorphine (0.032 μg/mL), caffeine (40 μg/mL), carbamazepine (40 μg/mL), cannabidiol (CBD, 0.02 μg/mL), cannabinol (CBN, 0.02 μg/mL), cetirizine, chlordiazepoxide, chlorprothixene, clozapine, citalopram, clonazepam, cocaine, codeine, cyclobarbital (40 μg/mL), demoxepam, desmethylmirtazapine, o-desmethyltramadol, o-desmethylvenlafaxine, diazepam, diclofenac (4 μg/mL), etodolac (40 μg/mL), fentanyl (0.032 μg/mL), flunitrazepam, gabapentin, hydromorphone, 10-hydroxycarbazepine (40 μg/mL), hydroxyzine, ibuprofen (40 μg/mL), ketamine, ketobemidone, lamotrigine (40 μg/mL), levamisole, lidocaine, lorazepam, lormetazepam (0.2 μg/mL), methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), metoclopramide, methamphetamine, methadone, metoprolol, methylphenidate, mianserin, mirtazapine, 6-monoacetylmorphine (6-MAM, 0.032 μg/mL), morphine, naproxen (40 μg/mL), nitrazepam, norbuprenorphine (0.032 μg/mL), nordazepam, nortriptyline, noscapine (0.032 μg/mL), olanzapine, orphenadrine, oxazepam, oxycodone, papaverine, paracetamol (80 μg/mL), pentobarbital (40 μg/mL), phenazepam (0.2 μg/mL), phenobarbital (40 μg/mL), phenytoin (40 μg/mL), pregabalin, promethazine, propofol, quetiapine, salicylic acid (80 μg/mL), sertraline, terbutaline (0.2 μg/mL), tetrahydrocannabinol (THC, 0.04 μg/mL), tetrahydrocannabinolic acid (THCA-A, 0.04 μg/mL), 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH, 0.2 μg/mL), THC-OH (0.04 μg/mL), tramadol, venlafaxine, vigabatrin, zolpidem, zopiclone and zuclopenthixol. Although the listed substances are not isobaric with senicapoc, the test was performed to prove that interferences were not generated due to in-source collision-induced dissociation. Finally, the selectivity was tested against drugs with a molecular weight (MW) close to the MW of senicapoc (MW = 323.3 ± 1): acetylfentanyl (MW = 322.5) and lysergic acid diethylamide (LSD, MW = 323.4). These drugs were selected from our stock of more than one thousand different substances and were tested at a plasma concentration of 1 μg/mL.

2.9.2. Matrix effects and true extraction recovery

The matrix effects (including ion suppression and ion enhancement effects) were investigated for 10 blank human plasma samples stored in FC and FX tubes according to the guidelines of Peters et al. [17]. A volume of 200 μL of the final sample extract was mixed with 10 μL of standard mixtures, resulting in levels that were equivalent to 1 and 100 μg/L senicapoc in the original samples. The samples were analysed in attenuating order along with blank samples and pure standards at the same concentration. The matrix effect from each sample was calculated from the peak areas (A) without IS correction using the closest standards in the series: matrix effect (%) = ($A_{\text{pure standard}} - A_{\text{spiked sample}}$) × 100/$A_{\text{pure standard}}$. The true extraction recoveries were determined from the same plasma samples spiked at 1 and 100 ng/mL of senicapoc before extraction. The standards that were used for the determination of the true recoveries were the same plasma samples that were spiked in the final extract: true extraction recovery (%) = $A_{\text{sample spiked before extraction}} \times 100/A_{\text{sample spiked after SPE}}$.

2.9.3. Precision and trueness

The repeatability standard deviation (SD_r) of the measured concentrations (i.e., the variability of independent analytical results obtained by the same operator using the same apparatus under the same conditions on the same test sample in a short interval of time) and the intra-laboratory reproducibility standard deviation ($SD_{R,intra\text{-}lab}$) of the measured concentrations (i.e., the variability of independent analytical results obtained on the same test sample in the same laboratory by different operators on different days) were determined using 5 different blank human plasma samples that were spiked at concentrations of 0.1, 1, 10, 100 and 250 ng/mL. The samples were stored in both FX and FC tubes. In addition, 4 human EDTA plasma samples and 5 porcine EDTA plasma samples containing senicapoc as a result of drug administration were included

Table 1
Mass spectrometry conditions in the ESI(+) mode. The bold and underlined ions were used as the quantifiers. The bold ions were used as the primary qualifiers.

Substance	Transition		DP (V)	CE (eV)	Relative abundance	Rt
	Q1 (*m/z*)	Q3 (*m/z*)		(min)		
Senicapoc	324	**200**/**228**/122	80	28/18/56	100/93/42	2.45
THC-OH-D_3	334	**201**	50	34		2.68
Diazepam-D_5	290	**227**	80	37		1.98

in the precision study. Duplicate analyses were performed on 8 different days. The repeatability and intra-laboratory reproducibility parameters were calculated in accordance with ISO standard 5725-2 [18]. The method trueness (i.e., the closeness of agreement between the average value obtained from a large series of test results and the accepted reference value) was determined from the results obtained for the spiked blank human plasma samples in the precision study. The trueness was also determined separately for 40 porcine EDTA plasma samples containing <0.1 – 88 ng/mL (41 ± 24 ng/mL (mean ± SD)) senicapoc due to oral administration of the drug. The samples were spiked at a concentration of 100 ng/mL. The trueness was expressed as the relative bias: relative bias = (mean test result of spiked sample – spiked concentration) × 100/spiked concentration.

2.9.4. Limits of detection and quantification

The limit of detection (LOD) was determined using 10 blank human plasma samples that were spiked prior to extraction to concentrations that were estimated to give S/N ratios of 3–6 based on initial experiments performed on donor plasma. The LOD was calculated as $2 \times t_{0.95} \times SD_B$ ($t_{0.95}$ = 1.645), where SD_B is the standard deviation of the results obtained from the spiked samples. The LLOQ was determined from precision studies at concentration levels of approximately $10 \times SD_B$. The acceptance criteria for the LLOQ were a maximum $RSD_{R,intra\text{-}lab}$ of 20 % and a bias within ± 20 % of the spiked concentration, which is an often-used performance criterion in clinical toxicology [17]. For the qualification of peaks, a maximum difference in ion ratios of ± 20 % compared to the calibrants and a maximum difference in the retention time (Rt) of 0.03 min compared to the mean Rt of calibrants were required. The upper limit of quantification (ULOQ) was defined by the highest calibrant.

2.9.5. Stability

The stability of the sample extracts of human plasma was tested over a period of 8 days. A series of calibrants prepared from plasma preserved with FX and FC mixtures were prepared according to the described procedure and stored at 5 °C, -20 °C and −80 °C. The calibrants were analysed on the day of preparation and after 3 and 8 days of storage together with freshly prepared calibrants. In addition, extracts from 4 human EDTA plasma samples and 5 porcine EDTA plasma samples containing senicapoc as a result of drug administration were stored at 5 °C, –20 °C and −80 °C for 8 days.

The stability of human plasma samples (n = 3) preserved with FX and FC mixtures was investigated over a period of 5 months. The samples were dry-spiked at 100 ng/mL with senicapoc (i.e., the solvent of the standard solution was evaporated before the drug was redissolved in plasma) and stored at 5 °C, –20 °C and −80 °C.

3. Results and discussion

3.1. Clean-up and LC–MS/MS

The plasma samples were extracted by protein denaturation using a mixture of MeOH and MeCN. Because there are no commercially available stable isotope-labelled (SIL) analogues of senicapoc, two different ISs with Rts on both sides of the Rt of senicapoc were selected (Table 1). This setup was applied to validate the IS correction for each sample. If the extract was analysed without clean-up, i.e., centrifugation was applied instead of filtration through a PL removal plate, a relatively high matrix suppression (at least 30 %) was observed for human plasma samples. Additionally, a significant variation in matrix effects between human plasma, porcine plasma and pure standards was observed (Table 2). Due to the lipophilicity of senicapoc, it was assumed that the suppression could be due to coeluting PLs. The PL elution profile was studied by a precursor ion scan of the *m/z* 184 ion in ESI(+), detecting phosphatidylcholine (PC), lysophosphatidylcholine (lysoPC) and sphingomyelin (SM) by the choline phosphate moiety, and by a neutral loss scan of a 141 Da group in ESI(+), detecting phosphatidylethanolamine (PE) and lysophosphatidylethanolamine (lysoPE) from the loss of the ethanolamine phosphate moiety [19]. From these scans, it appeared likely that the matrix effects could be due to the heavy overlapping profiles of PLs (Figs. 2 and 3). The Rt ranges of the PL classes were confirmed by analyses of similar PLs isolated from egg yolk. The bulk parts of PC, lysoPC, PE and lysoPE eluted in the Rt range of 1.8–3.5 min, while SM eluted in the range of 3–4 min. Even the use of an SIL analogue of senicapoc as an IS, if available, may not compensate efficiently for the matrix effects because slight differences in the Rt between the analytes and the SIL-ISs are often observed. In addition the sensitivity would be reduced.

To remove the suppression from PLs, the crude extract was passed through a stationary phase containing bonded zirconia, which binds PLs through a Lewis acid-base reaction between the zirconium atoms and the phosphate moiety of the PLs. After that treatment the matrix effect for human plasma was reduced to 6 ± 4% and 5 ± 4% at concentrations of 1 ng/mL and 100 ng/mL, respectively. Simultaneously, the differences between IS-corrected areas of calibrants based on human plasma, porcine plasma and pure standards were reduced to a negligible level (Table 2).

The method was validated using an analytical column with a stationary phase of biphenyl as this phase generally improves the selectivity of analyses of aromatic substances compared to a C18 stationary phase. However, similar absolute and relative Rts might be obtained with a C18 column if the gradient is modified appropriately. If, for example, an UPLC HSS C18 (1.8 µm, 2.1 mm I.D. × 100 mm) (Waters, Milford, MA) was used, similar Rts were obtained using the following changes to the gradient: after injection, the mobile phase was changed from 60 % A (40 % B) through a linear gradient to 90 % B over 1.8 min; increased to 100 % B over 0.1 min and maintained at 100 % for 3.9 min before equilibration. The ionisation efficiencies of the mobile phases based on MeCN and MeOH was not significant different (less than 5%). MeCN was selected because it produced less backpressure. An FA concentration in the mobile phases of 0.1 % was used to obtain a robust analysis. A change in the range of 0.05 to 0.15 % produced a change in sensitivity of less than 10 %.

Several product ions of the protonated molecular ion ($[M+H]^+$) of senicapoc were produced in the collision cell, including *m/z* 200, 228, 122, 183, 104, 279, 218 and 246, which are listed in the order of decreasing sensitivity. *m/z* 200 was selected as the quantifier, and *m/z* 228 was selected as the primary qualifier from the signal/noise ratios of the transitions. Diazepam-D_5 and THC-OH-D_3

Table 2
The effect of the PL removal step. Calibrants were prepared in human plasma, porcine plasma and water at the concentrations of 0.1, 2, 50, 100, 150, 200, 250 and 300 ng/mL. Linear calibration curves with concentration as an independent variable (y = ax + b) were created by weighted (1/x) regression analysis of the IS normalised peak areas. The average of two experiments is presented.

Calibrant matrix type	PL removal included	IS	a (± SD)	b (± SD)	R^2
Human plasma	Yes	THC-OH D_3	0.0321 ± 0.0006	0.070 ± 0.096	0.998
Porcine plasma	Yes	THC-OH D_3	0.0321 ± 0.0012	0.152 ± 0.172	0.998
Water	Yes	THC-OH D_3	0.0324 ± 0.0005	0.111 ± 0.086	0.999
Human plasma	No	THC-OH D_3	0.0251 ± 0.0003	0.040 ± 0.048	0.999
Porcine plasma	No	THC-OH D_3	0.0321 ± 0.0006	0.132 ± 0.103	0.998
Water	No	THC-OH D_3	0.0364 ± 0.0005	0.073 ± 0.075	0.999
Human plasma	Yes	Diazepam D_5	0.0583 ± 0.0006	0.131 ± 0.099	0.999
Porcine plasma	Yes	Diazepam D_5	0.0584 ± 0.0015	0.153 ± 0.254	0.996
Water	Yes	Diazepam D_5	0.0588 ± 0.0008	0.091 ± 0.088	0.999
Human plasma	No	Diazepam D_5	0.0355 ± 0.0005	0.063 ± 0.078	0.999
Porcine plasma	No	Diazepam D_5	0.0464 ± 0.0005	0.097 ± 0.084	0.999
Water	No	Diazepam D_5	0.0538 ± 0.0007	0.061 ± 0.121	0.999

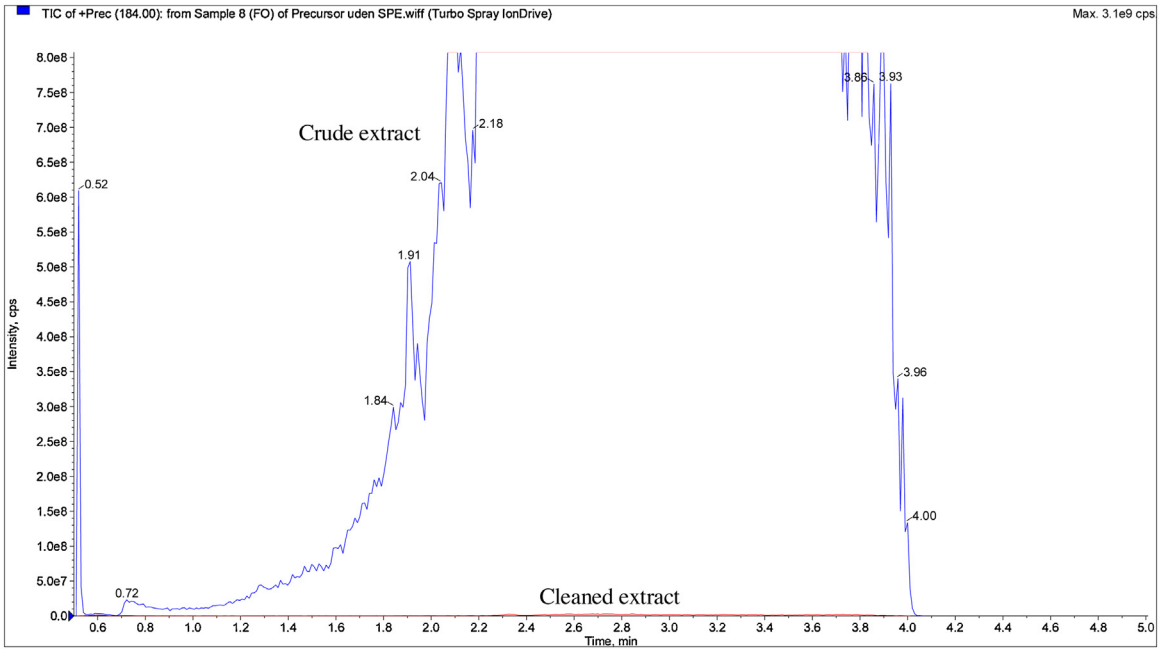

Fig. 2. Phospholipid scan profiles in ESI(+) of blank plasma extracts prepared with and without clean-up: precursor ion scan of *m/z* 184, scan range *m/z* 400-1000, DP 60 V, CE 30 eV. Senicapoc eluted at 2.45 min.

were selected as the ISs, as they eluted close to senicapoc with relative Rts of 0.82 and 1.09 compared to senicapoc. From the analyses of 24 different porcine plasma samples containing senicapoc from drug administration and the analyses of 10 different blank human plasma samples each spiked with 1, 50, 100 and 250 ng/mL senicapoc, a relative difference in results of -0.2 ± 4% (mean ± SD) was obtained by using the two ISs separately. On that basis, an acceptance criterion of a maximum of an 8% difference (20 % at LLOQ) between the results obtained by the separate use of the two ISs was established for routine analyses. A larger difference could be due to the unsuccessful removal of PLs. Without the removal of PLs, the differences were in the range of 17–30 %. This difference was increased considerably if the flush time with 100 % mobile phase B was reduced to for example 1 min. If the acceptance criterion was not fulfilled the sample was reanalysed again. The mean result of the results obtained by the two ISs was reported as the final result. Raw chromatograms are shown in Fig. 4.

3.2. Method performance parameters

The mean true extraction recoveries for human plasma were 104 ± 10 % and 99 ± 6% at concentrations of 1 ng/mL and 100 ng/mL, respectively. The LODs of the quantifier and qualifier ions were both 0.03 ng/mL. The LLOQ criteria were fulfilled at a concentration of 0.1 ng/mL (Table 3). Generally, the $RSD_{R,intra\text{-}lab}$ values obtained on human and porcine plasma were below 8% (Table 3). The absolute mean bias determined on human plasma did not exceed 2% at concentrations of 1–100 ng/mL (Table 3). For porcine plasma, a bias of 3 ± 4% was obtained when samples with a natural content of senicapoc (<0.1–88 ng/mL) were spiked with 100 ng/mL senicapoc (n = 40). No interferences from endogenous substances or frequently detected drugs and metabolites that could impact the accuracy of the method at the LLOQ level were observed. All investigated drugs, except cannabinoids, had a shorter Rt than that for senicapoc. The method performance parameters were not dependent on the stabilisation mixture used (data not shown).

The calibration curves were initially created using second-order polynomial regression models ($y = ax^2 + bx + c$). The P-values of the quadratic term (a) were 0.4–0.6, which indicates that the terms were not significant at the 90 % confidence level. Thus, linear regression models were considered sufficient for quantification in the concentration range of 0.1–300 ng/mL. A weighting factor of 1/x was introduced because the variance of the residuals increased with increases in the concentration of senicapoc. The relative error

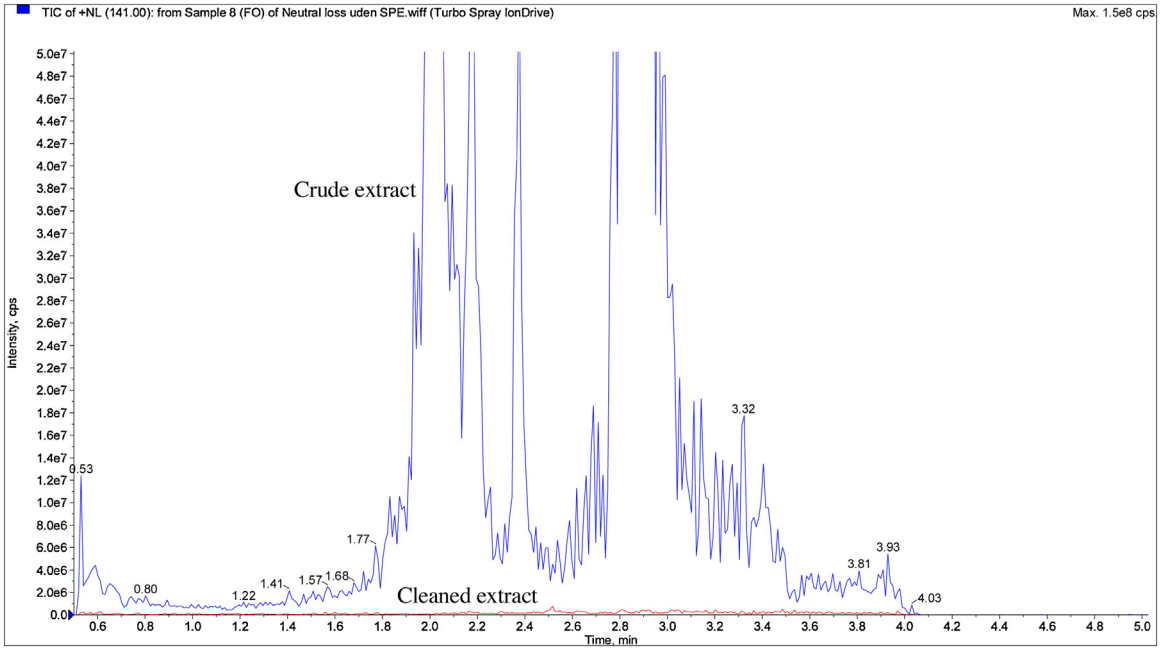

Fig. 3. Phospholipid scan profiles in ESI(+) of blank plasma extracts prepared with and without clean-up: neutral loss scan of a 141 Da group, scan range *m/z* 400-1000, DP 60, CE 30 eV. Senicapoc elutes at 2.45 min.

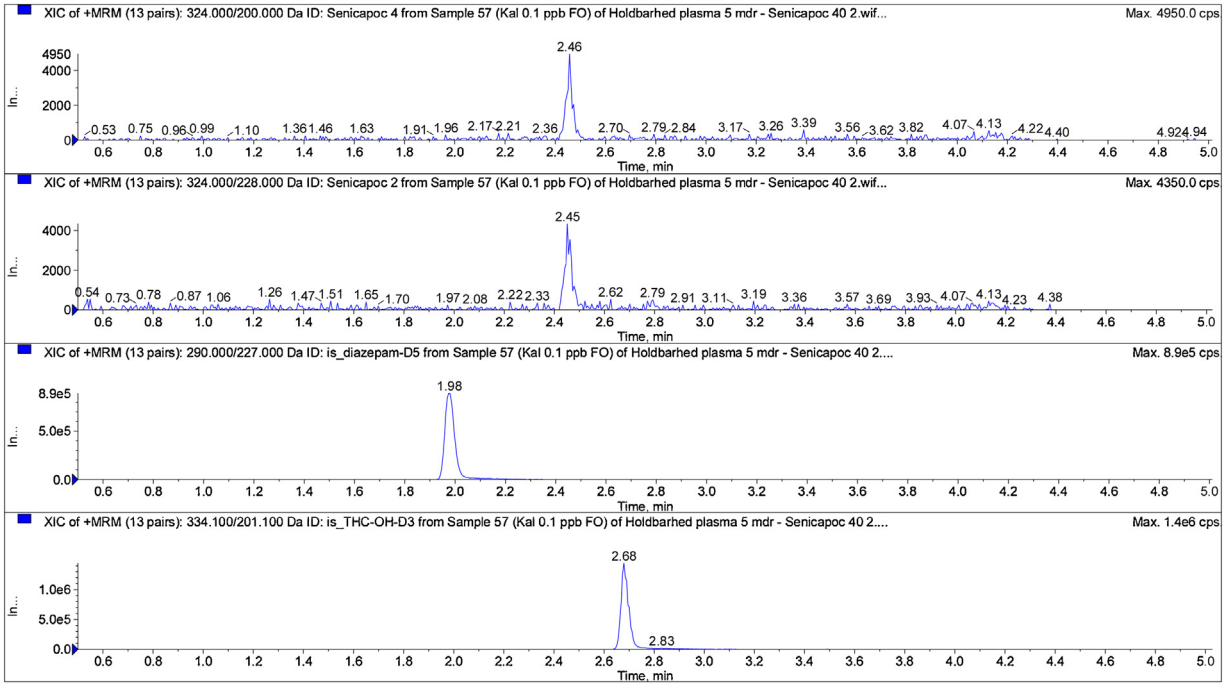

Fig. 4. Chromatograms of the quantifier and primary qualifier ions of a blank human plasma sample spiked with 0.1 ng/mL senicapoc together with the quantifier ions of diazepam-D_5 and THC−OH-D_3.

at low-concentration points was reduced by this weighting. No detectable carry-over of senicapoc from plasma samples spiked at 300 ng/mL to blank control samples was observed.

3.3. Stability of the analytes in plasma and extracts

For crude peaks of senicapoc (without IS correction) the differences between slopes of freshly prepared calibrants and calibrants stored at 4, –20 and −80 °C for 8 days were at a maximum of 5%. This was irrespective of the stabilisation mixture used. For IS-normalised peaks, the differences in slopes were less than 5%, irrespective of the IS used. The results obtained on plasma extracts from humans and animals administered senicapoc did not change during 8 days storage at 4, –20 and −80 °C (the differences were less than 5 % compared to the initial results).

Plasma samples spiked with senicapoc to a concentration of 100 ng/mL were stable for at least 5 months when stored at 4, –20 and −80 °C. The mean recoveries at each temperature level were within 96–103 %, and the recoveries were independent of the stabilisation mixture used. The storage was interrupted by thaw/freeze

Table 3
Method precision and trueness determined at different senicapoc concentration levels in spiked human plasma and in plasma from pigs administered senicapoc.

Origin of substance	Anal. conc. mean (ng/mL)	RSD_r [a] (%)	$RSD_{R,intra-lab}$ [b] (%)	Rel. bias mean ± SD (%)
Blank human plasma 1 + 0.1 ng/mL	0.108	5	8	8 ± 3
Blank human plasma 2 + 1 ng/mL	0.98	3	5	−2 ± 2
Blank human plasma 3 + 10 ng/mL	9.9	7	7	−1 ± 2
Blank human plasma 4 + 100 ng/mL	99	4	5	−1 ± 2
Blank human plasma 5 + 250 ng/mL	255	4	4	2 ± 2
Porcine plasma 1	4.5	3	4	
Porcine plasma 2	8.0	4	5	
Porcine plasma 3	22	3	4	
Porcine plasma 4	64	4	5	
Porcine plasma 5	72	5	5	
Human plasma 1	33	6	7	
Human plasma 2	93	5	7	
Human plasma 3	122	5	6	
Human plasma 4	177	3	5	

[a] Relative standard deviation of repeatability.
[b] Relative standard deviation of intra-laboratory reproducibility.

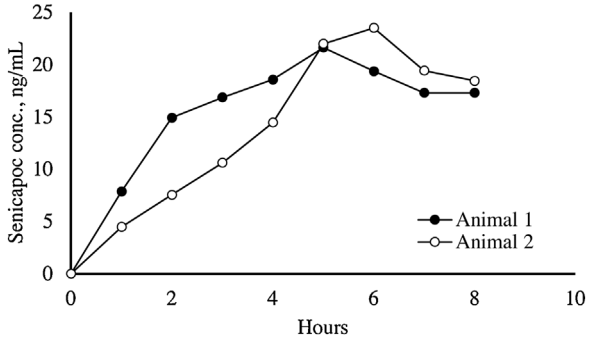

Fig. 5. Senicapoc concentration measured in plasma obtained from animals orally administered 50 mg of senicapoc. Blood samples were collected before the administration (baseline, 0 h) and 1–8 h after the administration of senicapoc.

processes after one week, two weeks, 6 weeks and 12 weeks of storage. The samples were kept at ambient temperature for approximately 2 h after each thawing.

3.4. Application of the method

In the animal experiment using two Landrace/Yorkshire/Duroc pigs, mean peak concentrations were observed 5–6 hours after administration (Fig. 5). The senicapoc plasma Cmax values were 21.6 and 23.5 ng/mL for animals 1 and 2, respectively. In the patient study, the patients had senicapoc plasma concentrations of 33, 93, 122 and 177 ng/mL (106 ± 60 ng/mL (mean ± SD)) 48 h after administration.

4. Conclusions

For clinical studies a UHPLC-MS/MS method was developed for selective quantification of senicapoc in plasma samples. The ESI of senicapoc was pronouncedly interfered by coeluting PLs present in the crude plasma extracts obtained by solvent precipitation of the proteins. The interfering PLs included PC, lysoPC, PE and lysoPE. Due to the broad elution profile of the PLs and the lipophilic properties of senicapoc, it was impossible to separate the analytes from PL interferences by rapid reversed phase chromatography. However, it was possible to remove the PLs without the loss of senicapoc by filtration through a sorbent with Lewis acid active sites. The developed UHPLC-MS/MS method showed high sensitivity because the main ion suppression substances were removed efficiently. Simultaneously, high-throughput performance was achieved by a minimised sample preparation procedure that included protein denaturation and filtration.

CRediT authorship contribution statement

Lambert K. Sørensen: Methodology, Validation, Investigation, Writing - original draft. **Asbjørn Petersen:** Resources, Investigation, Writing - original draft. **Asger Granfeldt:** Resources, Investigation, Writing - original draft. **Ulf Simonsen:** Supervision, Investigation. **Jørgen B. Hasselstrøm:** Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

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