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Research paper

In vitro release of propofol and binding capacity with regard to plasma constituents

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ABSTRACT

Purpose: New evidence suggests that the anesthetic effect of parenteral propofol emulsions varies between commercial preparations. We examined and compared different propofol preparations to determine propofol release and binding capacity with regard to plasma lipoproteins and albumin.
Methods: We created a novel assay consisting of microtiter plates coated with either low-density lipoprotein (LDL) or albumin to analyze propofol binding kinetics. Using high performance liquid chromatography (HPLC), we measured propofol release from the oily phase and the corresponding amount of propofol bound to the plates in a time-dependent manner and at equilibrium conditions attained after 30 min of incubation at 37 °C. The concentrations of free propofol in the aqueous phase of different propofol preparations – Diprivan, and the generic formulations Propofol "Fresenius" (1% and 2% propofol) and Propofol-Lipuro – were analyzed using ultracentrifugation or dialysis for phase separation. Finally, we investigated the effect of isolated lipoprotein fractions on propofol release.
Results: Propofol bound to LDL-coated plates with approximately twofold higher affinity than to albumin-coated plates. No significant differences in total propofol release were observed between preparations. Moreover, similar amounts of free propofol were observed in the aqueous phase of all products tested (1% propofol preparations: 18 μg/ml; 2% propofol preparations: 35 μg/ml), except for the medium-chain and long-chain triglyceride (MCT/LCT) preparation studied, in which the concentration of free propofol was lower. Lipoproteins had no effect on propofol release, except for high-density lipoprotein (HDL), which triggered almost 100% release from the oily phase at HDL concentrations above 1000 μg/ml.
Conclusions: No differences were observed between the binding/release capacity and lipoprotein interactions of any of the propofol preparations tested. We propose that clinical observations of inconsistent propofol activity are related to variations in the lipoprotein profile, enzyme activity or genetic disorders of individual patients, rather than to the propofol preparation itself.

1. Introduction

Propofol (2,6-di-isopropylphenol) is a commonly used lipophilic anesthetic, which induces rapid onset of hypnosis. As propofol is nearly insoluble in water, it is administered in lipid droplets in an oil-in-water emulsion [1]. In general, this emulsion is composed of soybean oil, additionally added triacylglycerols for medium-chain and long-chain triglyceride (MCT/LCT) formulations, ovolecithin, glycerol, oleic acid, sodium hydroxide, and water. Once injected into the bloodstream, propofol is rapidly released and transported to the brain. The transport is probably mediated by binding to plasma constituents such as lipoproteins, albumin or erythrocytes [1,2]. The mechanism is not fully understood, although the hypnotic effect is known to involve the GABA-system. Propofol binds to the $GABA_A$ receptor, a chloride ion channel, and increases the binding of GABA to the receptor. This in turn leads to an opening of chloride channels, an increased influx of chloride ions, which induces hyperpolarization of the cell, and subsequent desensitization [3].

Since the narcotic effect occurs immediately after administration, the process of release must be very fast. Blood analysis has revealed that 48% of propofol is bound in serum, 51% is bound to erythrocytes and only 1% is present in an unbound state, due to the low water solubility of propofol. In serum, 98% of propofol is bound to serum proteins (mainly albumin), and high-density-, low-density- and very low-density-lipoproteins (HDL, LDL and VLDL), and passes the blood–brain barrier in a very short time period [2,4,5]. To some extent, propofol shows a distinct liver metabolism and becomes hydroxylated in the *para*-position. Propofol is conjugated to sulfates and glucuronides, and is excreted by the kidneys.

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Although patients wake up very quickly after the infusion, complete excretion of propofol takes several hours because of its slow elimination from lipophilic body compartments [6].

In this paper, we report on the *in vitro* release behavior of propofol from its lipophilic emulsion phase by binding to either albumin or lipoprotein fractions. For this purpose, we have developed a novel assay consisting of microtiter plates coated with streptavidin, in combination with biotinylated lipoproteins or albumin. Additionally, the concentration of free propofol in the aqueous phase of the emulsion was determined, as it may be an important parameter correlated, for example, with pain on injection [7–9].

This comparative study was initiated after reports of inter-individual differences of propofol pharmacokinetics in a clinical trial. Possible differences in the doses of propofol preparations required to maintain anesthesia were reported in various patients [10]. After preliminary tests of physical and chemical properties, no differences could be found between preparations employed in our study. Therefore, we started to consider other comparison criteria such as the kinetics of propofol release from the oily phase of injectable emulsions, binding ability to blood components, and the amount of free propofol in the aqueous phase of the emulsions.

2. Materials and methods

2.1. General materials

All chemicals were purchased from Sigma–Aldrich (Vienna, Austria) or VWR (Vienna, Austria). D-Biotin-*N*-hydroxy-succinimide ester was obtained from Roche Diagnostics (Vienna, Austria). Table 1 shows the propofol products investigated in this study. They were all purchased in a public pharmacy, except for Propofol "Fresenius" which was kindly donated by Fresenius Kabi Austria GmbH, Graz, Austria.

Blood samples for the plasma pool were collected from 25 normolipidemic healthy volunteers following overnight fasting.

The dialysis membrane used was a Medicell Membrane MWCO: 12,000–14,000 (Bartelt, Graz, Austria), unless otherwise stated.

2.2. Methods

2.2.1. Lipoprotein isolation

The lipoprotein fractions VLDL, LDL and HDL were isolated from a plasma pool by multiple-step ultracentrifugation. To prevent clotting, blood was collected in vials containing EDTA at a final concentration of 0.1%. Cellular components were removed by centrifugation (Jouan CR 412, LabX, Midland, Canada) at 3000 rpm (1500*g*) for 10 min at 4 °C. The plasma in the supernatant was withdrawn and treated with 60% sucrose to a final concentration of 6 g/l, and immediately frozen at −70 °C.

Aliquots of the cryoprotected plasma were thawed and centrifuged for 24 h at 44,000 rpm (140,000*g*) and 4 °C at its own density (Sorvall Ultra Pro 80, rotor: T-865, Kendro, Vienna, Austria). VLDL was removed in the top layer, and the remaining phase (containing LDL, HDL and the lipoprotein-free fraction) was adjusted to a density of 1.063 g/cm^3 by addition of solid KBr. After repeated ultracentrifugation LDL was removed from the top layer. In order to separate HDL from the lipoprotein-free plasma, the density of the remaining solution was increased to 1.21 g/cm^3 by addition of solid KBr, and again centrifuged for 72 h at 140,000*g* and 4 °C.

The separated fractions were dialyzed overnight at 4 °C against a $NaHCO_3$ buffer solution (50 mM $NaHCO_3$, 0.9% NaCl, 0.01% EDTA, pH 8.0) with buffer changes every 8 h and stored under argon at 4 °C.

2.2.2. Biotinylation of LDL

Total cholesterol concentration (taken as sum of free cholesterol and cholesteryl esters) was determined using an enzymatic assay (CHOD-PAP, Roche Diagnostics, Germany), assuming a total cholesterol content of 50 wt% for LDL [11]. The concentration of LDL was adjusted to 2 mg/ml (i.e. 4 mg/ml total cholesterol) by dilution with 50 mM $NaHCO_3$ buffer.

Biotinylation of LDL was performed as described [12]. Briefly, 10 μl of a biotin stock solution (10 mg/ml D-biotin-*N*-hydroxy-succinimide ester dissolved in dimethylformamide) was added per milliliter of LDL and the mixture was stirred for 1 h at room temperature. The biotinylation process was stopped by addition of 8 μl of 1 M NH_4Cl per milliliter of LDL solution and the mixture was dialyzed against 10 mM Tris/HCl buffer, pH 7.4 (0.9% NaCl, 0.01% EDTA) for 24 h (with buffer changes every 8 h) to remove excess NH_4Cl. Under these experimental conditions, 11 biotin molecules are bound per LDL particle [12,13].

2.2.3. Coating of microtiter plates

Streptavidin-coated microtiter plates (Nunc, Wiesbaden, Germany) were washed three times with Sörensen buffer (10 mM Na_2HPO_4/KH_2PO_4, pH 7.4). The wells were then filled with 200 μl of biotinylated LDL or biotinylated BSA (bovine serum albumin, 2 mg/ml, Immuno Pure, Pierce Technology, New York, USA). After 2 h of incubation (Eppendorf Thermomixer Comfort) at 300 rpm, the liquids were removed and the wells were washed twice with Sörensen buffer, and subsequently filled with buffer and stored at 4 °C.

The binding capacity for biotin is about 12 pmol/well (8.44 pmol/cm^2), according to the manufacturer's manual, which corresponds to about 1 pmol LDL (corresponding to 2.5 μg assuming an average molecular weight of 2.5×10^6 g/mol) or 1.5 pmol BSA (corresponding to ~0.1 μg) per well.

2.2.4. Propofol release

The coated microtiter plates were heated to 37 °C and each well was filled with 200 μl propofol emulsion, which was diluted to 1:400 (v/v) with PBS (phosphate-buffered saline pH 7.4, 0.2 g/L KCl, 0.2 g/L KH_2PO_4, 8 g/L NaCl, 1.44 g/L $Na_2HPO_4{\cdot}2H_2O$), vortexed (10 s) and immediately filled into each well. The 1:400 dilution was chosen to reflect the dilution factor when the emulsion is injected in the human body (10 ml propofol emulsion diluted in 4 l of human blood). The ratio between LDL/lipid added by the emulsion will account to 1:20 (w/w) LDL to lipid. In case of BSA the weight ratio was 1:500 BSA to lipid. The microtiter plates were incubated for 0–50 min at 37 °C and 300 rpm and samples were drawn every 5–10 min, followed by four rigorous washing steps using PBS. Further experiments were performed after reaching an

Table 1
Propofol products used in this study

Product	Charge	Composition[a]
Propofol 2% LCT "Fresenius"	E020092	LCT, 20 mg/ml propofol, 10% lipid
Propofol 1% LCT "Fresenius"	E050041	LCT, 10 mg/ml propofol, 10% lipid
Propofol 2% MCT/LCT "Fresenius"	E010027	MCT/LCT, 20 mg/ml propofol, 10% lipid
Propofol 1% MCT/LCT "Fresenius"	D120080	MCT/LCT, 10 mg/ml propofol, 10% lipid
Diprivan 2% (Astra Zeneca)	T3017B	LCT, 20 mg/ml propofol, 10% lipid
Diprivan 1% (Astra Zeneca)	BM716	LCT, 10 mg/ml propofol, 10% lipid
Propofol-Lipuro 1% (Braun)	4072A183	MCT/LCT, 10 mg/ml propofol, 10% lipid

The preparations are marked as long-chain triglyceride emulsions (LCT) and medium chain mixed with long-chain triglyceride emulsions (MCT/LCT). Also the propofol concentration and lipid content for each formulation is indicated.

[a] Further excipients are identical for all preparations, including: soybean oil, ovolecithin, glycerol, oleic acid, sodium hydroxide, and water.

equilibrium which was obtained after 30 min of incubation. LDL- or BSA-bound propofol was extracted and dissolved with 200 μl pure isopropyl alcohol within 5 min. Due to preliminary experiments these extractions led to 100% recovery. Aliquots of the alcoholic solution of propofol were then injected into an HPLC system to quantify the amount of propofol.

2.2.5. Propofol in the aqueous phase

To determine the propofol concentration in the aqueous phase of the emulsion, separation of the two phases was performed using either ultracentrifugation or dialysis of undiluted and 1:400 diluted propofol samples.

Fifty milliliters of propofol emulsion was dialyzed against 1 ml glycerol solution (2.25%) in water for 5 h at room temperature. An aliquot of this glycerol solution (20 μl) was diluted with isopropyl alcohol to 1 ml, and injected into an HPLC system.

Ultracentrifugation was performed at 30,000 rpm (~100,000*g*) at 4 °C for 5 h (Sorvall Combi Plus, Rotor TH 641, Inula, Vienna, Austria). PCR-vials were filled with 200 μl propofol emulsion and after centrifugation the aqueous phase was extracted with a syringe. An aliquot of 20 μl of this phase was diluted with isopropyl alcohol to 1 ml and analyzed by HPLC.

A free fatty acid assay (Half Micro Test, Roche Diagnostics, Vienna, Austria) was used to confirm quantitative phase separation. This assay is based on the cleavage of triglycerides into glycerol and free fatty acids through lipase activity. The concentration of free fatty acids in propofol products is extremely low and does not interfere with this enzymatic assay.

2.2.6. Influence of lipoproteins on propofol release

The concentrations of the lipoprotein fractions were estimated from their total cholesterol content, which was determined enzymatically (CHOD-PAP, Roche Diagnostics, Germany), assuming a total cholesterol content of 20, 50 and 20 wt% for VLDL, LDL and HDL, respectively. Propofol emulsions (final dilution 1:400 v/v) were prepared containing 0–2 mg/ml of lipoproteins in PBS buffer, reflecting physiological conditions [11]. After 30-min incubation at 37 °C, the samples were centrifuged (30,000 rpm, 4 °C, 5 h) and the propofol content in the aqueous phase measured as described above.

Due to the fact that we have just anticipated to outline fundamental effects of the influence of lipoprotein fractions on propofol release, we have performed these experiments only with one preparation, namely Propofol 2% LCT "Fresenius". In addition, a control experiment was conducted using lipoprotein-free plasma (LPFP), which was prepared according to the procedure described in Section 2.2.1.

2.2.7. Analytical procedure

The isocratic HPLC used for these analyses contained a fluorescence detector (Merck Hitachi L-7480, Germany) with an excitation wavelength of 270 nm and an emission wavelength of 310 nm, and a Hypersil ODS, 200× 2.1 mm, 5 μm column. The mobile phase was a mixture of 40% NaH_2PO_4 buffer, pH 3.0, and 60% acetonitrile. The column temperature was held constant at 40 °C, the flow rate was 0.5 ml/min and each run was stopped after 10 min. The propofol peak appeared after 4.5 min.

2.2.8. Statistical analysis and data handling

Data are expressed as means ± standard deviation (SD). A Kruskal–Wallis test was performed to determine statistical significance, assumed at $p < 0.05$ using SigmaStat v3.5. Graphs were created using SigmaPlot v9.01 and curves were fitted using an equation for exponential rise ($y = a \cdot (1 - e^{-bx})$), which often is applicable for pharmaceutical release processes.

3. Results

3.1. Propofol release

We tested seven propofol preparations (Table 1). Diprivan (Astra Zeneca) and Propofol "Fresenius" (Fresenius Kabi, Austria) were available as 1% and 2% propofol emulsions, corresponding to 10 and 20 mg/ml propofol, respectively. Propofol-Lipuro (Braun) was only available as a 1% emulsion. Propofol "Fresenius" was also classified as LCT (long-chain triglycerides) and MCT/LCT (medium-chain and long-chain triglycerides) preparations.

LDL- or BSA-coated microtiter plates were filled with different propofol emulsions (1:400 diluted) and incubated at 37 °C for 50 min. After incubation, propofol bound to the coated wells was extracted with alcohol and quantified by HPLC. First, we analyzed the release kinetics of propofol from the oily phase into the aqueous compartment, from which the drug was bound to LDL or albumin. All propofol preparations achieved an equilibrium between propofol bound to the coated wells, and propofol remaining in the diluted emulsion after 30 min (only shown for Propofol 2% LCT "Fresenius" in Fig. 1).

The equilibrium values between the different 1:400 diluted propofol products did not vary significantly ($n = 4–7$), meaning that the concentrations of propofol bound to LDL after 30 min of incubation were almost the same for all 1% preparations as well as for the 2% preparations. In all 2% preparations, 300–350 ng propofol per milliliter diluted propofol emulsion was bound to LDL, whereas 150–180 ng/ml was bound in all 1% preparations. The same pattern was observed with BSA-coated wells, except that the amount of bound propofol was approximately 50% lower than in the LDL-coated wells; 150–180 ng/ml for the 2% preparations and only 70–90 ng/ml for the 1% preparations. The results and a comparison between the different propofol products are shown in Fig. 2.

3.2. Propofol in the aqueous phase

To determine the amount of propofol dissolved in the aqueous phase, undiluted and diluted propofol emulsions were separated into lipid and aqueous phases. High concentrations of triacylglycerols in the aqueous phase of the undiluted samples indicated insufficient phase separation using ultracentrifugation, while dialysis led to good phase separation of all preparations. Both dialysis

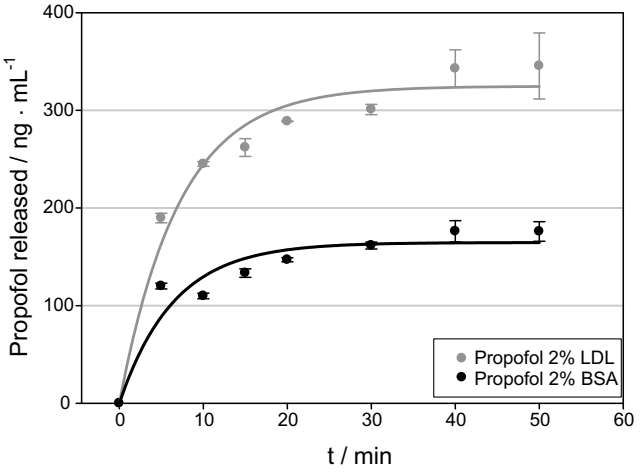

Fig. 1. Propofol release (propofol 2% LCT "Fresenius") and binding kinetics on microtiter plates coated with LDL or BSA. Propofol (1:400 in PBS) bound to the wells was extracted from the coating, dissolved in isopropyl alcohol, and analyzed by HPLC. Values are means ± SD ($n = 3$).

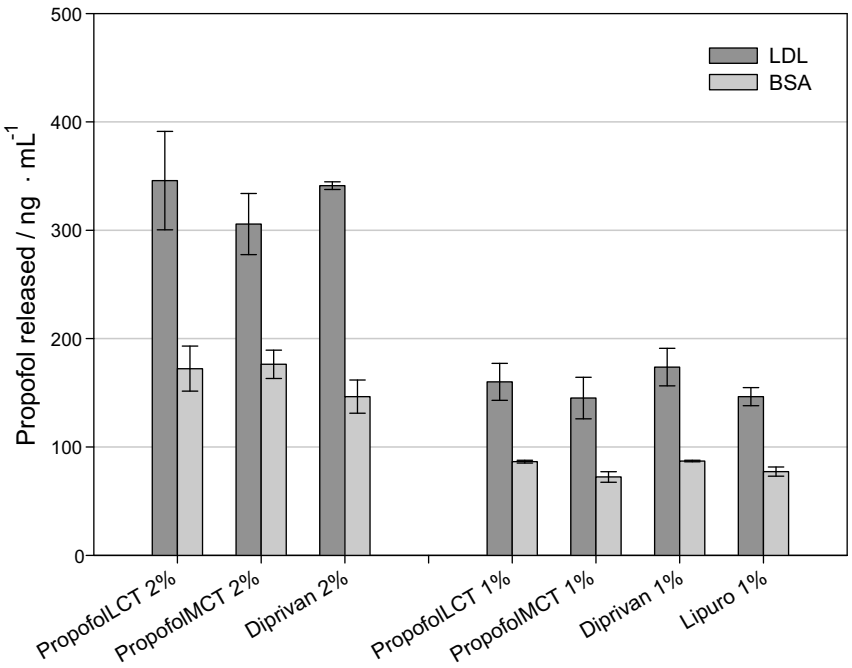

Fig. 2. Propofol released from different preparations (1:400 in PBS; 50 μg/ml for 2% formulations, 25 μg/ml for 1% formulations) at 37 °C after 30 min of incubation in microtiter plates coated with LDL or BSA. Propofol was extracted from the coating, dissolved in isopropyl alcohol, and analyzed by HPLC. Values are means ± SD (n = 4–7).

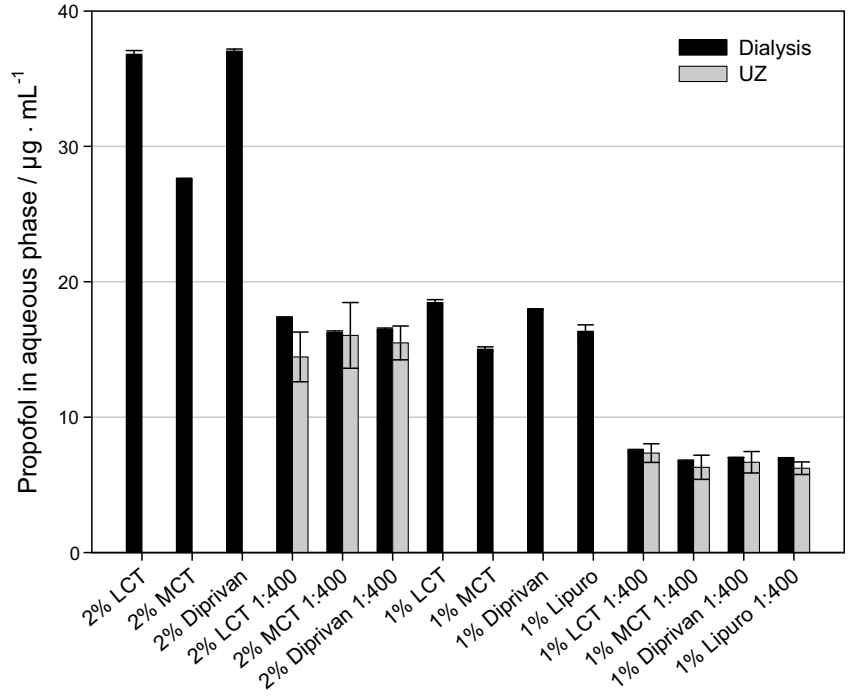

Fig. 3. Concentration of free propofol in the aqueous phase (μg/ml) of diluted (1:400 in PBS) and undiluted samples. Samples were separated using ultracentrifugation (UZ) for 5 h at 100,000g, or dialysis for 5 h against a glycerol solution in water (2.25%). Values are means ± SD (n = 3).

and ultracentrifugation achieved good separation of the diluted samples. Propofol concentrations in the aqueous phase are shown in Fig. 3.

Following phase separation and drug-content analysis, all preparations were the same except for the undiluted 1% and 2% propofol preparations from Propofol "Fresenius", in which significant differences between LCT and MCT/LCT preparations were obtained. The concentration of free propofol in the aqueous phase of the undiluted 2% preparations was found to be 36.81 μg/ml in the LCT, but only 27.57 μg/ml in the MCT/LCT preparations. These values correspond to 0.18% free propofol in LCT and 0.14% in MCT/LCT preparations. For the undiluted 1% preparations: 18.47 μg/ml (0.18%) and 15.02 μg/ml (0.15%) free propofol were detected in the LCT and MCT/LCT preparations, respectively.

After dilution, these differences were not observed any longer. Interestingly, the concentrations of free propofol in the diluted

samples were found to be much higher than that calculated by a 1:400 dilution factor: about 15 μg/ml and 7 μg/ml for the 2% and 1% propofol preparations, respectively. This corresponds to 30% free propofol in the 2% preparations and 28% in the 1% preparations. No differences in propofol concentrations were found between the Fresenius Kabi, Astra Zeneca, and Braun 1% preparations (see Fig. 3).

3.3. Influence of lipoproteins on propofol release

To see whether different lipoprotein fractions of human serum affect propofol release into the aqueous phase, we added 0–2 mg/ml of VLDL, LDL or HDL fractions to a 1:400 diluted 2% propofol LCT emulsion. Again, the propofol concentration in the aqueous phase was measured after ultracentrifugation, and neither VLDL nor LDL significantly affected propofol release (propofol concentration in the aqueous phase was about 15 μg/ml regardless of lipoprotein content).

In contrast, HDL containing samples showed an increase in propofol concentrations in the aqueous phase, with rising HDL concentrations up to a theoretical maximum of 50 μg/ml propofol. This corresponds to 100% release from the oily phase, which was already achieved at 1.5 mg/ml HDL (Fig. 4).

Finally, 1:400 diluted propofol emulsions were incubated with lipoprotein-free plasma (LPFP) in the same way as described for lipoproteins, to see whether plasma components other than lipoproteins might affect propofol release. Interestingly, the LPFP induced a concentration-dependent propofol release from the oily phase to the aqueous compartment, in which plasma proteins were present (Fig. 5).

4. Discussion

In comparison to solid drug formulations, for which *in vitro* dissolution and drug-release measurements are standardized, *in vitro* drug release from parenteral emulsions into the plasma is difficult to evaluate. The aim of this study was to develop an *in vitro* assay to analyze the release of lipophilic substances from lipid emulsions, and to determine their partition between the oily emulsion phase and possible binding sites in the blood, namely, lipoproteins or albumin. Propofol served as model drug, since variation in the composition of the emulsion might have an influence on the onset of anesthesia and pain of injection [1,2,4,8].

We first measured the kinetics of propofol release/binding by incubating BSA- or LDL-coated microtiter plates with propofol preparations (1:400 dilutions), for different time periods ranging from 5 to 50 min (Fig. 1). With this experimental design, we simulated the dilution of the emulsion in the plasma after injection. The quantity of bound propofol as detected by these assays did only increase within the first 20 min and reached a plateau after this time, indicating that equilibrium conditions are achieved. That saturation of the assay is not the reason for the plateau can be deduced from the results obtained for the 1% propofol preparations in comparison to the 2% preparations. LDL-coated microtiter plates showed a higher propofol-binding capacity than BSA-coated plates. This observation correlates also to the data published by Zamacona et al. who demonstrated that the binding rate of propofol to LDL is higher than to BSA [4]. In addition, with LDL-coated microtiter plates, we observed a combined first-order release and binding kinetic. It is most likely that the transition of propofol from the oily phase to the lipoprotein-binding site occurs by direct contact and interaction between the LDL and the oil droplets. This was supported by investigations of a propofol raw emulsion (data not shown), which consists mainly of large oil droplets with a mean diameter of 7 μm, instead of the final emulsions, which have a mean diameter of approximately 200 nm. More propofol was bound to the LDL-coating using the large oil droplets instead of the smaller droplets, although one would suppose that the smaller droplets have a larger specific surface and thus the contact area between LDL and oil droplets should be larger. We presume that in case of large droplets an increased coating thickness is responsible for this effect. This assumption was also confirmed by an oily film observed on the wall after emptying the wells, which could be solubilized with isopropyl alcohol. Following these preliminary experiments we established a thorough washing procedure to remove as much residual oil as possible and prevent such artefacts.

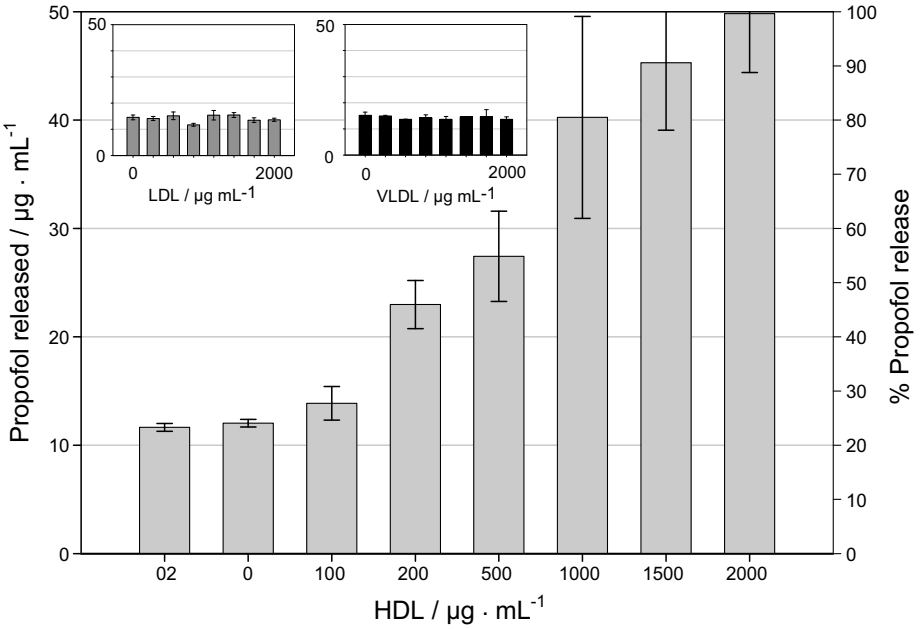

Fig. 4. Concentration of propofol of diluted (1:400 in PBS) propofol 2% LCT "Fresenius" emulsions in the aqueous phase (μg/ml), following incubation with different concentrations of lipoprotein fractions (HDL, LDL, and VLDL) at 37 °C for 30 min. Samples were prepared by ultracentrifugation (100,000*g*). The amount of propofol in the aqueous phase was determined by HPLC. Values are means ± SD ($n = 2$).

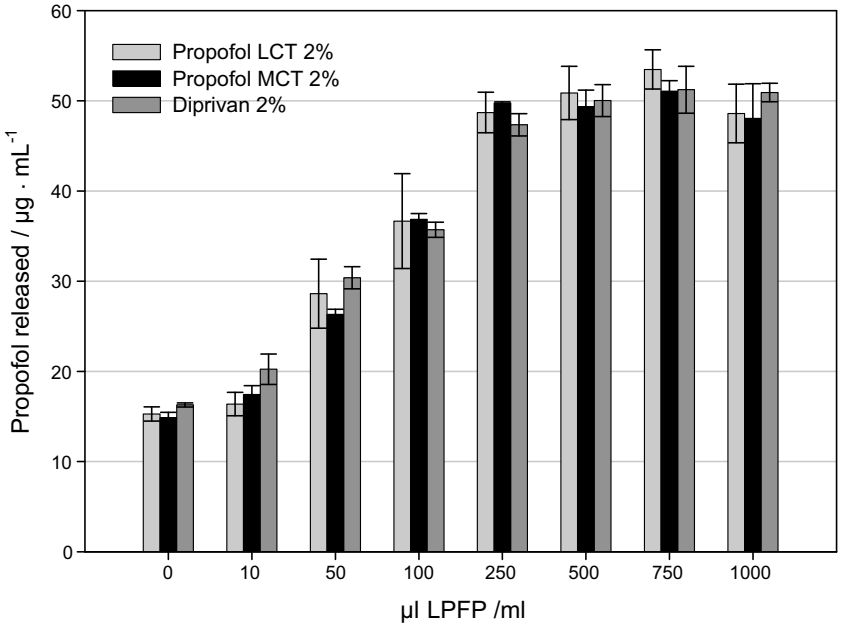

Fig. 5. Concentration of propofol in diluted (1:400 in PBS) emulsions in the aqueous phase (µg/ml) after incubation with increasing portions of lipoprotein-free plasma (LPFP). Samples were prepared by ultracentrifugation (100,000*g*). The amount of propofol in the aqueous phase was determined by HPLC. A propofol concentration of 50 µg/ml theoretically corresponds to a release of 100%. Values are means ± SD (n = 3–5).

Comparison of the release and binding kinetics of the original and generic propofol preparations (Diprivan 2% and Propofol 2% LCT "Fresenius") revealed no differences within the first 30 min of the assay (data not shown). Thus we decided to use the amount of propofol released and bound to the coated wells after 30 min. However, most likely the kinetics in blood will be much faster and direct conclusions from our experimental setup to the physiological situation are problematic.

As shown in Fig. 2, we found no significant differences in propofol release and binding among the different preparations (1% and 2% preparations). Though, the quantity of bound propofol in BSA- and LDL-coated wells was quite different, as discussed previously.

During the characterization of our assay, we asked whether the amount of propofol bound to the LDL-coating correlated with the amount in the aqueous phase of the undiluted emulsion. The product specification supplied by Fresenius Kabi, Austria, indicated that the amount of propofol dissolved in the aqueous phase should be between 30 and 40 µg/ml for 2% propofol preparations, and thus approximately 99.8% of the drug is dissolved in the oily phase. These data are also supported by Levitt and Schnider who published a partition coefficient (K_{oil}) with 4700 [6]. Since the analysis of the partition requires a complete phase separation followed by content analysis with HPLC, we separated the undiluted propofol emulsion by dialysis. In general, our data confirmed those of the supplier: the generic Propofol 2% LCT "Fresenius", as well as the original Diprivan 2% contained approximately 37 µg/ml propofol in the aqueous phase. Only the Propofol 2% MCT/LCT "Fresenius" preparation had significantly less free propofol in the aqueous phase (approximately 28 µg/ml). Since it was shown that the amount of free propofol can be related to a decrease of pain of infusion [7–9], this could be an advantage for the application of Propofol 2% MCT/LCT "Fresenius" preparation.

Phase separation of the 400-fold diluted samples was achieved by both ultracentrifugation and dialysis; however, following dilution, the lower solubility of propofol in the aqueous phase of the Propofol 2% MCT/LCT "Fresenius" preparation was not observed. This is a strong indication that after injection and immediate dilution in the bloodstream, the anesthetic potency of propofol will be the same for LCT and MCT/LCT preparations.

Furthermore, a direct comparison between dialysis and centrifugation methods was applied in our study in order to exclude a possible influence of phospholipid vesicles on propofol release. No differences were found between both separation methods, indicating negligible influence of phospholipid vesicles, which will not pass the dialysis membrane but might not be separated by centrifugation.

Using a 400-fold dilution, we calculated that the concentration of propofol (Diprivan 2%) in the aqueous phase should be 0.0925 µg/ml. However, we found much higher values, i.e. approximately 16–18 µg/ml in all diluted 2% preparations (Fig. 3). Since we had to separate both phases by ultracentrifugation or dialysis, which takes several hours, we may speculate on the possible effects on the dissolution kinetic. So far it can be assumed that due to the large surface of the oily phase, a shift of the partition between both phases occurs immediately after dilution, resulting in a much higher propofol concentration in the aqueous phase than predicted by simple calculation. Equivalent data were also found for the 1% propofol preparations (Fig. 3). Washington et al. also showed that dilution of drug emulsion systems leads to a diffusion of the drug out of the carrier until the partition equilibrium is re-established [14–16].

It has been suggested that albumin, lipoproteins and lipases are responsible for the propofol release into the plasma fraction, and that propofol becomes bound to different blood components immediately after injection [2,17]. Our studies using coated microtiter plates, different lipoprotein fractions and LPFP strongly support this view.

Our experiments with separated lipoprotein fractions showed that VLDL and LDL did not significantly influence propofol release, whereas the amount of propofol released by HDL reached almost 100% at concentrations above 1000 µg/ml HDL. Therefore, propofol appears to preferentially interact with HDL, which could be explained by the high binding capacity and/or surface activity of apolipoproteins A-I and A-II to lipophilic substances [18,19]. These proteins occur predominantly in HDL and not in LDL or VLDL. Our data show that propofol can be bound to LDL to some extent but that no pronounced propofol release from the emulsion to the aqueous phase is triggered by LDL addition.

Finally, for control purposes, we investigated the effect of LPFP on propofol release. LPFP also induced an increase in propofol release, which supports our data from BSA-coated microtiter plates where we demonstrated binding of propofol to BSA. At concentrations above 100 μg/ml LPFP, sufficient binding sites are provided by plasma constituents to allow almost 100% binding and release.

In summary, this part of our study was devoted to the effects of lipoproteins on the concentration of propofol released from the emulsion in the aqueous phase. The highest impact was observed for HDL. Thus it seems reasonable to speculate that variations in HDL levels in blood, which might occur in some genetic diseases and metabolic disorders [20–23], could directly interfere with propofol kinetics in the bloodstream.

5. Conclusions

In conclusion, the novel *in vitro* assay described in this study did not detect any significant differences between the release kinetics of propofol followed by binding to BSA or LDL in generic and original propofol preparations. However, in combination with content measurements of free propofol in the aqueous phase, one could use such an assay to investigate the influence of the lipid composition of the oil phase on the binding to LDL *in vitro*. Here, an example was shown for LCT preparations in comparison with MCT/LCT preparations. We have also seen that different plasma fractions had an influence on the propofol release from the oily phase, as well as the total content of propofol in the aqueous phase. Lipoprotein fractions, especially LDL, but also albumin, were shown to bind propofol.

Thus, we conclude that the anesthetic properties of propofol depend on many different parameters and it can still not be excluded that formulation related parameters contribute to physiological effects. However, it seems to be more feasible that the required dosage for rapid onset of sleep may vary from patient to patient, due to variations in serum protein or lipoprotein composition, genetic disorders, or differences in lipase activities due to diverse diseases.

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