






# Analysis of the ketamine binding to total plasma protein from domestic cats\*

## *Análise da ligação da cetamina às proteínas plasmáticas totais de gatos domésticos*

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### ABSTRACT

Ketamine is a versatile veterinary, clinical, and hospital drug. Evaluating the binding parameters of ketamine to total plasma proteins from domestic cats provides necessary information in determining the value of the pharmacokinetic parameter named distribution volume, which is used for the consequent prospection of drug concentrations in clinical trials. This work aimed to evaluate the binding rate of ketamine to total plasma proteins, the binding constant, and the binding mode to serum albumin in cats. After approval of the project by CEUA/UEM (protocol 3292020621), a plasma pool from six animals (n=6) was reinforced with ketamine concentrations aiming for ultrafiltration with a 10 kDa cutoff membrane device. The drug levels before and after ultrafiltration were analyzed using the liquid chromatography-mass spectrometry technique (LC-MS/MS). The results were calculated using a Scatchard plot to calculate the binding rate and binding constant to albumin. Docking simulations identified the most likely albumin-binding sites. The ketamine binding rate was 65% when the drug reached a plasma concentration above 300 ng·mL<sup>-1</sup>, and the binding constant ( $K_b$ ) was 2×10<sup>6</sup> mol·L<sup>-1</sup> with a positive Hill coefficient ( $n_H$ ) of 2.3. These results show a good correlation between the physicochemical parameters of the drug with structural evaluation by docking simulations and coherence with the values reported by other methodologies in recent literature. This work brought data on the ketamine-binding behavior in cats, an important parameter for future pharmacokinetic evaluations in the search for better protocols for the clinical use of this drug in veterinary medicine.

**Keywords:** Drug development. Analgesia. *Felis catus*. Albumin.

### RESUMO

A cetamina é um medicamento versátil amplamente utilizado nos ambientes clínico e hospitalar veterinário. A avaliação dos parâmetros de ligação da cetamina às proteínas plasmáticas totais de gatos domésticos fornece informações necessárias na determinação do parâmetro farmacocinético denominado volume de distribuição, o qual é utilizado para a prospecção de concentrações do medicamento em estudos clínicos. O objetivo deste trabalho foi avaliar a taxa de ligação da cetamina às proteínas plasmáticas totais, sua constante ( $K_b$ ), e modo de ligação à albumina sérica em gatos. Após a aprovação do projeto pelo CEUA/UEM (protocolo 3292020621), uma mistura de plasma de seis animais (n=6) foi reforçada com concentrações de cetamina visando ultrafiltração com membrana de 10 kDa. Os níveis do fármaco antes e depois da ultrafiltração foram analisados pela técnica de Cromatografia Líquida acoplada a Espectrometria de Massas (LC-MS/MS) e os resultados utilizados para calcular a taxa de ligação e a constante de ligação à albumina pelo gráfico de Scatchard. Simulações de docking identificaram os mais prováveis sítios de ligação à proteína. A taxa de ligação da cetamina foi de 65% quando o fármaco atingiu concentrações plasmáticas acima de 300 ng·mL<sup>-1</sup>, e a constante de ligação foi de 2×10<sup>6</sup> mol·L<sup>-1</sup> com coeficiente de Hill ( $n_H$ ) de 2,3. Estes resultados mostram uma boa correlação entre os parâmetros físico-químicos do fármaco com a avaliação estrutural por meio de docking, além de coerência com os valores relatados por outras metodologias. Este trabalho traz informações sobre o comportamento de ligação da cetamina em proteínas plasmáticas de gatos, parâmetro importante para futuras avaliações farmacocinéticas na busca de melhores protocolos para uso clínico deste medicamento na área veterinária.

**Palavras-chave:** Desenvolvimento de fármacos. Analgesia. *Felis catus*. Albumina.

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## Introduction

Ketamine is an anesthetic drug commonly used in veterinary medicine routine due to its versatility and consequent variety of applicability in the clinical environment (Zeiler et al., 2014). The main reasons for this medicine are its significant sedative and anti-nociceptive effects and its known anesthetic, analgesic, anti-inflammatory, and antidepressant properties (Zanos et al., 2018).

The effects of ketamine are mediated mainly by acting as a non-competitive antagonism of the *N*-methyl-*D*-aspartate (NMDA) receptor in the central nervous system (Portmann et al., 2010). This drug has relative lipid solubility and a short latency period, as it quickly crosses the blood-brain barrier, especially when administered intravenously. After passing through the more perfused tissues, it redistributes to less perfused tissues, reducing central effects (Li et al., 2019).

Commercially, ketamine is presented for clinical use as a racemic mixture with the *R*(-) and *S*(+) enantiomers (Casoni et al., 2015). It is extensively metabolized *in vivo*, forming its primary metabolite, norketamine, followed by the production of secondary metabolites (Zanos et al., 2018). The ketamine metabolism starts from demethylation, where the liver enzymes CYP2B6 and CYP3A4 from cytochrome P<sub>450</sub> are mainly responsible for this process (Rao et al., 2016).

The enzymes responsible for this drug's metabolism act differently on the two isomers. CYP2B6 demethylates ketamine enantiomers with almost equal efficiency, and CYP3A4 demethylates the *S*(+) enantiomer more rapidly (Portmann et al., 2010). Therefore, part of the individual variability of its metabolism is attributed to differences in the expression of cytochrome P<sub>450</sub> enzymes in the individual's body (Hijazi & Boulieu, 2002b).

Despite being commonly administered by intramuscular or intravenous routes, using ketamine by alternative routes has shown to be very promising (Kronenberg, 2002; Niesters et al., 2014; Jonkman et al., 2017). The intranasal route, for example, has been widely used in medical practice, especially in pediatric patients, and is increasingly applied to the routine of veterinary medicine. Human medicine has described this route as an effective and safe method for procedures requiring analgesia and sedation in pediatric patients (Poonai et al., 2017), even in hospital emergency sectors (Guthrie et al., 2021). In addition, the use of intranasal ketamine has demonstrated efficacy in the control of chronic and neuropathic pain (Huge et al., 2010; Singh et al., 2018; Shteamer et al., 2019).

In the field of veterinary medicine, a study evaluated the use of intranasal ketamine in cats associated with midazolam (Marjani et al., 2015). The treatment promoted effective sedation in animals of this species compared to intramuscular administration, avoiding the discomfort caused by this route. This reinforces the versatility and ease of this drug's application in different contexts.

Drugs with more positive octanol/water partition coefficients tend to bind strongly to plasma proteins, and ketamine has a partition coefficient value of 2.3 and showed a binding rate to total plasma proteins close to 47% in adult human patients (Dayton et al., 1983). The correct measurement of this parameter is important because the portion of the drug that does not bind to plasma proteins influences the determination of the pharmacokinetic parameter known as distribution volume. This parameter indicates how much a drug can leave the blood flow and reach the tissues. Therefore, it is a reference for estimating the drug concentration in tissues at its site of action (Dinis-Oliveira, 2017). Considering the different pharmacokinetic phases of distribution, metabolism, and excretion, it is known that only the portion of the drug that is not bound to plasma proteins can cross cell membranes and go through these steps. Conceptually, the elimination phase comprises metabolic processes, where the drug is transformed into metabolites to be excreted or by excretion processes of the unmodified drug, either by renal and/or biliary excretion. As stated, only the free drug can undergo these pharmacokinetic processes (Smith et al., 2010). The reason is that only the free drug can cross cell membranes and exert its effect, and the portion that remains bound to specific sites of plasma proteins does not manifest the expected effects in the body (Trainor, 2007; Smith et al., 2010; Yamasaki et al., 2013).

In dogs, the binding rate of ketamine for plasma proteins reached about 27%, bound especially to plasma glycoproteins

(Dayton et al., 1983; Dinis-Oliveira, 2017), which showed lower affinity than that observed in humans. In horses, the rate was similar to that found in humans and presented an average of 50% of binding when administered intravenously (Kaka et al., 1979). The importance of the affinity level of a given drug to plasma proteins is reinforced when there is an association of drugs during the patient's therapy (Yamasaki et al., 2013), which is a common condition during clinical and hospital routines. The process of competitive displacement can occur during drug association and ends in changes in the degrees of specific drug affinity for the binding site in the protein (Otagiri, 2005). Alterations in the affinity levels of a drug for a specific region of the protein result in different concentrations of the portion of free drug in plasma and have a direct impact on the identification of its systemic effects (Smith et al., 2010).

Based on this context, this study aimed to evaluate the binding rate of ketamine to total plasma proteins from domestic cats by the ultrafiltration method, measured by the high-performance liquid chromatography technique coupled to tandem mass spectrometry with electrospray ionization source (HPLC-ESI-MS/MS). The results of this work may contribute to the development of new ketamine administration routes in animals, as well as to evaluate more adequate clinical protocols using this drug. Furthermore, the values of the binding rate, as well as the binding constant for ketamine to total plasma proteins, have not yet been reported in the literature for cats using this technique.

## Material and Methods

### Biological material

This work was carried out after approval by the Ethics Committee in the Use of Animals (CEUA) of the State University of Maringá, protocol 3292020621. Blood samples were collected from six healthy, mixed-breed cats of both genders aged between one and five years. Samples were collected by puncture of the jugular vein and added to previously heparinized microtubes. Plasma was immediately separated by centrifugation at 3,500 rpm for 12 min in a clinical benchtop centrifuge (Metroterm). In the end, the plasma samples were combined in a single plasma pool solution of 35 mL. The concentration of total proteins in the cat plasma pool ( $\text{g}\cdot\text{dL}^{-1}$ ) was determined by the Biuret method kit, as recommended by the Labtest Diagnostics' manufacturer.

### Construction of the LC-ESI-MS/MS calibration curve and optimization of detection and ionization parameters

A solution of racemic ketamine ( $R(-)$  and  $S(+)$ ) (PubChem CID 3821) at  $300 \text{ ng}\cdot\text{mL}^{-1}$ , dissolved in methanol,

was used to optimize the ionization parameters by an HPLC equipment coupled to a triple quadrupole mass spectrometer and electrospray ionization source, model LCMS-8050 (Shimadzu). Then, a calibration curve was constructed in 20 mM potassium phosphate buffer, pH 7.2, at concentrations ranging from 40 to  $850 \text{ ng}\cdot\text{mL}^{-1}$ . All analyses were performed on a C18 Chim-pack XR-ODS III column ( $75 \text{ mm} \times 2 \text{ mm}$  internal diameter,  $1.6 \mu\text{m}$  particle size). Water (A) and methanol (B) with 0.1% formic acid were solvents. The LC flow was isocratic with 85% methanol at  $0.1 \text{ mL}\cdot\text{min}^{-1}$ . The analyses were performed in triplicate, and the values of concentrations obtained at each point corresponded to the average of two injections of  $10 \mu\text{L}$ . The TIC chromatograms in MRM mode were processed by the LabSolutions Insight program (Shimadzu), and the area corresponding to each chromatographic band was used to construct the calibration curve. The coefficient of variation was used to assess the quality of data points in the calibration curve. All solvents used in the test were LCMS grade (Merck), and the racemic ketamine standard was provided by the König company.

### Plasma sample preparation by ultrafiltration method for dosage of ketamine

Considering the need for a direct dosage of the ketamine from plasma, that is, without using extraction procedures, total ketamine ( $C_T$ ) and free ketamine ( $C_F$ ) species were performed in two different ways.

For the dosage of total ketamine ( $C_T$ ), two series of microtubes with varying concentrations of the drug were prepared. The first series was the Plasma series, and the second was the Phosphate Buffer series. In the Plasma series, ketamine concentrations ranging from  $48.8$  to  $780.5 \text{ ng}\cdot\text{mL}^{-1}$  were prepared in  $400 \mu\text{L}$  of plasma pool. In the Phosphate Buffer series, the same ketamine concentration range between  $48.8$  to  $780.5 \text{ ng}\cdot\text{mL}^{-1}$  was prepared in  $400 \mu\text{L}$  of 20 mM Phosphate Buffer, pH 7.2.

Then, all microtubes from both series were incubated in a water bath at  $30 \text{ }^\circ\text{C}$  for 20 minutes and transferred to a 0.5 mL Amicon Ultra® concentrator with a 10 kDa regenerated cellulose filter membrane cutoff. The sample was centrifuged at  $30 \text{ }^\circ\text{C}$  for 2 h at 3,500 g. After this period, the filtrate volume ( $\sim 100 \mu\text{L}$ ) was transferred to inserts in LCMS vials to measure the corresponding ketamine concentrations.

The Phosphate Buffer series was built for two reasons. First, the total ketamine (CT) that would have been added to the Plasma series (filter) should be dosed without requiring extraction. Second, as there were no plasma proteins in the

Phosphate Buffer series tubes, the ketamine concentration of the filtering should be equal to that of the filtrate, and with this procedure, verify if the concentrator membrane could somehow retain the ketamine and interfere with the results. Thus, the ketamine concentrations added to the Phosphate Buffer equal those added to the plasma and make up the fraction of  $C_T$ .

The Plasma series was used to obtain free ketamine that did not bind to plasma ( $C_F$ ) after ultrafiltration. Thus, the difference between the concentration of free ketamine measured in microfiltered plasma ( $C_F$ ) and the concentration of total ketamine measured in the Phosphate Buffer ( $C_T$ ) series gives the concentration of ketamine that has bound to plasma proteins ( $C_B$ ). Sample preparation was carried out based on the methodology proposed by (Diniz et al., 2008).

### Measurement of ketamine binding rate to plasma proteins

The areas of the TIC chromatograms generated after passing the samples through the LC-ESI-MS/MS were used to determine the ketamine concentrations by applying the linear equation constructed with the calibration curve. The concentration values obtained were used to calculate the binding rate of ketamine (%) to plasma proteins according to Equation 1.

$$\text{Binding (\%)} = \frac{C_T - C_F}{C_T} \times 100 \quad (1)$$

where  $C_T$  is the total concentration of ketamine added to plasma and  $C_F$  is the concentration of free ketamine in plasma after microfiltration.

### Determination of the binding constant

The  $C_F$  values were used to calculate the binding constant ( $K_b$ ) using the Scatchard method (Tinoco et al., 2013), where the Hill equation (Equation 2) was fitted to the experimental data points (Bordbar et al., 1996).

$$v = g \frac{(K_b [C_F])^{n_H}}{1 + (K_b [C_F])^{n_H}} \quad (2)$$

where  $v$  is the fraction of the drug bound by the macromolecule,  $g$  is a factor of maximum amplitude of the binding curve,  $[C_F]$  is the free drug concentration,  $K_b$  is the binding constant, and  $n_H$  is the Hill coefficient (cooperativity).

### Bioinformatics

The crystallographic structure of cat serum albumin at 3.4 Å resolution (Yokomaku et al., 2018) was obtained

from the Protein Data Bank (PDB) and used in molecular docking simulations using the default configurations of the program Autodock-Vina (Trott & Olson, 2010), implemented in PyRx-0.9 graphical interface (Dallakyan & Olson, 2015). In this procedure, the structure of Ketamine (PubChem CID 3821) was docked four times in each of the seven binding sites described for albumin (Fasano et al., 2005), and the average scores for each site were compared. We assume that the sites with the best scores are most likely for ketamine to bind.

### Results

Table 1 shows the final parameters after optimization for ionization and detection of ketamine by HPLC-ESI-MS/MS. This procedure is important to detect the lowest drug concentrations above the detection limit (noise) inherent to the assay.

TPolar solvents like water may make ionizing the substance of interest difficult. Thus, it is important to optimize the detection parameters, with the drug dissolved in pure methanol, and maintain a high concentration of this solvent in the mobile phase along LC analysis. However, it maintains a percentage of water that guarantees the solubilization and separation of other components from the plasma during the passage through the column.

After the optimization procedures of the LC and MS methods, the calibration curve for ketamine dosage was constructed, whose results are shown in Figure 1, where the average variation coefficient was below 9.0% (Table 2). The calibration curve was constructed in a pH 7.2 phosphate buffer to approximate the conditions of peripheral plasma

Table 1 – Final parameters obtained after optimization for detection of ketamine by the LC-ESI/MS/MS equipment

Parameter	Condition
LC flow	0.1 mL·min <sup>-1</sup>
Solvent B concentration (methanol)	85%
Column oven temperature	40 °C
Sample injection volume	10 µL
Nebulizing gas	3 L·min <sup>-1</sup>
Drying gas	10 L·min <sup>-1</sup>
Heating gas	10 L·min <sup>-1</sup>
CID gas (argon)	270 kPa
ESI interface temperature	200 °C
Desolvation temperature	526 °C
DL temperature	250 °C
Heat Block temperature	400 °C
Precursor ion $m/z$ rate	238.1
Product ions $m/z$ rate	125.05, 132.10, 209.95
Event time	0.309 s
Dwell time	0.100 s

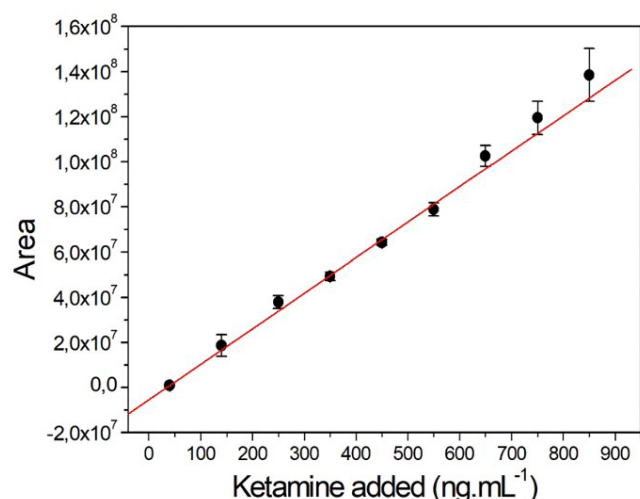


Figure 1 – Calibration curve for ketamine dosage in 20 mM potassium phosphate buffer, pH 7.2. Each point represents the mean of three replicates with two injections each (six chromatograms). Linear regression parameters are  $y = a + bx$ ,  $a = -537,382.00 \pm 186,285.35$ ;  $b = 157,273.32 \pm 2290.73$ ;  $R=0.99926$ .

Table 2 - Variation coefficients were obtained for each mean concentration data point in constructing the ketamine calibration curve

Concentration (ng.mL <sup>-1</sup> )	Mean area	Standard deviation	Variation coefficient (%)
40	$0.91 \times 10^7$	$1.52 \times 10^6$	16.68
140	$1.87 \times 10^7$	$4.78 \times 10^6$	25.62
250	$3.79 \times 10^7$	$2.85 \times 10^6$	7.51
350	$4.93 \times 10^7$	$1.81 \times 10^6$	3.67
450	$6.42 \times 10^7$	$1.38 \times 10^6$	2.15
550	$7.89 \times 10^7$	$2.94 \times 10^6$	3.72
650	$1.03 \times 10^8$	$4.64 \times 10^6$	4.53
750	$1.19 \times 10^8$	$7.39 \times 10^6$	6.19
850	$1.39 \times 10^8$	$1.17 \times 10^7$	8.47
<b>Mean:</b>			<b>8.73</b>

as closely as possible but without being concentrated in salts to impair the ionization of ketamine or cause damage to the ionization interface. After constructing the calibration curve, we proceeded with the dosage of the samples added to ketamine, whose results are shown in Figure 2A.

The concentration of  $C_T$ , represented in Figure 2A, was obtained by adding ketamine to the phosphate buffer, which was then ultrafiltered in an Amicon concentrator and dosed both in the filtering and the filtrate elements of this series. Since there was no significant difference ( $p < 0.05$ ) between the concentration of  $C_T$  in the filtering and the filtrate, we concluded that ketamine did not bind to the regenerated cellulose membrane of the concentrator when evaluated in phosphate buffer. This provided security for  $C_F$  quantification in the microfiltered plasma series as the

difference between  $C_T$  (added to plasma), and  $C_F$  (measured in microfiltered plasma) can be attributed entirely to bound ketamine ( $C_B$ ). Thus, applying Equation 1 to the corresponding ketamine concentration values resulted in a binding rate close to 65% to the cat's total plasma proteins when a plasmatic concentration peak above  $300 \text{ ng} \cdot \text{mL}^{-1}$  was reached (Figure 2B).

To determine the total protein binding constant of ketamine ( $K_b$ ), measuring the concentration of total proteins in the plasma pool was necessary, performed with a clinical diagnostic kit by the Biuret method. As a result, the concentration of total proteins in the plasma pool used in this assay, without the addition of drugs (pure plasma), was  $8 \text{ g} \cdot \text{dL}^{-1}$  ( $8.36 \times 10^4 \text{ mg} \cdot \text{L}^{-1}$ ), a value that is within the reference values for this marker ( $6.0$  to  $8.0 \text{ g} \cdot \text{dL}^{-1}$ ) (Weiss & Wardrop, 2010).

The ketamine binding constant to plasma proteins was calculated using the Scatchard plot. For this, the molecular mass of cat serum albumin (*Felix catus*), recorded in the UniProt database (entry P49064), was considered, which is  $65,845.05 \text{ Da}$ . Then, Equation 2 was adjusted to the experimental data points to obtain the parameters  $K_b$  and  $n_H$ , as shown in Figure 3.

The fit of Equation 2 to the experimental data points in Figure 3 provided a binding constant  $K_b$  of  $2 \times 10^6 \text{ mol} \cdot \text{L}^{-1}$  and a Hill coefficient ( $n_H$ ) of 2.3. The result of the docking simulations can be seen in Table 3, which evidences the existence of three sites of high affinity for ketamine. The location of the ketamine binding sites in cat serum albumin can be seen in Figure 4.

## Discussion

From a pharmacological point of view, it is much more interesting to determine a drug's binding rate to total plasma proteins than to albumin alone. Binding to total proteins can provide a more realistic view of bioavailability. At the same time, the binding rate of a drug to albumin generates information that is more focused on the thermodynamic and biophysical points of view.

The ketamine binding rate to cat total plasma proteins has already been reported by equilibrium dialysis (Hanna et al., 1988), showing a mean binding percentage of 37.54%, and also by the ultrafiltration method associated with gas chromatography (Hijazi & Bouliou, 2002a). This last experiment evaluated the binding of the isolated form of the drug (66% of binding) and in the presence of metabolites (60% of binding), both at  $30^\circ\text{C}$ . This value agrees with the rate found in our study using ketamine isolated (65%). The difference between our work and the

Table 3 – Mean scores from the docking simulations of ketamine in the seven binding sites described for albumin in the 5yxe PDB entry. The more negative the value, the higher the score

Binding site (subdomain)	Box center at x, y and z	Box dimension at x, y and z	Mean score
IB	32, -1, 42	13, 13, 13	-6.9 ± 0.00
IIB	00, 03, 21	14, 13, 14	-6.8 ± 0.05
IIA (Sudlow's site I)	08, -2, 35	13, 13, 13	-6.8 ± 0.00
IA	13, 06, 51	13, 18, 13	-5.0 ± 0.00
IIB	44, -7, 09	13, 13, 15	-4.1 ± 0.22
IIIA (Sudlow's site II)	18, -7, 16	15, 13, 13	-3.2 ± 0.00
IIIA (Sudlow's site II)	24, -1, 13	14, 14, 14	0.8 ± 0.10

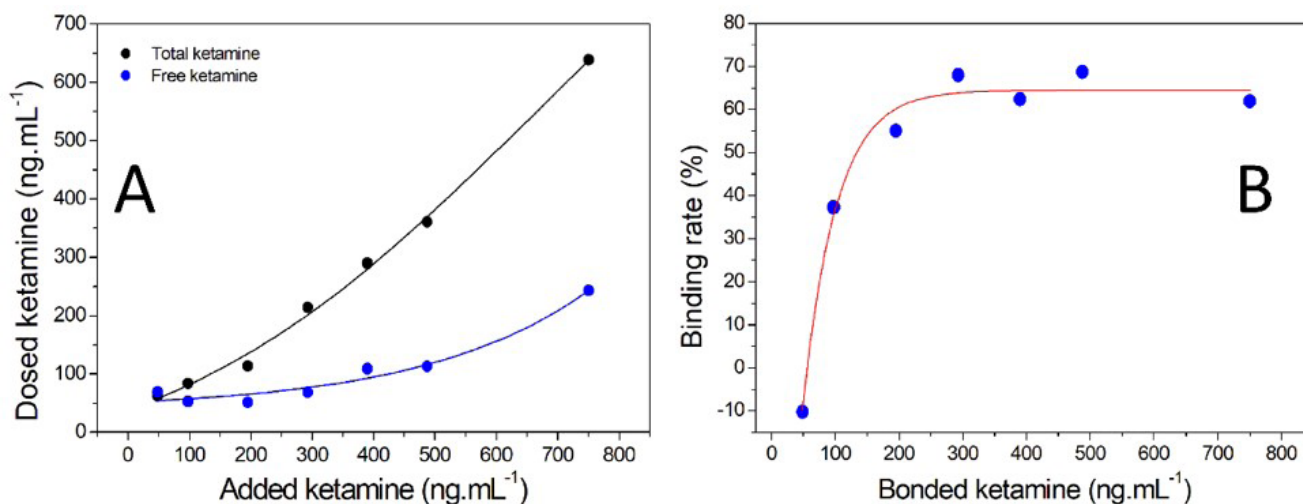


Figure 2 – (A) Ketamine levels are measured in a phosphate buffer (filtering element representing CT, black circles) and in microfiltered plasma (filtered element representing  $C_F$ , blue circles); (B) Ketamine binding rate in total plasma proteins of domestic cats as a function of drug concentration, according to Equation 1. The size of the circles is the size of the standard deviation.

previous is our use of a much more sensitive methodology and the determination of the binding constant ( $K_b$ ), which demonstrated positive cooperativity in the binding of ketamine ( $n_H = 2.3$ ). The values of binding constants of a drug to plasma proteins can be used to estimate possible interaction effects between drugs in the pharmacokinetic distribution phase (Ye et al., 2016).

Comparatively, the values reported in the literature for the binding rate of ketamine to total plasma among other species, such as equines and humans, were similar to each other, reaching about 50% of the binding percentage in both species (Kaka et al., 1979; Dayton et al., 1983; Williams et al., 2004). In dogs, the binding rate of ketamine to total plasma proteins was 53.1%, measured by the equilibrium dialysis method (Kaka et al., 1979). In another assay with the canine species, lower values of binding rate to total plasma proteins were found by the same method (33%). However, the pH presented an average value of 7.36 (Dayton et al., 1983).

However, a drug's binding rate to plasma proteins is already known to be influenced by the medium's pH (Dayton et al., 1983; Kragh-Hansen, 1990). A pH below the physiological

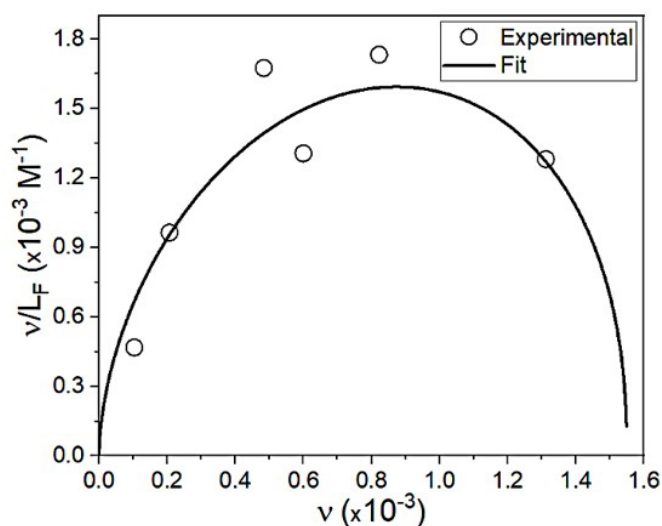


Figure 3 – Scatchard plot for the binding of ketamine to cat total plasma proteins. The solid line shows the fit to the experimental data points through Equation 2, with a correlation coefficient (R) of 0.94.

value for a given species tends to decrease the drug's affinity for plasma proteins and, consequently, the drug's distribution

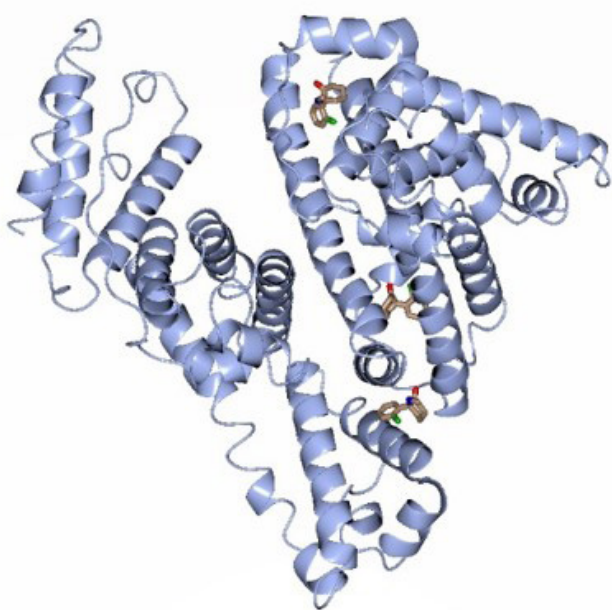


Figure 4 – Ribbon model of cat serum albumin (cyan) showing the location of the three high-affinity binding sites for ketamine (brown). Source: Laboratory of Structural Biochemistry – DTC/UEM (2023).

volume in the organism (Hinderling & Hartmann, 2005). This condition makes the pH a parameter of interest for correlating the drug's binding affinity to plasma proteins.

The binding rate of a drug to total plasma proteins is also influenced by the serum albumin concentration, which can be noticed by comparing the affinity of a drug in plasma or in serum, where the last one is rich in albumin, and results in similar binding rates (Colclough et al., 2014; Yang et al., 2014). The plasma albumin concentration can be altered in cases of metabolic diseases such as liver diseases, kidney diseases, or injuries such as burns, which are clinical conditions responsible for altering the concentrations of this protein in the plasma (Otagiri, 2005; Fanali et al., 2012).

It is known that highly hydrophobic compounds tend to bind much more strongly to plasma proteins than hydrophilic substances (Arnott & Planey, 2012). The solubility of a compound can be determined by the octanol/water partition coefficient ( $XlogP3$ ), in which hydrophobic compounds tend to have positive values. In contrast, hydrophilic compounds tend to have negative values. Thus, comparatively, a significantly hydrophobic drug like propofol (PubChem CID 4943) has an  $XlogP3$  of 3.8 and a binding rate of 88.7% in human plasma. In contrast, a much less hydrophobic drug like morphine (PubChem CID 5288826), has an  $XlogP3$  of 0.8 with a total plasma protein binding rate of 31.6% in horses (Combie et al., 1983; Altmayer et al., 1995; Roberts et al., 2013). Ketamine has an intermediate  $XlogP3$  of 2.2 (PubChem CID 3821). Thus, the degree of

solubility of this drug was consistent with the binding rate to plasma proteins measured for cats in this work (65%).

The ketamine binding constant to plasma proteins was described in this work as being  $2 \times 10^6 \text{ mol} \cdot \text{L}^{-1}$ . Comparatively, other ligands showed binding constants to albumin of similar values. For example, the flavonoids have binding constants ranging from  $1 - 15 \times 10^4 \text{ M}^{-1}$  (Dufour & Dangles, 2005), and the drug rofecoxib (PubChem CID 5090) has  $K_b$  of  $3,450 \times 10^6 \text{ M}^{-1}$  at  $31^\circ \text{C}$  (Qi et al., 2008) and  $XlogP3$  of 2.3. However, the binding constants of small molecules to albumin generally range between  $10^3 - 10^8 \text{ M}^{-1}$  (Kragh-Hansen, 1990).

Albumin is the total plasma protein found in concentrations of around  $46.7 \text{ g} \cdot \text{L}^{-1}$  in cats (Taylor et al., 2010). The same occurs in human plasma, which has albumin as the primary plasma protein component, but with more significant variations in concentrations, from  $26.5$  to  $46.5 \text{ g} \cdot \text{L}^{-1}$  (Schaller et al., 2008). The albumin concentration is more constant in healthy cats since it is in a narrower concentration range, which helps to explain why the values of binding rates and binding constant reported in the literature are very similar to ours. These values were cited at the beginning of this discussion.

The positive Hill coefficient is related to the positive cooperativity in the binding process of a ligand to a protein. This means that binding the first ketamine molecule to the first site of a protein facilitates binding at other sites (Tinoco et al., 2013). We assume that the cooperativity is due to ketamine binding to albumin, as this is the major plasma protein and is also capable of binding more than one drug at the same time since it is known that albumin has more than one binding site (Carter & Ho, 1994). Positive cooperativity in the binding of ketamine to albumin is described for the first time in this work.

Albumin has six subdomains in which up to seven equivalents of long-chain fatty acids at multiple binding sites with different affinities bind (Fasano et al., 2005). The results of our docking simulations indicate three high-affinity sites for ketamine located in subdomains IB, IIB, and IIA, respectively. These sites were identified by the highest ketamine docking score (Table 1). In addition to the higher score, another factor that indicates that the ligand binds to these sites is the binding mode (pose). When all simulations recover the same pose, with a low root mean square deviation (rmsd), it indicates a drug-like interaction mode. In cases where this does not happen, that is, each simulation presents a different pose with high rmsd, it is doubtful that the drug should interact with this site. The rmsd for ketamine poses in simulations in those three sites was less than  $0.1 \text{ \AA}$ .

The three sites identified in the binding of ketamine to albumin are consistent with those observed in other structures of this protein co-crystallized in the presence of other drugs that also occupy, at least, one of these same sites (PDB entries: 5v0v, 1h9z, 2bxc, 2bx8). This indicates that these are the preferred sites for drugs to bind to albumin. These results also match those observed in our binding experiments, where a Hill coefficient of 2.3 was found. This can also be interpreted as just over two ketamine molecules binding to cat serum albumin (Tinoco et al., 2013).

## Conclusions

The analytical methodology used in this work was able to provide reliable information on the ketamine binding rates to plasma proteins from domestic cats, and the data generated here allowed us to establish the binding constant ( $K_b$ ) of  $2 \times 10^6 \text{ mol} \cdot \text{L}^{-1}$  and Hill coefficient ( $n_H$ ) of 2.3. Docking simulations on the crystallographic structure of cat serum albumin suggest up to three high-affinity sites for ketamine, which corroborates the data obtained experimentally by the Scatchard plot. These data can be used to assess the

risks of the dislocation of ketamine binding to proteins caused by other drugs and to estimate the potential of drug interactions in the pharmacokinetic distribution phase.

## Conflict of Interest

All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report.

## Ethics Statement

All actions reported in this manuscript were carried out following the procedures set out in Federal Law No. 11,794 of October 8, 2008, and in Decree No. 6,899 of July 15, 2009, approved by the Ethics Committee on the Use of Animals (CEUA) of the Universidade Estadual de Maringá, under Process n° 3292020621.

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## References

- Altmayer P, Büch U, Büch HP. Propofol binding to human blood proteins. *Arzneimittelforschung*. 1995;45(10):1053-6. PMID:8595056.
- Arnott JA, Planey SL. The influence of lipophilicity in drug discovery and design. *Expert Opin Drug Discov*. 2012;7(10):863-75. <http://doi.org/10.1517/17460441.2012.714363>. PMID:22992175.
- Bordbar AK, Saboury AA, Moosavi-Movahedi AA. The shapes of Scatchard plots for systems with two sets of binding sites. *Biochem Educ*. 1996;24(3):172-5. [http://doi.org/10.1016/0307-4412\(95\)00122-0](http://doi.org/10.1016/0307-4412(95)00122-0).
- Carter DC, Ho JX. Structure of serum albumin. *Adv Protein Chem*. 1994;45:153-203. [http://doi.org/10.1016/S0065-3233\(08\)60640-3](http://doi.org/10.1016/S0065-3233(08)60640-3). PMID:8154369.
- Casoni D, Spadavecchia C, Adami C. S-ketamine *versus* racemic ketamine in dogs: their relative potency as induction agents. *Vet Anaesth Analg*. 2015;42(3):250-9. <http://doi.org/10.1111/vaa.12200>. PMID:25041216.
- Colclough N, Ruston L, Wood JM, MacFaul PA. Species differences in drug plasma protein binding. *MedChemComm*. 2014;5(7):963-7. <http://doi.org/10.1039/C4MD00148F>.
- Combie JD, Nugent TE, Tobin T. Pharmacokinetics and protein binding of morphine in horses. *Am J Vet Res*. 1983;44(5):870-4. PMID:6869996.
- Dallakyan S, Olson AJ. Small-molecule library screening by docking with PyRx. *Methods Mol Biol*. 2015;1263:243-50. [http://doi.org/10.1007/978-1-4939-2269-7\\_19](http://doi.org/10.1007/978-1-4939-2269-7_19). PMID:25618350.
- Dayton PG, Stiller RL, Cook DR, Perel JM. The binding of ketamine to plasma proteins: emphasis on human plasma. *Eur J Clin Pharmacol*. 1983;24(6):825-31. <http://doi.org/10.1007/BF00607095>. PMID:6884418.
- Dinis-Oliveira RJ. Metabolism and metabolomics of ketamine: a toxicological approach. *Forensic Sci Res*. 2017;2(1):2-10. <http://doi.org/10.1080/20961790.2017.1285219>. PMID:30483613.
- Diniz A, Escuder-Gilabert L, Lopes NP, Villanueva-Camañas RM, Sagrado S, Medina-Hernández MJ. Characterization

- of interactions between polyphenolic compounds and human serum proteins by capillary electrophoresis. *Anal Bioanal Chem.* 2008;391(2):625-32. <http://doi.org/10.1007/s00216-008-2046-4>. PMID:18418586.
- Dufour C, Dangles O. Flavonoid-serum albumin complexation: determination of binding constants and binding sites by fluorescence spectroscopy. *Biochim Biophys Acta.* 2005;1721(1-3):164-73. <http://doi.org/10.1016/j.bbagen.2004.10.013>. PMID:15652191.
- Fanali G, di Masi A, Trezza V, Marino M, Fasano M, Ascenzi P. Human serum albumin: from bench to bedside. *Mol Aspects Med.* 2012;33(3):209-90. <http://doi.org/10.1016/j.mam.2011.12.002>. PMID:22230555.
- Fasano M, Curry S, Terreno E, Galliano M, Fanali G, Narciso P, Notari S, Ascenzi P. The extraordinary ligand binding properties of human serum albumin. *IUBMB Life.* 2005;57(12):787-96. <http://doi.org/10.1080/15216540500404093>. PMID:16393781.
- Guthrie AM, Baum RA, Carter C, Dugan A, Jones L, Tackett T, Bailey AM. Use of Intranasal ketamine in pediatric patients in the emergency department. *Pediatr Emerg Care.* 2021;37(12):e1001-7. <http://doi.org/10.1097/PEC.0000000000001863>. PMID:31290798.
- Hanna RM, Borchard RE, Schmidt SL. Plasma protein binding of ketamine and metabolite I in the cat. *J Vet Pharmacol Ther.* 1988;11(1):115-7. <http://doi.org/10.1111/j.1365-2885.1988.tb00129.x>. PMID:3379662.
- Hijazi Y, Bouliou R. Protein binding of ketamine and its active metabolites to human serum. *Eur J Clin Pharmacol.* 2002a;58(1):37-40. <http://doi.org/10.1007/s00228-002-0439-4>. PMID:11956671.
- Hijazi Y, Bouliou R. Contribution of CYP3A4, CYP2B6, and CYP2C9 isoforms to N-demethylation of ketamine in human liver microsomes. *Drug Metab Dispos.* 2002b;30(7):853-8. <http://doi.org/10.1124/dmd.30.7.853>. PMID:12065445.
- Hinderling PH, Hartmann D. The pH dependency of the binding of drugs to plasma proteins in man. *Ther Drug Monit.* 2005;27(1):71-85. <http://doi.org/10.1097/00007691-200502000-00014>. PMID:15665750.
- Huge V, Lauchart M, Magerl W, Schelling G, Beyer A, Thieme D, Azad SC. Effects of low-dose intranasal (S)-ketamine in patients with neuropathic pain. *Eur J Pain.* 2010;14(4):387-94. <http://doi.org/10.1016/j.ejpain.2009.08.002>. PMID:19733106.
- Jonkman K, Duma A, Velzen M, Dahan A. Ketamine inhalation. *Br J Anaesth.* 2017;118(2):268-9. <http://doi.org/10.1093/bja/aew457>. PMID:28100533.
- Kaka JS, Klavano PA, Hayton WL. Pharmacokinetics of ketamine in the horse. *Am J Vet Res.* 1979;40(7):978-81. PMID:507501.
- Kragh-Hansen U. Structure and ligand binding properties of human serum albumin. *Dan Med Bull.* 1990;37(1):57-84. PMID:2155760.
- Kronenberg RH. Ketamine as an Analgesic: parenteral, oral, rectal, subcutaneous, transdermal and intranasal administration. *J Pain Palliat Care Pharmacother.* 2002;16(3):27-35. [http://doi.org/10.1080/J354v16n03\\_03](http://doi.org/10.1080/J354v16n03_03). PMID:14640353.
- Li R, Zhong K, Jiang J, Zhan Y, Chen X. Enantioselective determination of ketamine in dog plasma by chiral liquid chromatography-tandem mass spectrometry. *Biomed Chromatogr.* 2019;33(9):e4578. <http://doi.org/10.1002/bmc.4578>. PMID:31077428.
- Marjani M, Akbarinejad V, Bagheri M. Comparison of intranasal and intramuscular ketamine-midazolam combination in cats. *Vet Anaesth Analg.* 2015;42(2):178-81. <http://doi.org/10.1111/vaa.12183>. PMID:24986665.
- Niesters M, Martini C, Dahan A. Ketamine for chronic pain: risks and benefits. *Br J Clin Pharmacol.* 2014;77(2):357-67. <http://doi.org/10.1111/bcp.12094>. PMID:23432384.
- Otagiri M. A Molecular functional study on the interactions of drugs with plasma proteins. *Drug Metab Pharmacokinet.* 2005;20(5):309-23. <http://doi.org/10.2133/dmpk.20.309>. PMID:16272748.
- Poonai N, Canton K, Ali S, Hendrikx S, Shah A, Miller M, Joubert G, Rieder M, Hartling L. Intranasal ketamine for procedural sedation and analgesia in children: a systematic review. *PLoS One.* 2017;12(3):e0173253. <http://doi.org/10.1371/journal.pone.0173253>. PMID:28319161.
- Portmann S, Kwan HY, Theurillat R, Schmitz A, Mevissen M, Thormann W. Enantioselective capillary electrophoresis for identification and characterization of human cytochrome P450 enzymes which metabolize ketamine and norketamine *in vitro*. *J Chromatogr A.* 2010;1217(51):7942-8. <http://doi.org/10.1016/j.chroma.2010.06.028>. PMID:20609441.
- Qi Z-D, Zhou B, Qi X, Chuan S, Liu Y, Dai J. Interaction of rofecoxib with human serum albumin: determination

- of binding constants and the binding site by spectroscopic methods. *J Photochem Photobiol Chem*. 2008;193(2-3):81-8. <http://doi.org/10.1016/j.jphotochem.2007.06.011>.
- Rao LK, Flaker AM, Friedel CC, Kharasch ED. Role of cytochrome P4502B6 polymorphisms in ketamine metabolism and clearance. *Anesthesiology*. 2016;125(6):1103-12. <http://doi.org/10.1097/ALN.0000000000001392>. PMID:27763887.
- Roberts JA, Pea F, Lipman J. The clinical relevance of plasma protein binding changes. *Clin Pharmacokinet*. 2013;52(1):1-8. <http://doi.org/10.1007/s40262-012-0018-5>. PMID:23150213.
- Schaller J, Gerber S, Kämpfer U, Lejon S, Trachsel C. Human blood plasma proteins: structure and function. West Sussex: Wiley; 2008. 538 p. <http://doi.org/10.1002/9780470724378>.
- Shteamer JW, Harvey RD, Spektor B, Curseen K, Egan K, Chen Z, Gillespie TW, Sniecinski RM, Singh V. Safety of intranasal ketamine for reducing uncontrolled cancer-related pain: protocol of a phase I/II clinical trial. *JMIR Res Protoc*. 2019;8(4):e12125. <http://doi.org/10.2196/12125>. PMID:31038469.
- Singh V, Gillespie TW, Harvey RD. Intranasal ketamine and its potential role in cancer-related pain. *Pharmacother J Hum Pharmacol Drug Ther*. 2018;38(3):390-401. <http://doi.org/10.1002/phar.2090>. PMID:29396996.
- Smith DA, Di L, Kerns EH. The effect of plasma protein binding on *in vivo* efficacy: misconceptions in drug discovery. *Nat Rev Drug Discov*. 2010;9(12):929-39. <http://doi.org/10.1038/nrd3287>. PMID:21119731.
- Taylor SS, Tappin SW, Dodkin SJ, Papasouliotis K, Casamian-Sorrosal D, Tasker S. Serum protein electrophoresis in 155 cats. *J Feline Med Surg*. 2010;12(8):643-53. <http://doi.org/10.1016/j.jfms.2010.03.018>. PMID:20655494.
- Tinoco I, Sauer K, Wang J, Puglisi J, Harbison G, Rovnyak D. Physical chemistry: principles and applications in biological sciences. 5th ed. Boston: Pearson; 2013. 724 p.
- Trainor GL. The importance of plasma protein binding in drug discovery. *Expert Opin Drug Discov*. 2007;2(1):51-64. <http://doi.org/10.1517/17460441.2.1.51>. PMID:23496037.
- Trott O, Olson AJ. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J Comput Chem*. 2010;31(2):455-61. <http://doi.org/10.1002/jcc.21334>. PMID:19499576.
- Weiss DJ, Wardrop KJ. Schalm's veterinary hematology. 6th ed. Hoboken: Wiley-Blackwell; 2010. 1206 p.
- Williams ML, Mager DE, Parenteau H, Gudi G, Tracy TS, Mulheran M, Wainer IW. Effects of protein calorie malnutrition on the pharmacokinetics of ketamine in rats. *Drug Metab Dispos*. 2004;32(8):786-93. <http://doi.org/10.1124/dmd.32.8.786>. PMID:15258102.
- Yamasaki K, Chuang VTG, Maruyama T, Otagiri M. Albumin-drug interaction and its clinical implication. *Biochim Biophys Acta*. 2013;1830(12):5435-43. <http://doi.org/10.1016/j.bbagen.2013.05.005>. PMID:23665585.
- Yang F, Zhang Y, Liang H. Interactive association of drugs binding to human serum albumin. *Int J Mol Sci*. 2014;15(3):3580-95. <http://doi.org/10.3390/ijms15033580>. PMID:24583848.
- Ye M, Nagar S, Korzekwa K. A physiologically based pharmacokinetic model to predict the pharmacokinetics of highly protein-bound drugs and the impact of errors in plasma protein binding. *Biopharm Drug Dispos*. 2016;37(3):123-41. <http://doi.org/10.1002/bdd.1996>. PMID:26531057.
- Yokomaku K, Akiyama M, Morita Y, Kihira K, Komatsu T. Core-shell protein clusters comprising haemoglobin and recombinant feline serum albumin as an artificial O<sub>2</sub> carrier for cats. *J Mater Chem B Mater Biol Med*. 2018;6(16):2417-25. <http://doi.org/10.1039/C8TB00211H>. PMID:32254458.
- Zanos P, Moaddel R, Morris PJ, Riggs LM, Highland JN, Georgiou P, Pereira EFR, Albuquerque EX, Thomas CJ, Zarate Junior CA, Gould TD. Ketamine and ketamine metabolite pharmacology: insights into therapeutic mechanisms. *Pharmacol Rev*. 2018;70(3):621-60. <http://doi.org/10.1124/pr.117.015198>. PMID:29945898.
- Zeiler GE, Dzikiti BT, Fosgate GT, Stegmann FG, Venter FJ, Rioja E. Anaesthetic, analgesic and cardiorespiratory effects of intramuscular medetomidine-ketamine combination alone or with morphine or tramadol for orchietomy in cats. *Vet Anaesth Analg*. 2014;41(4):411-20. <http://doi.org/10.1111/vaa.12136>. PMID:24576259.

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