Do the enantiomers of ketamine bind enantioselectively to human serum albumin?

Sebastian Schmidt, Ulrike Holzgrabe [*]

Institute for Pharmacy and Food Chemistry, Am Hubland, D-97074 Wuerzburg, Germany

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ABSTRACT

The binding of drugs to plasma proteins is an important process in the human body and has a significant influence on pharmacokinetic parameter. Human serum albumin (HSA) has the most important function as a transporter protein. The binding of ketamine to HSA has already been described in literature, but only of the racemate. The enantiomerically pure *S*-ketamine is used as injection solution for induction of anesthesia and has been approved by the *Food and Drug Administration* for the therapy of severe depression as a nasal spray in 2019. The question arises if there is enantioselective binding to HSA. Hence, the aim of this study was to investigate whether there is enantioselective binding of *S*-and *R*-ketamine to HSA or not. Ultrafiltration (UF) followed by chiral capillary electrophoretic analysis was used to determine the extent of protein binding. Bound fraction to HSA was 71.2 % and 64.9 % for enantiomerically pure *R*- and *S*-ketamine, respectively, and 66.5 % for the racemate. Detailed binding properties were studied by Saturation Transfer Difference (STD)-, waterLOGSY- and Carr-Purcell-Meiboom-Gill (CPMG)-NMR spectroscopy. With all three methods, the aromatic ring and the *N*-methyl group could be identified as the structural moieties most strongly involved in binding of ketamine to HSA. pK_{aff} values determined using UF and NMR indicate that ketamine is a weak affinity ligand to HSA and no significant differences in binding behavior were found between the individual enantiomers and the racemate.

1. Introduction

Binding of drugs to proteins, such as albumin, plays an important role in drug therapy. It can lead to low unbound drug levels in cases of very high binding and interactions between simultaneously administered drugs, if there is competition for the same binding site (Rahman et al., 1993; Wani et al., 2020; Yamasaki et al., 2013). The purpose of protein binding is the transport of xenobiotics and endogenous substances. Human serum albumin (HSA) is the most important plasma protein, with an amount of 60 % of total plasma proteins. In addition, it also maintains the colloid osmotic pressure of the blood, has antioxidant and antithrombotic functions and stabilizes the endothelium (Spinella et al., 2016). HSA is synthetized in the liver, has a molecular weight of 66 kDa and an average serum concentration of 600 μM in healthy adults. HSA concentration may be reduced by diseases such as liver cirrhosis leading to hypoalbuminemia which can affect the protein binding of drugs (Doweiko and Nompleggi, 1991; Tillement et al., 1978).

Binding of drugs to HSA is very well described. HSA has two main binding sites, which are known as Sudlow`s site I and Sudlow`s site II according to their discoverer Sudlow (Sudlow et al., 1975, 1976). In addition to these two binding sites, it also has many other sites where xenobiotics and endogenous substances are bound to be transported (Kragh-Hansen, 1981). HSA binds mainly anionic (Sudlow`s site I) and neutral (Sudlow`s site II) substances but not exclusively (Routledge, 1986). Other plasma proteins such as alpha-1-acid glycoprotein (AGP) bind mainly basic substances.

The anesthetic ketamine that has been on the market for over 50 years (Kohtala, 2021), is of basic structure. It is used as an injection solution for the induction of anesthesia either in racemic form, or as the enantiomerically pure *S*-ketamine. Both enantiomers differ in their anesthetic effect due to different affinity to the NDMA receptor, with *S*-ketamine being the eutomer (White et al., 1980). It also received approval by the *Food and Drug Administration* in 2019 for the treatment of severe depression as a nasal spray (FDA, 2019).

Protein binding of ketamine to HSA has been described in the past, with a wide range of 15–80 % (Dayton et al., 1983; Hijazi and Boulieu, 2002; Pedraz et al., 1985). Since enantioselective binding of basic drugs such as antihistaminic drugs to HSA has already been described, it is of interest whether ketamine enantioselectively binds to HSA or not (Martínez-Gómez et al., 2007).

In this study, protein binding was determined by ultrafiltration (UF) in combination with chiral capillary electrophoresis (CE). CE is ideally suited for the separation of enantiomers by adding chiral selectors such as cyclodextrins. It is possible to separate enantiomers quickly without waste of resources. Hence, the chiral CE method of Schmidt et al. was applied here (Schmidt and Holzgrabe, 2023). NMR measurements were performed to confirm the results of the UF and to elucidate binding properties. Ligand screening techniques such as Saturation Transfer Difference-NMR spectroscopy (STD-NMR) and waterLOGSY-NMR spectroscopy, both based on the nuclear overhauser enhancement effect (NOE), and Carr-Purcell-Meiboom-Gill (CPMG)-NMR spectroscopy measurements, applying relaxation filters, were used to characterize binding with two orthogonal principles. A non-linear fit was used for the UF, while an isotherm model was used for the NMR measurements to determine the affinity constant K_{aff}. The aim of this work was to investigate a possible enantioselective binding of ketamine to HSA and to characterize its binding properties.

2. Materials and methods

2.1. Chemicals

Racemic ketamine hydrochloride, sodium chloride, potassium chloride, albumin from human serum (> 96 % agarose gel electrophoresis), disodium hydrogen phosphate, tripotassium phosphate, *R*-ketamine hydrochloride, tris-(hydroxymethyl)-aminomethane (TRIS), and sodium azide were purchased from Sigma Aldrich (Darmstadt, Germany). *S*-Ketamine hydrochloride in form of Ketanest® was obtained from Pfizer (New York City, US). α-Cyclodextrin was purchased from Wacker Chemie (Munich, Germany), neostigmine bromide from Roche (Grenzach, Germany), 0.1 M sodium hydroxide solution from VWR chemicals (Darmstadt, Germany), 0.1 M hydrochloric acid solution from Bernd Kraft (Duisburg, Germany), deuterated water, tetramethyl silane, and deuterated hydrochloric acid from Deutero GmbH (Kastellaun, Germany). 85 % Phosphoric acid and potassium dihydrogen phosphate were obtained from Merck (Darmstadt, Germany) as well as an in-house purification system for deionized water.

2.2. Instrumentation

pH measurements were performed using a pH meter from Metrohm (Filderstadt, Germany). Capillary electrophoresis was performed using a P/ACE MDQ system from Sciex (Darmstadt, Germany) equipped with a photodiode array detector. NMR data were acquired on a Bruker III Avance spectrometer operating at 400.13 MHz equipped with a PABBI inverse probe (Karls-ruhe, Germany). The samples of the UF were incubated in a thermomixer and centrifuged with a centrifuge 5702, both from Eppendorf (Hamburg, Germany).

2.3. Capillary electrophoresis

The method described by Schmidt et al. was used to separate the ketamine enantiomers by means of CE (Schmidt and Holzgrabe, 2023). Therefore, a fused silica capillary from BGB Analytik Vertrieb (Rheinfelden, Germany) with an internal diameter of 50 μm, a total length of 70 cm and an effective length of 60 cm was chosen. Samples were injected at a pressure of 1.0 psi for 10.0 s. New capillaries were conditioned with 1.0 M sodium hydroxide solution, 2.0 M hydrochloric acid and deionized water at a pressure of 30.0 psi in this order, each for 10 min. Subsequently, the capillary was rinsed with background electrolyte

(BGE) for 2 min and a voltage of 20.0 kV for 20 min was applied. Before each sample injection, the capillary was rinsed 1.0 min with a 0.1 M sodium hydroxide solution, 1.0 min deionized water and 2.0 min with BGE at a pressure of 20.0 psi. Analytes were detected at a wavelength of 194 nm. A constant voltage of 30 kV and a temperature of 15 °C was applied. The BGE consisted of 275 mM TRIS dissolved in deionized water, adjusted with 85 % phosphoric acid to a pH of 2.50, and 50 mM α-cyclodextrin. Data were analyzed with 32 Karat software 8.0 from Sciex (Darmstadt, Germany).

2.4. Ultrafiltration

A PBS buffer consisting of 12 mM phosphate salts, 137 mM sodium chloride and 2.7 mM potassium chloride was used as the incubation medium, thus representing the physiological properties of the blood well. The pH was adjusted to 7.40 with a 0.1 mM sodium hydroxide solution. 900 μM HSA, 800 μM neostigmine bromide, 2.5 mM racemic ketamine and 1.25 mM *R*-ketamine stock solutions were prepared by dissolving the required amount in PBS buffer. The drug Ketanest® was diluted with PBS buffer, resulting in a 1.25 mM stock solution for *S*-ketamine. The protein and drug stock solutions were mixed with the respective amount of PBS buffer in 5 ml Eppendorf caps, resulting in six different ratios in the range from 0.2 to 0.5 of drug to protein, based on the respective enantiomer. Both the individual enantiomers and the racemate were incubated for 45 min. The minimum drug concentration was 120 μM and the maximum was 600 μM. HSA concentration was kept constant at 600 μM. The mixtures were incubated at 37 °C for 45 min. Before being transferred into the Amicon® Ultra 4.0 ml ultracentrifugation unit with a molecular cut-off of 10 kDa (Merck, Darmstadt, Germany), 500 μM neostigmine bromide was added as an internal standard. Subsequently, centrifugation was performed at 4400 rpm for 15 min. The filtrate was diluted in vials and measured by means of CE. Each drug-protein ratio measurement was performed in triplicate. Data fitting was performed with GraphPad Prism 9.5.1 from GraphPad Software (Boston, MA, US).

2.5. NMR conditions

The solvent used was a partially deuterated 30 mM phosphate buffer (90 % H_2O/10 % D_2O, V/V) consisting of tripotassium phosphate, 25 mM sodium chloride, 0.02 % sodium azide and 100 μM tetramethyl silane. The buffer was adjusted with deuterated hydrochloric acid to a pD of 7.40. 3.125 mM stock solutions of racemic ketamine, *S*-ketamine, and *R*-ketamine, respectively, and a 125 μM HSA stock solution was prepared by weighing the required amount of substance and dissolving it in the buffer. The samples were prepared by mixing the individual stock solutions with the buffer. For screening, one sample was measured with HSA only (50 μM), one sample with ligand only (1 mM), and a mixture of both (50 μM HSA, 1 mM ligand). The difference in chemical shifts were evaluated for the determination of the pK_{aff}. Therefore, different drug-protein ratios were measured at a constant HSA concentration of 25 μM. Ligand concentration ranged from 25 μM to 2.5 mM.

For STD-NMR measurements, the pulse frequency stddiffesgp.3 was used, coupled with an excitation sculpting pulse frequency to suppress the water signal at 4.703 ppm. The number of scans was 8 with 16 dummy scans. The saturation pulse was 400 Hz and the saturation time 3 s.

The sequence cpmg_esgp2d was applied for the relaxation filter measurements. The CPMG filters were 2 and 200 ms, respectively. The number of scans was 128 and the number of dummy scans was 4.

For the waterLOGSY experiments, the sequence ephogsygopno.2 was used. The number of scans was 256 with 4 dummy scans. The mixing time for the transfer of saturation by the NOE was 1.7 ms.

For K_{aff} determination using the chemical shift, ^{1}H spectra with water signal suppression at 4.703 ppm through excitation sculpting were recorded with a scan number of 1024 and 4 dummy scans. HSA

concentration was kept constant at 50 μM and different drug-protein ratios between 1:1 and 50:1 were mixed in the solvent. All measurements were performed at 300 K and a spectral width of 15.98 ppm with an applied loop counter of 1.

NMR data was evaluated with TopSpin 4.0.9 from Bruker (Karlsruhe, Germany). Data fitting was performed with GraphPad Prism 9.5.1 from GraphPad Software (Boston, MA, US).

3. Results and discussion

3.1. Protein binding determination by means of ultrafiltration

UF was performed to determine the extent of protein binding of ketamine to HSA. The experiment was divided into two parts. First, the drug was incubated with the protein and then the drug-protein complex was separated by a membrane filter. Free unbound drug is filtered, which is then analyzed. Using cyclodextrin-modified CE, the enantiomers could be separated and individually quantified, respectively, or determined as racemate. An example electropherogram is given in Fig. 1.

Both ketamine enantiomers and neostigmine could be baseline separated within 30 min. Neostigmine was used as internal standard because no binding to HSA has been reported in the literature (Ammon, 2001). Nevertheless, to avoid unknown binding processes of neostigmine to HSA, the internal standard was not added to the solution until immediately before filtration. A protein normally shows several binding sites of different composition. As already mentioned in the introduction, albumin has the Sudlow`s site I and Sudlow`s site II, mainly to bind anionic and neutral substances. Ligands can either bind to one binding site only, or multiple binding to more binding sites is also possible. In both cases, the greater the excess of ligand, the more binding sites can be occupied. The binding occurs under an equilibrium reaction which can be described by Eq. (1).

$$\text{ligand} + \text{protein} \rightleftharpoons \text{ligand} - \text{protein complex} \qquad (1)$$

To characterize the binding behavior, the affinity constant K_{aff} can be determined from the equilibrium reaction using the law of mass action. For this purpose, different drug-protein ratios were incubated together, and the free, unbound part of the drug is analyzed, using a constant

Fig. 1. Electropherogram of racemic ketamine with neostigmine bromide as internal standard after incubation with 600 μM HSA; electrophoretic conditions: 275 mM TRIS buffer adjusted with 85 % phosphoric acid to a pH value of 2.50, 50 mM cyclodextrin, 15 °C, 30 kV, injection at 1.0 psi for 10 s, fused silica capillary with a total length of 70 cm and an effective length of 60 cm, internal diameter of 50 μm, wavelength 194 nm.

number of binding sites to which the drug binds. If a straight line is obtained with a slope of 1, the drug binds only to one binding site. The presence of one binding site can also be verified using Eq. (5).

$$K_{aff} = \frac{f_{bound}}{1 - f_{bound}} * \frac{1}{\text{protein}_{total}} \qquad (5)$$

K_{aff} is constant over all measured drug-protein ratios if the drug binds only to one binding site (Volpp and Holzgrabe, 2019). As shown in Fig. 2 and S1, K_{aff} remains constant, which proves the existence of only one binding site for both enantiomers. For better usability, the pK_{aff} is used, which corresponds to the negative decadic logarithm of the K_{aff}.

The confirmation of the presence of one binding site allows the use of a non-linear model according to Asensi-Bernardi (Asensi-Bernardi et al., 2010). In cases where n (number of binding sites) = 1, Eq. (6) can be fitted for K_{aff}.

$$\text{ligand}_{free} = \frac{-\left(1 - K_{aff}*\text{ligand}_{total} + n*K_{aff}*\text{protein}_{total}\right) \pm \sqrt{\left(1 - K_{aff}*\text{ligand}_{total} + n*K_{aff}*\text{protein}_{total}\right)^2 + 4 * K_{aff}*\text{ligand}_{total}}}{2* K_{aff}} \qquad (6)$$

protein concentration and different ligand concentrations. The experimental conditions were kept as physiological as possible: an HSA concentration of 600 μM, incubation at 37 °C and low drug protein ratios of 0.2 to 0.5 were used (Asensi-Bernardi et al., 2010). The extent of protein binding was determined by quantifying the unbound ligand concentration (ligand$_{free}$). Eq. (2) and Eq. (3) are used to determine the bound fraction (f_{bound}). Results are presented in Table 1.

$$\text{ligand}_{bound} = \text{ligand}_{total} - \text{ligand}_{free} \qquad (2)$$

$$f_{bound} \, [\%] = \frac{\text{ligand}_{bound}}{\text{ligand}_{total}} * 100 \qquad (3)$$

The drug bound per mole of protein can be defined as r and is characterized by Eq. (4).

$$r = \frac{\text{ligand}_{bound}}{\text{protein}_{total}} \qquad (4)$$

If this term is plotted as r/1-r in logarithmic form against the logarithmic free ligand concentration, information can be obtained about the
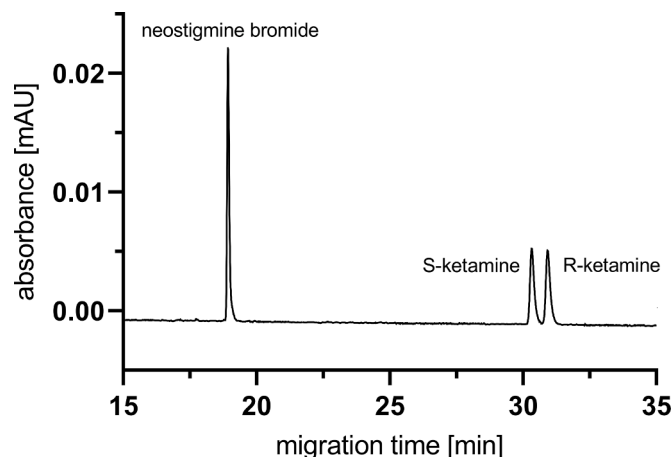
The fit for enantiomerically pure *S*-ketamine as well as the correlation between experimental and calculated values of the fit are shown in Fig. 3. The data for *R*-ketamine and the racemic mixtures are shown in Fig. S2, the obtained values are given in Table 1.

The protein binding of all enantiomers, whether enantiomerically pure or in the racemic mixture, and the racemate are between 64.9 and 71.2 %. Comparing the enantiomerically pure *R*- and *S*-ketamine, *R*-ketamine shows a slightly, but significantly higher binding. However, a clinically relevant impact is not expected. No significant differences between the enantiomers have been observed in the racemic mixtures as a kind of average was measured. In the literature, binding of ketamine of 15–33 % to HSA and human plasma, respectively, and 45 % to AGP (Dayton et al., 1983), 60 % to HSA (Hijazi and Boulieu, 2002), 30–60 % to HSA and 60–80 % to human serum (Pedraz et al., 1985) have been reported. Except for Dayton et al., the binding percentages obtained, fall into theses wide ranges. Dayton et al. focused primarily on binding of ketamine to human plasma. Nevertheless, binding of ketamine to bovine serum albumin, HSA, AGP and γ-globulin was also studied. Additionally,

Table 1

Comparison of the determined protein binding by means of ultrafiltration, mean value of pK_{aff} calculated according to Eq. (5) and pK_{aff} fitted with the non-linear fit according to Eq. (6); in racemate: incubation of the racemate with HSA; enantiomerically pure: incubation of the respective enantiomer with HSA.

	protein binding [%]	pK_{aff} (Eq. (5))	pK_{aff} (non-linear fit)
R-ketamine (enantiomerically pure)	71.2 ± 1.2	2.38 ± 0.03	2.25
S-ketamine (enantiomerically pure)	64.9 ± 1.5	2.51 ± 0.03	2.39
R-ketamine (in racemate)	66.4 ± 2.8	2.48 ± 0.06	2.38
S-ketamine (in racemate)	67.5 ± 2.8	2.46 ± 0.06	2.36
ketamine (racemate)	66.5 ± 3.4	2.48 ± 0.07	2.20

enantioselective binding to AGP was investigated, with the result that both *S*- and *R*-ketamine bind equally with 45 %. Thus, ketamine does not bind enantioselectively to AGP. As our results show, this is also the case for binding to HSA. pK_{aff} values obtained with Eq. (5) and the non-linear fit are in a similar range, but they are lower than the pK_{aff} value of 3.01 reported by (Pedraz et al., 1985). In our work, drug-protein ratios were measured no greater than 0.5, while Pedraz et al. measured up to twice the amount of drug compared to protein. Hence, a different model was chosen for the data fit resulting in different pK_{aff} values. Asensi-Bernardi et al. demonstrated the advantages of using low-drug protein ratios, which is why we chose this approach (Asensi-Bernardi et al., 2010).
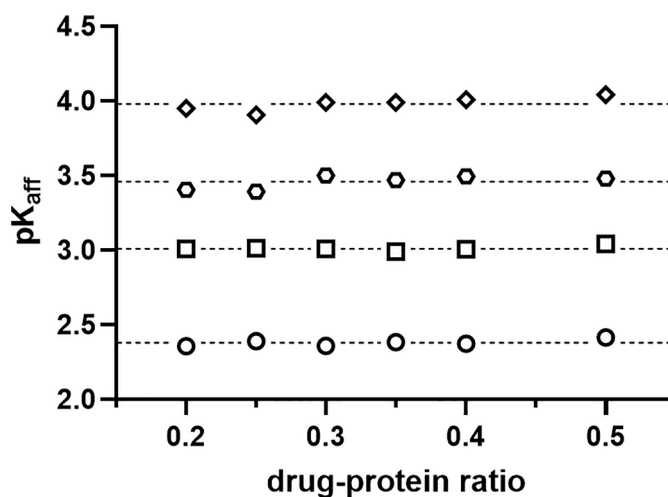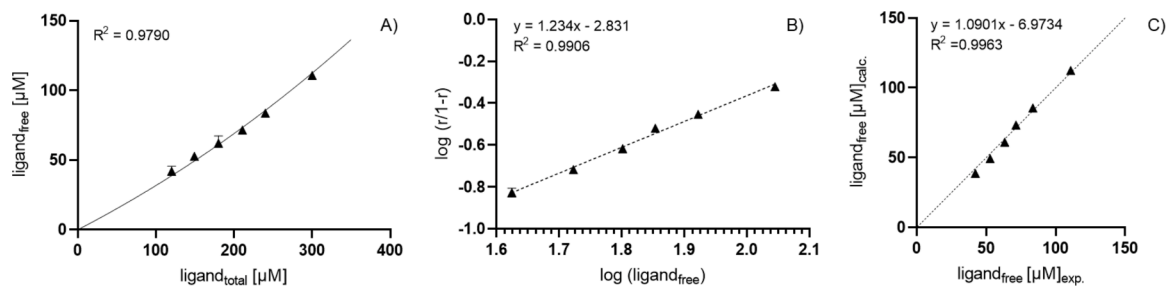

Fig. 2. pK_{aff} of ○ R-ketamine (enantiomerically pure), □ S-ketamine (enantiomerically pure), ◇ R-ketamine (in racemate), ○ S-ketamine (in racemate) of different drug-protein ratios at a constant HSA concentration of 600 μM; for better presentation, a y-offset of 0.5 was used, the mean value of the respective pK_{aff} is shown in Table 1; in racemate: incubation of the racemate with HSA; enantiomerically pure: incubation of the respective enantiomer with HSA.

### 3.2. Characterization of binding properties by means of NMR spectroscopy

NMR is an excellent tool for drug screening in drug development (Stockman and Dalvit, 2002). Different measurement methods can be used to investigate structure-affinity relationships and chemical structures of a drug involved in binding. The main advantage is the possibility to apply lower target concentrations compared to UF. In addition, titration experiments can also be used to determine affinity constants. In this study, STD-NMR, waterLOGSY and CPMG relaxation filters were applied as screening methods. ^{1}H NMR spectra of different drug-protein ratios were used to determine the affinity constant. Fig. 4 shows a ^{1}H NMR spectrum of ketamine and the assignment of the individual protons to their respective signals.

STD-NMR is based on the NOE. When a drug binds to a protein, which is saturated with a pulse frequency, the saturation is transferred to the protons of the drug involved in the protein binding. By comparison of a spectrum recorded with (on-resonance spectrum) and without (off-resonance spectrum) the saturation pulse frequency, a difference spectrum is obtained. Signals that are seen in the difference spectrum are protons of the ligand involved in binding. It is important that no proton of the ligand is hit by the saturation pulse and thus falsifies the measurement. For each protein the appropriate saturation pulse must be found. Fig. S3 shows, that only the NHC**H**$_3$ protons are hit slightly, and HSA is fully saturated by the pulse. This effect was considered for the evaluation. The obtained STD signal is dependent on saturation time, rebinding processes, binding kinetics, and drug respective protein concentration (Walpole et al., 2019). With shorter saturation times, it can be assumed that complete saturation has not yet been reached and the signal is therefore independent. In this study, a short saturation time of 3 s was chosen to minimize the influence. Fig. 5A shows the difference spectrum (black) and the off-resonance spectrum (red) of *S*-ketamine. In the difference spectrum, the NHC**H**$_3$ protons and the aromatic protons of ketamine are the most intense signals, suggesting that these structure moieties are primarily involved in binding to HSA. The cyclohexanone protons show fewer intensive signals.

Another screening technique based on the NOE is waterLOGSY. The difference here is that the protein is not saturated with a pulse, but the bulk water in the solution is (Claridge, 2016). When a ligand binds to a protein, the saturation is transferred from the bulk water, via various

Fig. 3. A) non-linear fit according to Eq. (6) between ligand$_{total}$ and ligand$_{free}$ of S-ketamine (enantiomerically pure) B) logarithmic check of the presence of one binding site between log (r/1-r) and log (ligand$_{free}$) with r being the quotient between ligand$_{bound}$ and protein$_{total}$ C) correlation between calculated and experimental data of ligand$_{free}$ of the non-linear fit.
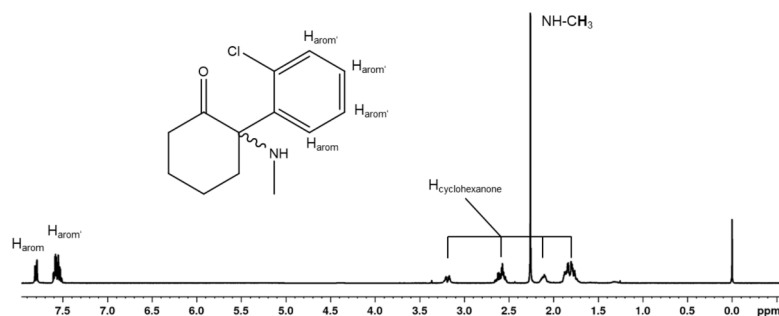
Fig. 4. ^{1}H NMR spectrum of racemic ketamine and the assignment of its protons; spectrum was recorded with water signal saturation at 4.703 ppm by excitation sculpting.
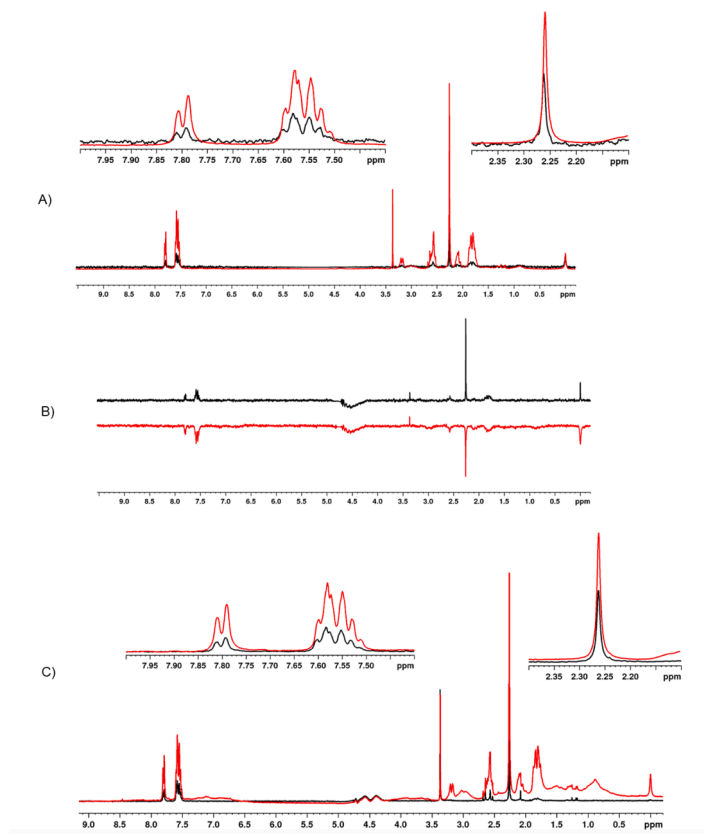
Fig. 5. A) STD-NMR: overlay of the off-resonance spectrum of 1 mM S-ketamine with 50 μM HSA (red) and the difference spectra (black) with a saturation pulse of 400 Hz and water signal suppression at 4.703 ppm by excitation sculpting B): waterLOGSY: overlay of 1 mM S-ketamine (black) with 1 mM S-ketamine + 50 μM HSA (red) C) CPMG NMR: overlay of the ^{1}H NMR with water signal suppression at 4.703 ppm by excitation sculpting of 1 mM S-ketamine with 50 μM HSA with a relaxation filter of 2 ms (red) and 200 ms (black).

processes such as spin diffusion, to the protein and beyond to the ligand. This transfer leads to a reduction in the intensity of the ligand signals up to negative deflections. It is important that the saturation can also be transferred directly to the ligand, which could distort the result. It is essential to conduct an experiment with only ligand and no protein to include possible intensity changes that are transferred by direct transmission of saturation through the water to the ligand and not the protein. Fig. 5B shows the waterLOGSY spectrum of S-ketamine without HSA (black) and with protein (red). The effect of binding and transfer of saturation respectively is so intense that the signals of the ligand are reduced in a way that they change into the negative. Again, the aromatic protons and NHCH$_3$ show the most intense signals. The cyclohexanone protons show only weak intensity changes. Hence, the results of the

waterLOGSY experiments are in line with those of the STD measurements.

CPMG spin lock filters were used as a third screening option. Large molecules relax much faster than smaller ones (Fernández and Wider, 2006). By setting suitable filter times, the signals of the protein can be attenuated until they are no longer visible. This effect is also transferred to bound ligands. If a ligand binds to a protein that is exposed to a relaxation filter, the signals of the protons involved in the binding are attenuated. First, it must be checked whether the signals of the HSA, are filtered out with a filter time of 200 ms or not. For this purpose, a pure sample of HSA is measured with a filter time of 2 and 200 ms, respectively. Pure ligand is also measured under these conditions to ensure that the ligand is not also attenuated. The results here are shown in Fig. S4.

HSA is completely filtered out at a filter time of 200 ms and ketamine remains unaffected. Fig. 5C shows the spectra of the mixture of HSA with *S*-ketamine at a filter time of 2 ms (red) and a filter time of 200 ms (black). The signals of the aromatic protons and of NHCH$_3$ are attenuated, while the signals of the cyclohexanone protons completely disappeared. STD, waterLOGSY, and CPMG spectra of *R*-ketamine and racemic ketamine are presented in Fig. S5 and S6. *S*-ketamine, *R*-ketamine and racemic ketamine show similar results. Aromatic and NHCH$_3$ protons are the most intense signals in STD NMR and have largest attenuations in waterLOGSY and CPMG. There was no difference between the individual enantiomers and the racemate. Since all three screening methods show the same result, it can be assumed that the aromatic protons and NHCH$_3$ are most strongly involved in the binding. As mentioned before, HSA has two main binding sites (Sudlow`s site I and Sudlow`s site II). Sudlow`s site I is characterized by its ability to bind mainly bulky anionic ligands, while Sudlow`s site II binds aromatic, anionic but also neutral substances (Sudlow et al., 1975, 1976). Since ketamine is still partially uncharged at physiological pH (pK$_a$ 7.5 (Tolksdorf, 1988)) and has an aromatic ring structure, Sudlow`s site II could be a possible binding site for ketamine. However, it must also be mentioned that HSA, as a transporter protein, has many uncharacterized binding sites that could bind ketamine.

To confirm the pK$_{aff}$ determined via UF, an orthogonal method was applied. Differences in the chemical shift of individual signals of various drug-protein ratios in the range from 1 to 100 drug to protein were investigated. When a ligand binds to a protein, it experiences a different chemical environment and a change in chemical shift. Since the H$_{arom}$ and NHCH$_3$ protons showed the largest difference and were well resolved in the screening, these signals were selected for the determination of K$_{aff}$. Various drug-protein ratios were measured, with the HSA concentration kept constant at 25 μM and a ligand concentration between 25 μM and 2.5 mM.

Table 2
Comparison of the determined pK$_{aff}$ fitted according to Eq. (8) for NHCH$_3$ and H$_{arom}$ and the coefficient of variation of the double reciprocal fit and the correlation of calculated and experimental data of Δδ.

|	pK$_{aff}$		R^2 fit		R^2 correlation	
	H$_{arom}$	NHCH$_3$	H$_{arom}$	NHCH$_3$	H$_{arom}$	NHCH$_3$
R-ketamine	2.08	2.02	0.9971	0.9997	0.9968	0.9997
S-ketamine	2.01	2.09	0.9948	0.9909	0.9991	0.9925
racemic ketamine	2.01	2.04	0.9949	0.9951	0.9970	0.9951

Fig. 6C shows the chemical shifts at different drug-protein ratios of *S*-ketamine. The differences of the chemical shifts were determined according to Eq. (7) with δ being the chemical shift. A sample of 50 μM ligand was used as reference.

$$\Delta\delta = \delta_{\text{drug-protein ratios}} - \delta_{\text{reference}} \tag{7}$$

An isotherm is achieved by plotting Δδ against ligand concentration after a double reciprocal data transformation as seen in Fig. 6A. Since the UF showed that the drug binds to one binding site, a model according to Eq. (8) was used for data fitting to determine K$_{aff}$ (Motulsky and Neubig, 2010). Fig. 6B shows the correlation of the experimentally determined and the calculated fits. Fig. S7 and S8 show data for *R*-ketamine and racemic ketamine.

$$\Delta\delta = \frac{\Delta\delta_{\max} * \text{ligand}}{K_{aff} + \text{ligand}} \tag{8}$$

In Table 2, pK$_{aff}$ values are given as the mean of the individual fits of H$_{arom}$ and NHCH$_3$, as well as the coefficients of variation of the fit and the correlation. pK$_{aff}$ values obtained with the NMR method are lower than those of the UF, but in the same order of magnitude. No significant difference can be observed between the evaluation of the H$_{arom}$ and NHCH$_3$ and between the individual enantiomers and the racemate.
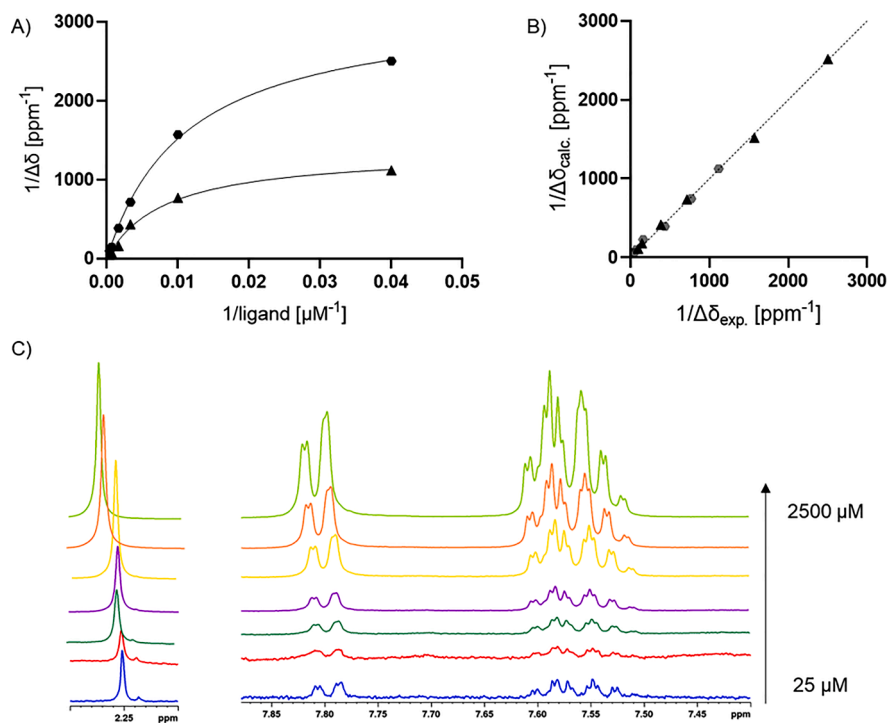
Fig. 6. A) double reciprocal fit for pK$_{aff}$ according to Eq. (8) for ▲NHCH$_3$ and ●H$_{arom}$ between Δδ and ligand concentration for S-ketamine B) correlation between calculated and experimental data of Δδ of the double reciprocal fit C) shift of signal at 2.26 and 7.56–7.80 ppm at different ligand concentration of S-ketamine at a constant protein concentration of 25 μM.

However, due to the rather low pK$_{aff}$, the enantiomers of ketamine can be classified as low affinity ligands to HSA.

4. Conclusion

The extent of protein binding of ketamine was successfully determined and agrees with previously reported values in literature. For the first time, enantioselective binding of *R*- and *S*-ketamine to HSA were investigated, both enantiomerically pure and in racemic mixtures. There was no significant difference between the enantiomers found. Consequently, the drug does not bind enantioselectively to HSA. Various NMR methods were used to investigate the structural binding behavior. It was found that mainly the aromatic protons and the protons of the *N*-methyl group are involved in the binding, indicating a hydrophobic binding site like the known Sudlow`s site II binding site. The pK$_{aff}$ values determined were lower than already published ones but confirmed by two orthogonal methods, indicating that ketamine is a weak affinity ligand to HSA.

CRediT authorship contribution statement

Sebastian Schmidt: Investigation, Methodology, Writing – original draft, Visualization, Conceptualization. **Ulrike Holzgrabe:** Conceptualization, Resources, Writing – review & editing, Supervision.

Declaration of Competing Interest

None of the authors of this paper does have a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

Data availability

Data will be made available on request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejps.2023.106640.

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