Supporting Information

A novel approach for characterizing propofol binding affinities to serum albumins from different species

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The Supporting information contains:

Tables S1-S3 present the information on the chemicals, and protein concentrations used in the HPLC experiments.

Tables S4-S8 describe the main components of the HPLC-MS system, MS settings for the multiple reaction monitoring (MRM) method, gradient HPLC parameters, and the preparation of the calibration standards.

Figure S1 shows the calibration curve using 10 µg/mL of propofol.
Table S1: Analyzed substances

| Substance                     | Abbreviation | Supplier     | Tracking number | Serial number |
|-------------------------------|--------------|--------------|-----------------|---------------|
| Propofol (2,6-Diisopropylphenol) | PR           | Sigma Aldrich| 218-206-6       | P185          |
| Bos taurus (Rind) Serumalbumin | BSA          | Sigma Aldrich| A2153-10G       | SLBQ0908V     |
| Canis familiaris (Hund) Serumalbumin | CSA      | Abcam        | ab119814        | GR3186902-7   |
|                               |              |              |                 | GR3231297-1   |
|                               |              |              |                 | GR3231320-1   |
| Homo sapiens (Mensch) Serumalbumin | HSA        | Sigma Aldrich| A1653-1G        | SLBT9727      |
| Rattus norvegicus (Ratte) Serumalbumin | RatSA     | Sigma Aldrich| A6272-500MG     | SLBD8890V     |
| Oryctolagus cuniculus (Kaninchen) Serumalbumin | RSA    | Sigma Aldrich| A0639-5G        | 105K7565V     |
| Ovis aries (Schaf) Serumalbumin | SSA         | Sigma Aldrich| A3264-1G        | SLBC5764V     |

Table S2: Chemicals

| Substance            | Supplier       | Tracking number | Serial number |
|----------------------|----------------|-----------------|---------------|
| H₂O                  | Promochem      | SO-9368-B025    | 1256 631      |
| Methanol             | Promochem      | SO-9356-B025    | 1230571       |
| Perchloric acid 70%  | Aldrich        | 311421-50mL     | SHBH9317      |
| Ammonium hydroxide 25%| Fluka Analytical | 44273-10X1ML-F | BCBM1912V    |
| Ammonium bicarbonate | Fluka Analytical | 40867-50G-F    | BCBJ0875V     |

Sample preparation

The samples were prepared for all albumins according to the following scheme. In three experiments, the samples were prepared with the albumin concentrations of 40 mg/mL, 20 mg/mL, 10 mg/mL, 6 mg/mL, 4 mg/mL, 2 mg/mL, 1 mg/mL, 0.5 mg/mL and 0 mg/mL. After adding 10 µg of propofol, the remaining concentration of propofol was determined. An HPLC-MS (High Performance Liquid Chromatography-mass spectrometry) was used for this. To save albumin, we opted for a halved approach at constant concentrations.

The first step in the preparation of the samples consisted of weighting 140 mg of the albumin to be examined in a 5 mL clear glass vessel. In order to reach the initial concentration of 40 mg/mL, the
lyophilized albumin was mixed with 3.5 mL of water. A magnetic stirrer ensured the complete dissolution without macroscopically visible remains. 0.7 mL of this 40 mg/mL starting solution was diluted with 2.1 mL of H₂O, which corresponded to a concentration of 10 mg/mL. A concentration of 4 mg/mL was achieved with 0.25 mL of the starting solution to 2.25 mL of H₂O. With 0.1 mL of the starting solution and 3.9 mL of H₂O, the albumin concentration was 1 mg/mL. After mixing the solutions by vortex, further sample processing was carried out in glass vials with Teflon-coated lids. Each albumin concentration was set up in triplicate according to the following scheme.

**Table S3:** Further dilution of the samples based on the previously prepared solutions

| Albumin solution | Total volume | H₂O volume | Albumin concentration |
|------------------|--------------|------------|-----------------------|
| 40 mg/mL         | 0.5 mL       | 0 mL       | 40 mg/mL              |
|                  | 0.25 mL      | 0.25 mL    | 20 mg/mL              |
| 10 mg/mL         | 0.5 mL       | 0 mL       | 10 mg/mL              |
|                  | 0.3 mL       | 0.2 mL     | 6 mg/mL               |
| 4 mg/mL          | 0.5 mL       | 0 mL       | 4 mg/mL               |
|                  | 0.25 mL      | 0.25 mL    | 2 mg/mL               |
| 1 mg/mL          | 0.5 mL       | 0 mL       | 1 mg/mL               |
|                  | 0.25 mL      | 0.25 mL    | 0.5 mg/mL             |
|                  | 0 mL         | 0.5 mL     | 0 mg/mL               |

Each of these 27 samples was then mixed with 0.05 mL of a 100 µg/mL propofol solution. To prepare this propofol solution, 10.0 mg of propofol was weighted out. The propofol was dissolved in 1 mL of methanol. To achieve a concentration of 100 µg/mL, 0.1 mL of this solution was mixed with 9.9 mL of a methanol-water mixture (ratio 1:1). The added water allowed the propofol solution to be pipetted exactly. The Propofol solutions were made in glass jars and stored in the dark when not in use. After adding the propofol to the albumins, the samples were mixed by vortex. After a three-minute contact time, the albumin in the samples was precipitated with 0.025 mL of 70% perchloric acid (HClO₄). This step allowed the albumin to be removed from the solutions. It became necessary because proteins would collect in the column of the liquid chromatograph and make it impermeable. The denatured albumin settled on the floor. After five minutes, 170 µL of the supernatant was pipetted into a glass insert. This could be placed in an Eppendorf tube so that the samples could be centrifuged at 22 °C and 10000 RPM for five minutes. The glass inserts were then placed in the glass vials of the HPLC-MS and analyzed.
Quantitative analysis of the samples

The samples were analyzed using HPLC-MS (High Performance Liquid Chromatography-mass spectrometry) to determine the remaining free propofol in the samples. The HPLC system consisted of a degassing unit from Shimadzu Prominence (DGU-20A5R), two liquid chromatography pumps from Nexera (LC-30AD), a sampler from Nexera (SIL-30AC), a column thermostat with integrated column switching valve from Prominence (CTO-20AC) and a communication module from Prominence (CBM-20A). The mass spectrometer was an 8030Plus triple quadrupole mass spectrometer with an APCI ion source (Shimadzu, Japan). The LabSolutions software was used to control the HPLC-MS system and to evaluate the chromatograms.

Table S4: Components of the LC-MS system

| Component                                           | Serial number       |
|-----------------------------------------------------|----------------------|
| Prominence DGU-20A5R degassing unit                 | L20704901317         |
| Nexera LC-30AD liquid chromatograph pump A           | L20555070826         |
| Nexera LC-30AD liquid chromatograph pump B           | L20555070829         |
| Nexera SIL-30AC autosampler                         | L20565070386         |
| Prominence CTO-20AC column oven with integrated column switching valve | L20215074847         |
| Prominence CBM-20A communication bus module         | L20234975576         |
| 8030Plus triple quadrupole mass spectrometer        | O10254900142JA       |
| LabSolutions software                               | Version 5.85         |

A Kinetex 5u EVO C18 100Å LC column was used for the chromatographic separation (100 x 2.1 mm; order number: 00D-4633-AN; Serial no. 735206-4) from Phenomenex (Aschaffenburg, Germany). The analytes were quantified in MRM mode (Multiple Reaction Monitoring). The MRP optimization function was used to select the propofol ion relevant for the analysis and to determine the optimal parameters for Q1 pre-bias, CE and Q3 pre-bias. The mass spectrometer was operated in negative mode. The APCI interface temperature was set to 400 °C and the voltage to –3.5 kV. The heat block temperature was set to 300 °C, the desolvation line temperature to 150 °C, the nebulizing gas flow to 2.5 L/min and the drying gas flow to 5.0 L/min.

Table S5: MS settings for the MRM method for propofol

| Channel | Precursor m/z | Product m/z | Q1 Pre Bias | CE  | Q3 Pre Bias |
|---------|---------------|-------------|-------------|-----|-------------|
| Ch1     | 177.05        | 177.15      | 34 V        | 9 V | 17 V        |
The settings for the MRM method and the source parameters were entered. The run time was 3.5 minutes. A gradient elution was carried out with eluent A (10 mM ammonium hydrogen carbonate solution with 0.1% by volume 25% ammonia solution; pH 9.10) and eluent B (methanol) as described in Table 4. The flow rate was set to 0.7 mL/min, the temperature of the column oven to 40 °C and that of the sampler to 20 °C.

Table S6: HPLC gradient program

| Time                  | Eluent B concentration |
|-----------------------|------------------------|
| 0.00 min bis 2.00 min | 55% zu 88%             |
| 2.10 min bis 2.50 min | 95%                    |
| 2.51 min bis 3.50 min | 55%                    |

The quantification was carried out with the method of the external standard, calculated with a linear calibration function over the peak area. For this, three calibrator solutions with the known propofol concentrations of 10, 5 and 2 µg per 1.15 mL were used. The volume of the calibrator solutions corresponded to that of the albumin solutions (1.15 mL). In order to save albumin, the entire test batch was halved. The calibrator solutions thus had a volume of 0.575 mL. The already described propofol solution with 100 µg/mL was used to prepare the calibrator solutions.

Table S7: Preparation of the calibrator solutions based on 100 µg / mL

|                   | Dilution in MeOH/H₂O 1:1 | Propofol concentration |
|--------------------|--------------------------|------------------------|
| Pre-Calibrator 1   | undiluted                | 100 µg/mL              |
| Pre-Calibrator 2   | 0.5/1                    | 50 µg/mL               |
| Pre-Calibrator 3   | 0.2/1                    | 20 µg/mL               |

0.05 mL of each of these calibrator solutions was used. The work-up was carried out analogously to the propofol albumin samples, in that 0.5 mL of H₂O and 0.025 mL of HClO₄ were added. This resulted in a volume of 0.575 mL per sample. Since both the calibrator solutions and the albumin solutions were halved, the ratio was preserved.

Table S8: Calibrator solutions used in the study

| Calibrator       | Concentration          | Dilution          |
|------------------|------------------------|-------------------|
| Calibrator 1     | 5 µg/0.575 mL          | 10 µg/1.15 mL     |
| Calibrator 2     | 2.5 µg/0.575 mL        | 5 µg/1.15 mL      |
**Figure S1:** Method calibration using 10 µg/mL of propofol