Oliver Wollner, B.Sc.

Method development and validation to determine free and protein-bound fractions of drugs in biological fluids with LC/MS analysis

MASTERARBEIT

zur Erlangung des akademischen Grades

Diplom-Ingenieur

Masterstudium Biotechnologie

eingereicht an der

Technischen Universität Graz

Betreuer

a.o.Univ.-Prof. Dipl.-Ing. Dr. techn. Michael Murkovic

Institute of Biochemistry
Department for Food Biotechnology

Graz, 03.04.2016 – 02.10.2016

EIDESSTATTLICHE ERKLÄRUNG

Ich erkläre an Eides statt, dass ich die vorliegende Arbeit selbstständig verfasst, andere als die angegebenen Quellen/Hilfsmittel nicht benutzt, und die den benutzten Quellen wörtlich und inhaltlich entnommenen Stellen als solche kenntlich gemacht habe. Das in TUGRAZonline hochgeladene Textdokument ist mit der vorliegenden Masterarbeit identisch.

Datum

Unterschrift

Table of content

Table of figures

Acknowledgments

Special thanks to my supervisor at Graz University of Technology Dr. Michael Murkovic for his guidance and support. He was always willing to share his precious time and helpful hints. Thank you for your patience and sharing of analytical knowledge. Special thanks as well to my supervisor at Joanneum Research Dr. Reingard Raml. She always gave me the chance to grow beyond myself and shared with me both - joy and frustration - when it came to evaluating experimental data. Nevertheless, I am really grateful to Joanneum Research for giving me the chance to write my master thesis there. The whole Health-Team was so friendly and kind, that I will always remember this incredible working place. Thank you also Dr. Frank Sinner, Dr. Thomas Pieber, Dr. Thomas Birngruber, and Dr. Christoph Magnes for making this happen. Thank you all for that opportunity and this interesting topic!

Furthermore, I want to thank the whole staff of Joanneum Research - too many to mention all - for always having an advice and helping me to orientate myself in the laboratory. I had a wonderful time with all of you and tremendously appreciate the nice atmosphere in our team. It brought me always motivation. It was a pleasure to be part of this group.

Finally, I want to thank the group of Prof. Madeo et al. at the Institute of Biochemistry of Karl-Franzens University Graz for giving me the chance to use their ultracentrifuge. Equal thanks go to the group of Prof. Daum et al. at the Institute of Biochemistry at Graz University of Technology for sharing their materials and devices with me for gel electrophoresis.

Last but not least I also want to thank my whole family and close friends for always being supportive during the last years. A special thanks goes to of my grandparents, my mother and my father and his wife for the additional monetary support that gave me the chance to entirely concentrate on my studies. The last acknowledgment goes to my fiancée Patty, thank you for giving me a reason.

omnis finis initium novum

Abbreviations

ABT	Agarose Bead Technologies
ADME	Absorption, distribution, metabolism and elimination
ADS	Alkyl-diol silica
AGP	Alpha-1-acid glycoprotein
BSA	Bovine serum albumin
conc	concentration
EDTA	Ethylenediaminetetraacetic acid
FA	Fatty acid
HPLC-MS	High-pressure liquid chromatography – mass spectrometry
HRP	Horseradish peroxidase
HSA	Human serum albumin
ISF	Interstitial fluid
kDa	Kilo Dalton
MWCO	Molecular weight cut-off
NADPH	Nicotinamide adenine dinucleotide phosphate (protonated)
OFM	Open flow microperfusion
PBS	Phosphate buffered saline
PDB	Protein data bank
PSA	Porcine serum albumin
RED	Rapid equilibrium dialysis
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPME	Solid-phase microextraction
SSC	Saline-sodium citrate
UC	Ultracentrifugation
UF	Ultrafiltration

Abstract

The analysis of drug binding by endogenous proteins plays a major role in pharmaceutical drug development. Current binding studies are mainly made with plasma as it is presumed that unbound drugs can move freely between tissues. As this hypothesis has its limits it would be necessary to take the interstitial fluid into account as well. The interstitium is the area where most of the drugs mediate their effect. This fluid, however, is difficult to access due to the narrow space between tissue cells, but modern sampling approaches have shown promising results. It is unclear whether binding essays can also function in fluids with a low-protein content such as the interstitium. In this thesis drug protein binding essays were evaluated with several biological fluids in order to highlight possible differences in drug binding capacities between tissues. Therefore, several techniques were investigated and compared. The rapid equilibrium dialysis was found to be the most suitable technique for drug-protein binding analysis and was additionally validated via various experiments. Moreover, it could be shown that ultrafiltration, a standard technique for plasma protein binding experiments, is not in line with the demands for low-protein fluid analysis.

Zusammenfassung

Die Analyse der Bindung von Wirkstoffen an endogene Proteine spielt eine wichtige Rolle in der pharmazeutischen Drogenentwicklung. Zurzeit werden Bindungsstudien hauptsächlich mit Plasma durchgeführt, da angenommen wird, dass sich ungebundene Wirkstoffe frei zwischen den Geweben verteilen können. Da diese Hypothese aber ihre Grenzen hat, wäre es notwendig, auch die interstitielle Flüssigkeit zu berücksichtigen. Das Interstitium ist der Bereich, in dem die meisten Medikamente ihre Wirkung vermitteln. Diese Flüssigkeit ist allerdings durch den engen Zellzwischenraum schwer zugänglich, doch die Probenentnahmen durch moderne Methoden zeigen mittlerweile vielversprechende Ergebnisse. Unklar ist ob Bindungsanalysen auch in Flüssigkeiten mit geringem Proteingehalt, wie dem Interstitium, funktionieren. In dieser Arbeit wurden Wirkstoff-Protein-Bindungen mit mehreren biologischen Flüssigkeiten untersucht, um mögliche Unterschiede in den Bindungskapazitäten zwischen den Geweben hervorzuheben. Dafür wurden mehrere Techniken untersucht und miteinander verglichen. Die schnelle Gleichgewichtsdialyse wurde als die geeignetste Technik für die Analyse von Wirkstoff-Protein-Bindungen identifiziert und durch mehrere Experimente validiert. Es konnte ebenfalls bestätigt werden, dass die Ultrafiltration, eine Standardmethode bei Plasma-Bindungsexperimenten, nicht den Anforderungen für Analysen von Proteinlösungen mit einem geringem Proteingehalt entspricht.

1 INTRODUCTION

1.1 Pharmacokinetics of drugs

Pharmacokinetics is a branch of pharmacology with interest in drug absorption, distribution, metabolism and elimination within an organism – in short ADME. Drug-protein interaction can have severe effects on the pharmacokinetic behaviors of drugs, especially in terms of the volume of distribution and the clearance of a drug. Pharmacokinetics is often combined with pharmacodynamics, the study of physiological and biochemical effects of drugs within the body.

Most common forms of drug administration are via enteral routes like oral application or parenteral as intravenous, intramuscular or subcutaneous injection. Also topical applications on the skin, ears or eyes for local symptoms are often used.

Once a drug enters the body it is usually distributed via the systemic blood circulation and a certain amount of drug is captured by endogenous proteins in the blood. Only the free/unbound drug is transported to all tissues and to the site for the desired receptor interaction. By these non-specific bindings the half-life and apparent volume of distribution of drugs can be affected. Few barriers like the blood-brain barrier or the stratum corneum in the skin generally limit the access of many drugs. In the case of oral

application the drug usually dissolves in the gastro-intestinal tract, is absorbed through the gut-wall and has to pass the liver before entering the bloodstream. Here degradation can occur even before distribution, which is called first-pass liver metabolism. (Waterbeemd, 2003)

Small and polar moleculres can be eliminated by the kidneys. As many drugs are bigger in size and often hydrophobic they undergo biotransformation in order to become more polar and water soluble. Through this metabolism drug activity can decrease, although in some cases substances can become toxic as well. On organ level the liver is the primary organ for drug metabolism, but lung, kidney, intestine, skin and placenta are capable of metabolizing reactions as well. On a biochemical level these reactions comprise phase I reactions to introduce polar functional groups like –OH, -NH2 or –SH and phase II reactions which conjugate the respective compound with an endogenous molecule such as glucuronic acid, sulfuric acid or acetic acid. Phase I reactions often involve NADPH as a reducing agent and oxidizing enzymes (monooxygenases) called cytochrome P450. Hereby drugs become easier to eliminate. (Routes, 2004)

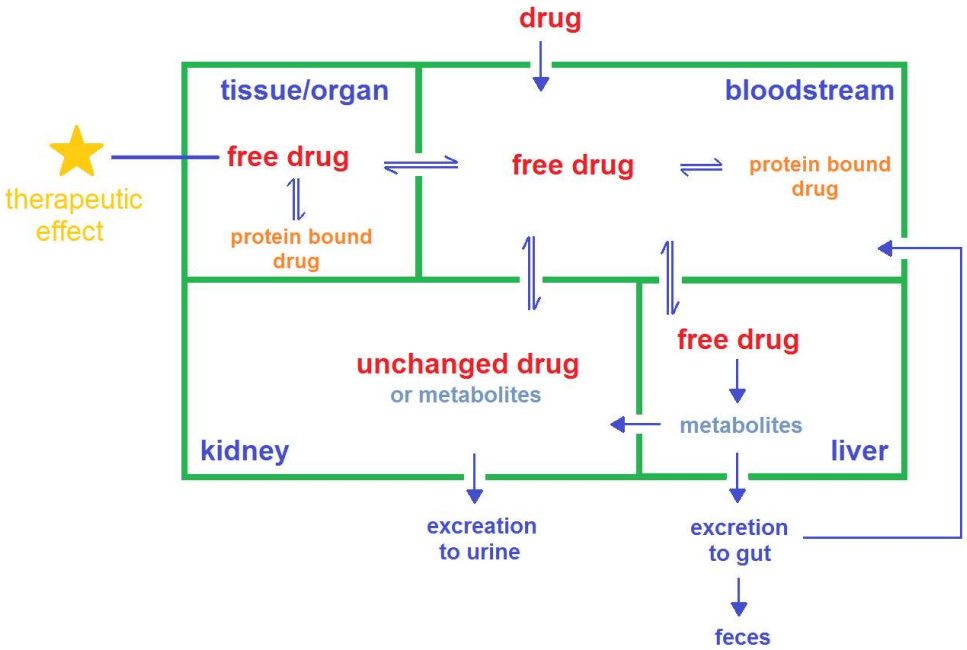


Figure 1: Absorption, distribution, metabolism and elimination (ADME) of a drug within an organism. A drug enters the bloodstream from the gut and can either be bound or free. The free drug can travel to tissues and organs to have a therapeutic effect or can be excreted by the liver or kidney. (Wang, 2011)

1.2 Importance of protein binding during drug development

Protein binding is an important parameter for drug safety and efficacy which has to be always investigated during drug development. Binding has a direct effect on drug dynamics as only the free drug promotes the pharmacological effect. Only an unbound drug can interact with receptors at the site of action. (Smith, 2010) Especially drugs with a narrow safety window are important to be investigated. If those drugs are known to be highly protein bound the dose of application will certainly be increased. Kidney or liver diseases, malnutrition, pregnancy or aging may alter protein binding. (Kommu, 2013) In the case of a disease which leads to plasma protein deficiencies or the percentage of drug binding was wrongly determined it can be dangerous as the free drug concentration alters as well and may rise to toxic concentrations.

In clinical studies of the pharma industry drug protein interactions are mainly tested by equilibrium dialysis or ultrafiltration experiments with plasma samples. Although the site of action is usually not the plasma, but the interstitial fluid as it fills the space between tissue cells where the corresponding receptors are located. On one hand plasma is used instead because of the free drug hypothesis, which proposes that at steady state, the free drug concentration should be the same on both sides of any biomembrane. Although it is known that biomembranes of higher organisms usually contain active transporters which lead to differences on both sides in terms of protein, ion, and lipid concentrations. Another fact which might confound the free drug hypothesis is that plasma and interstitial fluid contain some different types of proteins and especially different protein amounts, which has an effect on how much a drug is bound. (Smith, 2010) On the other hand plasma is used because of the lack of a suitable extraction method for interstitial fluid and in comparison plasma is much easier to sample.

1.3 Drug-binding proteins

Protein binding in plasma is mainly mediated by human serum albumin (HSA), alpha-1-acid glycoprotein (AGP) and lipoproteins. Human plasma contains approx. 60-80 g/l proteins, where albumin is the most abundant with around 50-60 % of total protein, while AGP accounts only for 1-3 %. (Shen, 2013)

Drug molecules in the blood or tissue fluids are either unbound or bound mainly to the proteins mentioned. This leads to the following equilibrium as the binding is a reversible process:

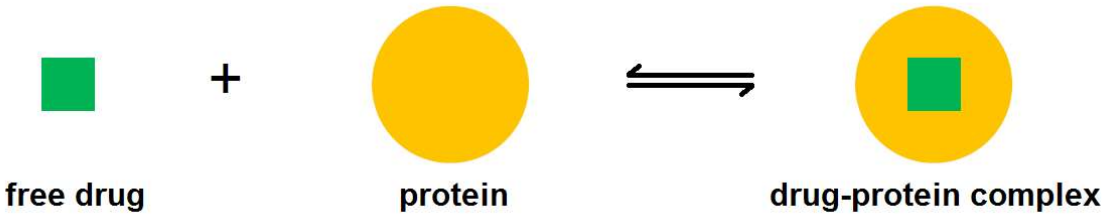


Figure 2: Drug-protein binding principle.

This equilibrium is specific for every drug and responds rapidly to environmental changes such as temperature, pH, and protein or drug concentration. As the conditions in the body fluids are usually strongly regulated and constant, the equilibrium of free and bound drug should be constant as well. Equilibria are often established within 100 ms. For a better overlook table 1 gives some information about the general properties of HSA, AGP and lipoproteins: (Nilsson, 2013)

Table 1: Main binding proteins and their properties.

	HSA	**AGP**	**Lipoproteins**
Molecular weight	66 kDa	41-43 kDa	6-550 kDa
Concentration in blood	35-50 g/l	0.5-1.4 g/l	2.5-3.6 g/l
Number of binding sites	6 (2 major)	7 (1 major)	-
Binding compound properties	mainly acidic	basic	lipophilic
Reference	Nilsson, 2013	Fournier, 2000	Dufaux, 1982

1.3.1 Albumin

As mentioned before human serum albumin (HSA) is the most abundant protein in plasma, comprising 50-60 % of total plasma protein. HSA consists of a single polypeptide chain with 585 amino acids. It contains mainly alpha-helices and has three homologous domains (I-III). Albumin is synthesized in the liver and exported to the blood, where it serves as a transporter and depot for many endogenous substances. It is also responsible for maintaining the colloid osmotic pressure, the force that pulls fluid into the capillaries. (Hein, 2010)

Albumin binds mainly acidic and neutral drugs. In general HSA contains two main binding sites – often described as Sudlow's site I and II – and four additional binding sites. Thereby HSA can simultaneously bind for example seven equivalents of fatty acids, as Sudlow's site II can bind two fatty acid molecules at once. Binding is usually accomplished by forming ion bridges or hydrogen bonds to amino acid residues present in the binding cavities. (Fasano, 2005)

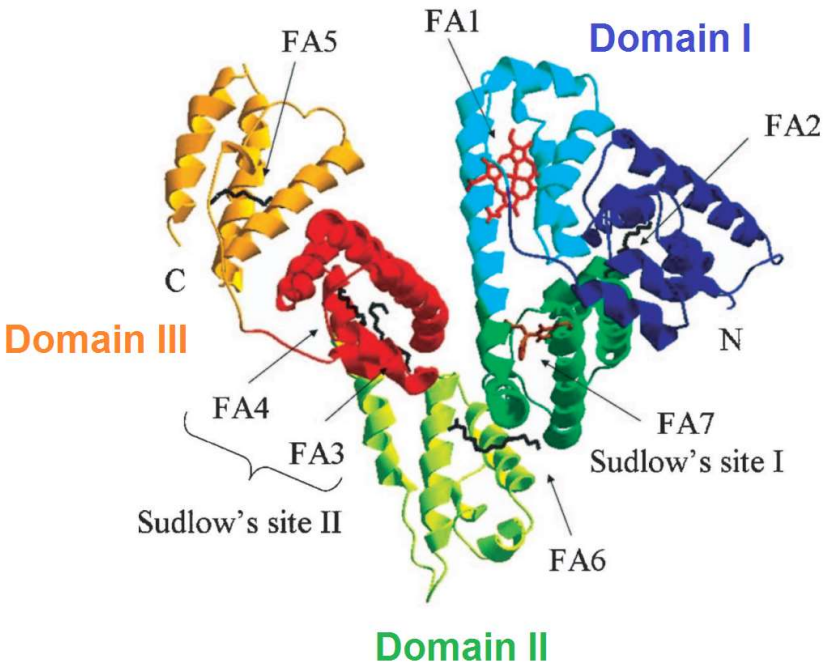


Figure 3: Cartoon structure of human albumin and its three domains. 7 fatty acids bindings sites (FA) are marked and the 2 main binding sites for drugs - Sudlow's site I and II. Example molecules are

visualized within the binding sites. (Fasano, 2005)

HSA can bind a wide range of endogenous and exogenous substances with varying specificity, including fatty acids, hormones, ions, amino acids and sugars. Ligands can compete at a single binding site or some even displace each other. It occurs also that different ligands bind to different binding sites within the same albumin molecule. But ligand-binding may also alter the affinity of albumin to other ligands by promoting conformational changes. The effect changes from substance to substance. (Margarson, 1998) Binding sites also differ between different species (Kosa, 1997).

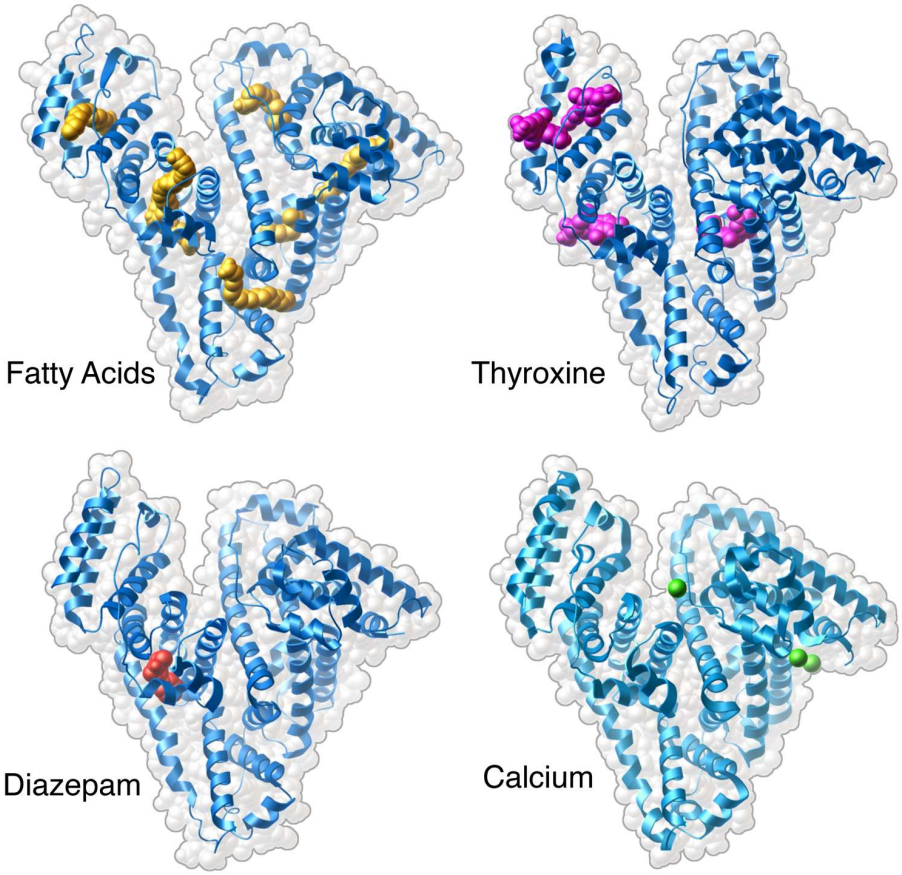


Figure 4: Example compounds are bound to human albumin. PDB entries 1HK1 (fatty acid), 2BXF (thyroxine), 3V03 (diazepam) and 1E7I (calcium).

Although human serum albumin is not glycosylated. Non-enzymatic glycation can occur due to the presence of reducing sugars like D-glucose in the bloodstream. The addition of a sugar molecule to an amine group like in lysine can have an effect on protein structure and

therefore also on drug binding, especially when the addition takes place near the binding sites. (Anguizola, 2013)

1.3.2 Alpha-1-acid glycoprotein

Alpha-1-Acid glycoprotein (AGP), also called orosomucoid, is a protein with many unusual properties such as a very low isoelectric point of 2.8-3.8 and a very high glycosylation content of 45 % of the total molecular weight. AGP consists of 183 amino acids, while two variants from different genes were detected showing differences in 22 amino acids. Additionally 12-20 different glycoforms occur in human serum. AGP is known to bind basic drugs such as lidocaine and amitriptyline. The biological function of AGP is still unknown, although some immunomodulating effects have been described. The gene for alpha-1-acid glycoprotein is regulated by mediators like glucocorticoids and cytokines such as tumor necrosis factor-α (TNFα), interleukin-1ß (IL-1ß), Interleukin-6 and IL-6 related cytokines. (Forunier, 2000)

AGP belongs to the acute phase reactant proteins, which means that its plasma concentration rapidly increases to a three to four-fold amount due to an immunological response or inflammation. (Shen, 2013)

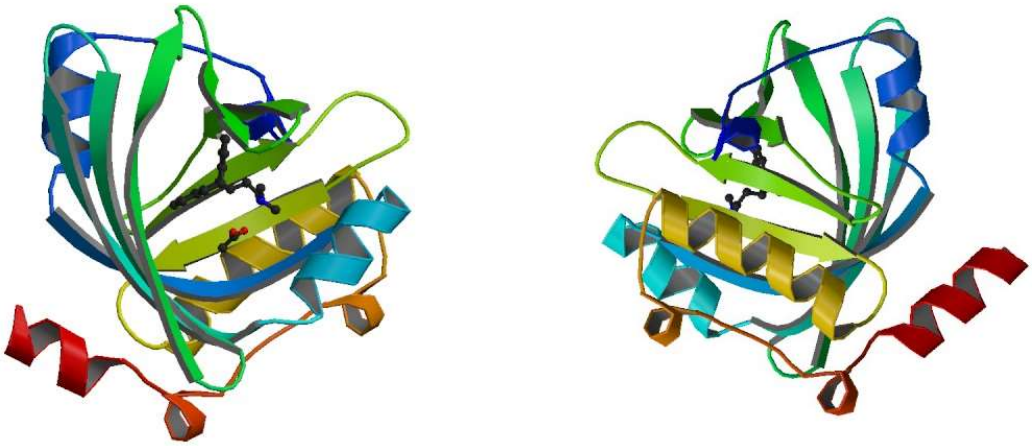


Figure 5: Cartoon structure of human alpha-1-acid glycoprotein and amitriptyline complex visualized from two angles. (PDB 3APV)

1.3.3 Lipoproteins

Lipoproteins are complex particles with a dualistic character. They have a hydrophobic core containing mainly triglycerides and cholesterol esters and a hydrophilic surface consisting of free cholesterol, phospholipids and apolipoproteins. (Feingold, 2000) The main function of lipoproteins is the transportation of lipids either from alimentary sources to the tissues or from cellular metabolism back to the liver. Lipoproteins are divided into seven classes due to their floating characteristics from ultracentrifugation. (Dufaux, 1982)

Because of the high lipid load of lipoproteins, drugs with similar properties may reside inside at the apolar core. In contradiction to the binding of drugs with albumin and alpha-1-acid glycoprotein, no specific binding site in lipoproteins for drug interaction has been published so far. As lipoprotein levels are highly dependent on physiological states the amount of bound drug might be affected as well. For example it has been shown that when a combination of an orally applied drug with a high-fat meal is applied, the bioavailability of a drug can be increased. Due to the high fat content the expression of lipoproteins will be enhanced and thereby the amount of lipoprotein-bound drug will increase as well. The plasma concentrations of the drug become notably higher than without a high-fat meal. (Wasan, 2008)

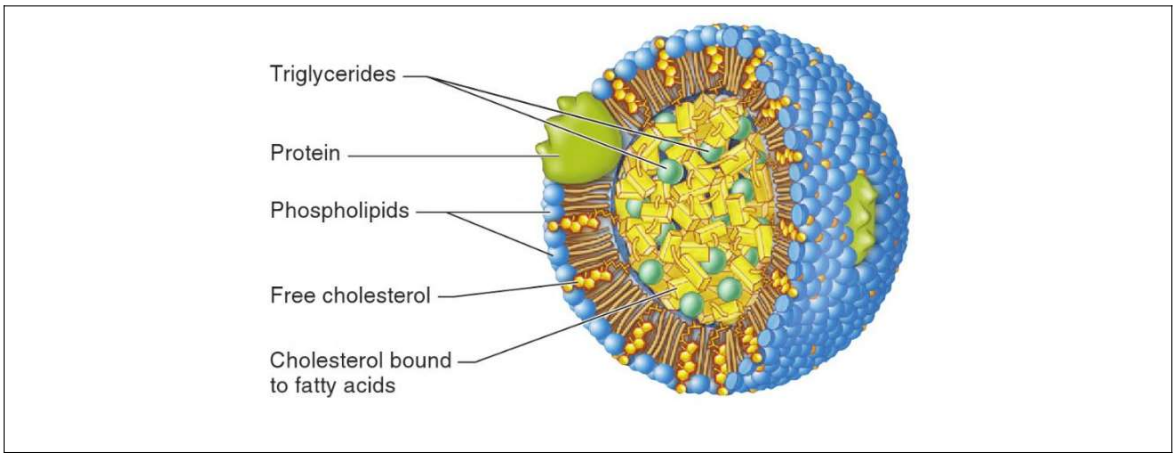


Figure 6: Lipoprotein structure consisting of triglycerides, apolipoproteins, phospholipids, free cholesterol and cholesterol bound to fatty acids. (Patton, 2015)

1.4 Body fluids as compartments with different binding capacities

1.4.1 Plasma as the main distributor of substances

Different body fluids act as compartments with their own binding capacities as environmental conditions differ as well. Plasma is the most studied biological fluid as it is easy to extract and the main distributor of substances in the body. Blood can be divided in a solid and a liquid fraction, while solid parts are erythrocytes, leukocytes and thrombocytes and the liquid part - called plasma - contains proteins, electrolytes, organic acids, sugars, lipids, hormones and other solved substances. Plasma is gained from blood samples by the addition of an anti-coagulant like heparin or EDTA in order to prevent clotting and is followed by centrifugation of the solid part. Serum instead is gained by centrifugation after blood clotting, which results in the same liquid part, but without fibrinogen - the main clotting factor. (Kim, 2007) In this thesis only EDTA-Plasma is used as fibrinogen can have an effect on drug-binding as well.

While plasma proteome analysis revealed 697 plasma proteins so far, only 14 of the most abundant proteins represent around 94 % of total protein mass. The range of plasma protein concentration spans more than 10 orders of magnitude. (Schenk, 2008)

1.4.2 Lymph as an approximation to the interstitial fluid

The lymph has three main functions, the removal of interstitial fluid, the absorption of fatty acids from the digestive system, and the transportation of leukocytes. If bacteria are taken up by the lymph, they are brought to lymph nodes where they get destroyed. Interstitial fluid is collected through lymph capillaries and returned to blood via lymphatic vessels. The lymphatic system is a separate system of vessels, consisting of capillaries on one hand which merge to bigger vessels until reaching the two main vessels – the thoracic ducts, which end at large veins near the heart (Figure 8). The lymphatic flow is mediated also by the heart, same as the blood flow. (Sadava, 2013)

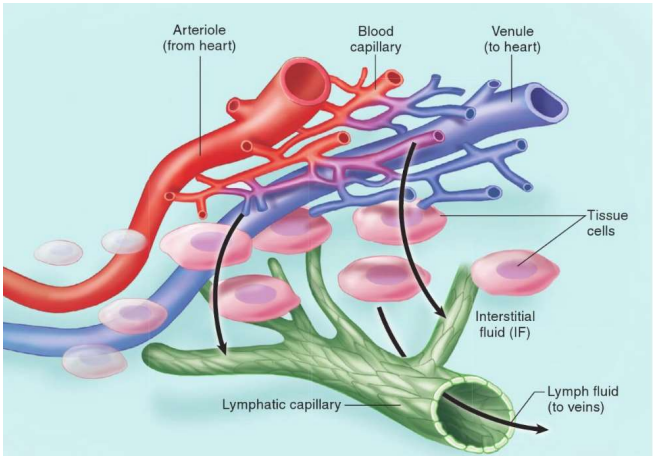


Figure 7: Fluid from plasma is entering the interstitial space through blood capillaries, becoming interstitial fluid (IF). The IF returns through lymphatic capillaries to the venous blood. (Patton, 2015)

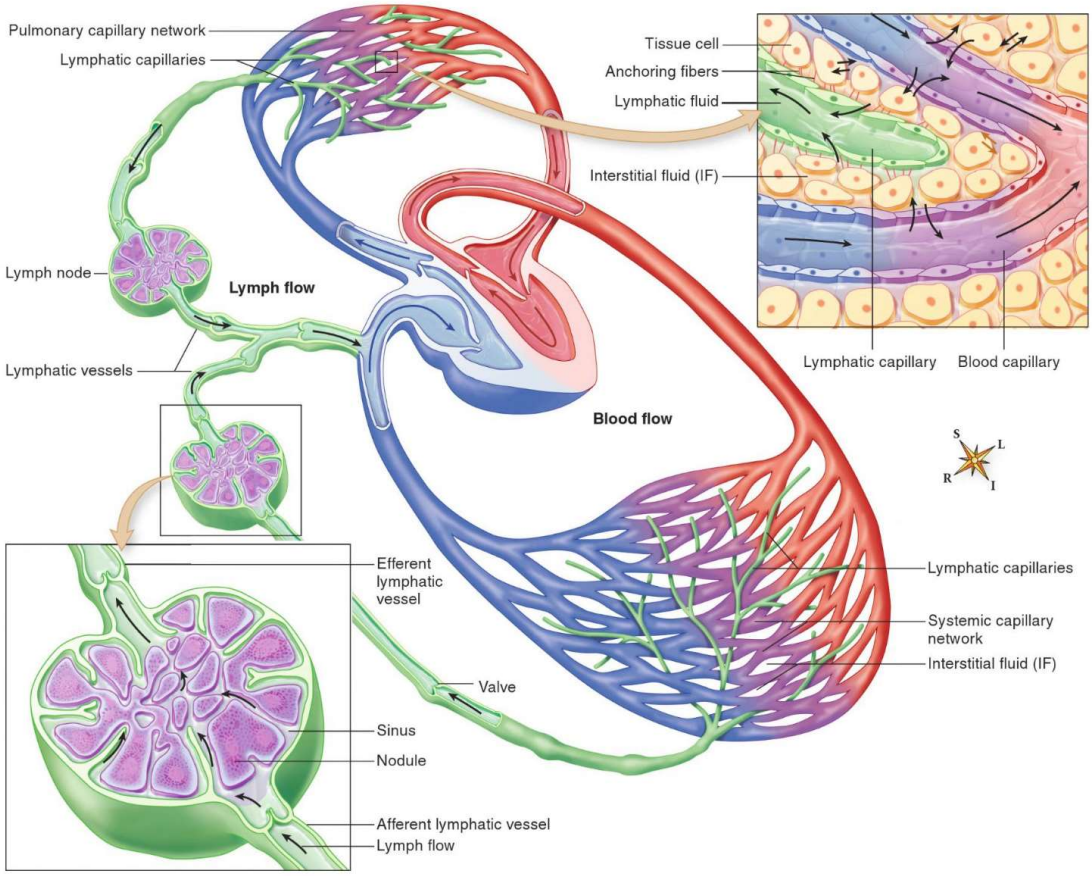


Figure 8: Circulation of lymphatic fluid. Fluid from the systemic and pulmonary capillary networks enters the interstitial space. Interstitial fluid (IF) exchanges materials with surrounding tissues and flows into the lymphatic capillaries. The lymph fluid is pumped through lymph nodes and to large lymphatic ducts. The lymph finally enters a vein and the fluid is returned to the bloodstream. (Patton, 2015)

The knowledge about the relationship between plasma, lymph and extracellular fluids yet is limited. In general the total protein content in the lymph is assumed to be half of that in plasma. Although for some protein species it occurs to be vice versa like lipoprotein levels are around 5 to 10 times higher in the lymph than in the plasma. (Anderson, 2002) In comparison to plasma, lymph contains also more leukocytes and more intracellular proteins from organelles, nucleus, and cytosol. Some differences might come from tissue cell debris which are delivered via the interstitial fluid to the lymph. This suggests that lymph and interstitial fluid could be rather similar. (Clement, 2013)

On the other hand it was found that the ratio of the protein content between serum and lymph is not a constant value. By investigating the protein content over time it could be shown that the lymph to serum ratio is changing enormously due to daytime and the activities of patients (Figure 9). These fluctuations could have a strong influence on experimental outcomes when samples are only taken at a single time point. (Lee, 2011)

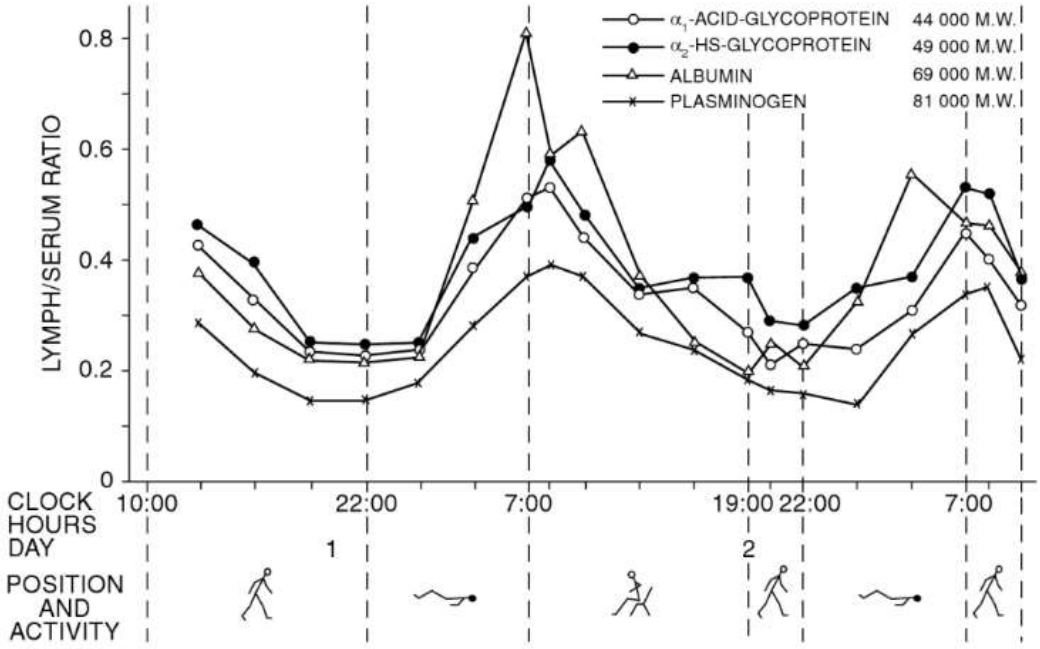


Figure 9: The lymph protein levels fluctuate during normal limb activity, expressed as a lymph to serum ratio. (Lee, 2011)

1.4.3 Interstitium as the area of effect

The interstitium is the connective space of tissue cells between blood and lymphatic vessels. Cells in the extracellular environment take up nutrients and oxygen over transcapillary filtration and release carbon dioxide and other waste molecules through the lymphatic system. The interstitial space is important for transporting signaling molecules between cells. In terms of immune regulation it takes a crucial part as well, as antigens and cytokines can drain from the interstitium to lymph nodes. Interstitial fluid (ISF) plays another important role for drug dynamics, as most drug-binding receptors are located on cell surfaces which are reached via the ISF. The interstitial space consists of a complex collagen fiber framework for scaffolding purposes, as well as elastin, glycosaminoglycans, electrolytes, and plasma proteins. The relative composition varies from tissue to tissue. (Wiig, 2012)

Although the interstitium accounts for around 80 % of total extracellular fluid – which is equal to approximately 11 l – the surrounding space of cells is usually microscopic. This bottleneck makes the sampling of interstitial fluid extremely difficult. (Sloop, 1987; Kool, 2007) However, the different approaches from ISF sampling have shown similarity between lymph and interstitial fluid as well. Due to this and those mentioned difficulties in ISF collection, lymph fluid is often used as an approximation to the ISF.

It is not sufficient to use plasma as an approximation to the interstitial fluid as the distribution of proteins is notably different. The albumin concentration is assumed to be lower in the interstitium than in plasma. (Sadava, 2013) The general problem with sampling extracellular fluid is, the difficulty to prove their similarity to the in-vivo situation as many sampling techniques disturb the in-vivo equilibrium.In addition, sampling of ISF takes significantly longer than e.g. blood sampling. For example a trauma, as through a syringe, prior to sampling leads to higher levels of cytokines and stress hormones and can directly affect certain protein levels connected to this regulation. (Desborough, 2000)

1.4.4 Extraction of interstitial fluid via open flow microperfusion

Open flow microperfusion (OFM) has already demonstrated its potential as a novel technique in terms of investigating dermal, adipose and brain tissues. It is a minimal invasive and membrane-free alternative to microdialysis, a standard technique for investigating the biochemistry of tissues. As a membrane-based sampling technique, microdialysis has problems with substances that have a high molecular weight or highly lipophilic properties. After long term sampling other problems such as membrane fouling and clotting can occur as well. The OFM overcomes these disadvantages by replacing the membrane to a catheter that has an exchange area with macroscopic openings. The catheter is placed into the tissue while a peristaltic pump is pushing and pulling a perfusate through the catheter. At the exchange area substances can displace unfiltered between the interstitial fluid (ISF) and the perfusate. Especially for quantifications of topically applied substances the technique can be used. Figure 9 visualizes this procedure. (Pieber, 2013)

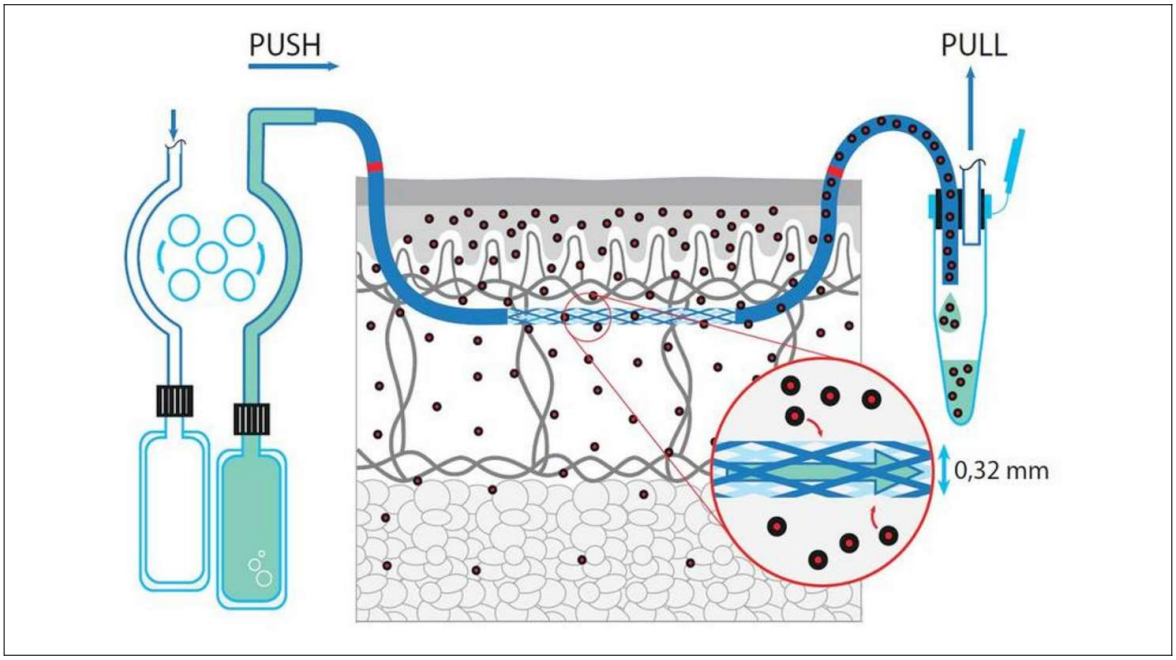


Figure 10: Principle of open-flow microperfusion (OFM). A perfusate is pumped by a peristaltic pump through a tube with an exchange area. Material from the interstitial space is diffusing into the tube and is collected. (Pieber, 2013)

Modifying this technique it can be used to sample ISF as well. One possible set-up is via recirculation. Therefore the perfusate is pumped in a circle, while always passing the

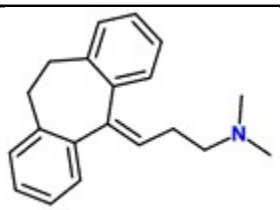
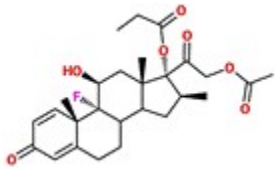
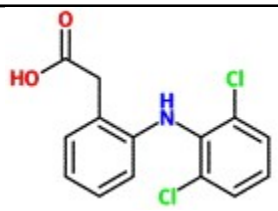
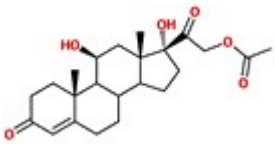
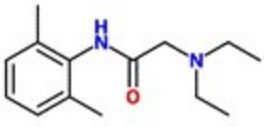
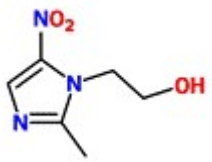
exchange area. After a certain number of recirculations the perfusate should be completely exchanged to the ISF. The amount of circles can be determined by monitoring the albumin content until it reaches a steady-state. Another suitable method for sampling ISF could be via suction as the pumps generate a negative pressure and ISF in sucked into the tube.

1.5 Methods for drug-protein binding analysis

1.5.1 Used Drugs

As a benchmark for the investigation of different methods for protein-drug binding studies six drugs with a varying range of properties and different binding percentages were used. The drugs are listed in the following table:

Table 2: A list of all used analytes with structure, physiological effects, percentage of bound drug in human plasma and logP- and pKa-value.

Name	**Structure**	**Effects**	**bound to proteins** (in human plasma)	**logP**	**pKa**
Amitriptyline		Antidepressant anxiolytic, mood elevating, analgestic, antidepressant, soporific	96 %	5.1	9.4
Clobetasol		Glucocorticoid antipruritic, anti-inflammatory, immunosuppressive, antimitotic	98 %	3.5	-
Diclofenac		Anti-inflammatory Drug antipyretic, analgestic, anti-inflammatory	99.7 %	4.51	4.15
Hydrocortisone		Glucocorticoid antipruritic, anti-inflammatory, immunosuppressive, antimitotic	> 90 %	1.61	2
Lidocaine		Local anesthetic antitussive, antiarrhythmic, local anesthetic	60-80 %	2.44	7.9
Metronidazole		Antibiotic antibacterial, antiprotozoal	< 5 %	-0.02	2.38

Data were acquired from DrugBank database and percentages from bound-drug in human plasma were found at New Zealand Medicines and Medical Devices Safety Authority. Additionally information about drug-binding could be found for few drugs as well. Therefore diclofenac should be mainly bound to albumin, lidocain mainly to alpha-1-acid glycoprotein and metronidazole should not be bound in general. For the other drugs no detailed information is available, but it could be assumed that they will interact simultaneously with different proteins.

1.5.2 Rapid Equilibrium Dialysis (RED)

The principle of equilibrium dialysis is that two chambers are separated by a semipermeable membrane. The sample chamber contains the biological fluid together with drugs which are either bound to proteins or free in the solution. Free drugs can migrate through the membrane, but proteins exceeding a size of 8 kDa cannot pass. This will lead to a distribution of free drugs between the two chambers and a new equilibrium will form. Although it is said that components that can cross the membrane are in equilibrium it can come to disparities of ions due to the Gibbs-Donnan effect. It occurs as proteins have a charge too and remain in only one chamber. It is not well studied whether it has an effect on the equilibrium or not. (Nilsson, 2013)

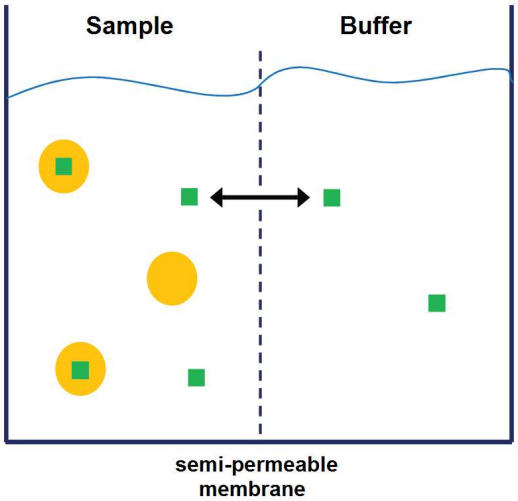


Figure 11: Principle of equilibrium dialysis. Small molecules like free drugs can pass the semi-permeable membrane, but proteins are retained.

General problems with techniques for drug protein binding studies are equilibrium disturbance and nonspecific adsorption. The biggest advantage of equilibrium dialysis is that limited adsorption. As the whole system is in equilibrium any loss due to adsorption will lead to a new equilibrium. A disadvantage is that the drug concentration will be distributed between the two chambers of the sample and the buffer leading to much lower concentrations when the volume of buffer is higher. This could become a problem if drug binding is strongly concentration-dependent or in the case of a highly-bound drug (>99 %) as the already small concentration will become more diluted. (Nilsson, 2013)

Usually equilibrium dialysis has to be performed between 16 and 24 hours or even longer. However, with the novel rapid equilibrium dialysis (RED) the experimental procedure can be shortened to 4 hours. This is achieved with a larger membrane surface as the sample chamber is completely surrounded by the membrane and buffer chamber.

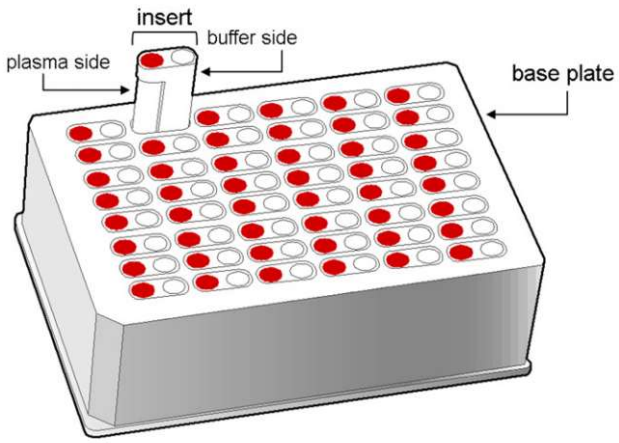


Figure 12: Rapid equilibrium dialysis device by Thermo Scientific.

The percentage of unbound drugs can be calculated by dividing the concentration of the buffer chamber by the concentration of the sample chamber.

$$\% \text{ unbound drugs} = [\text{conc buffer}] / [\text{conc sample}] * 100$$

$$\% \text{ bound drugs} = 100 - \% \text{ unbound drugs}$$

1.5.3 Ultrafiltration (UF)

During ultrafiltration an equilibrated solution is squeezed through a membrane by centrifugal forces. Only free drugs can pass the membrane, but proteins bigger than 10 kDa are retained. The percentage of free drugs in UF can be determined by the unbound concentration from the UF experiment divided by the total concentration in the matrix analyzed prior to the UF experiment.

% unbound drugs = [conc filtrate] / [conc total] *100

% bound drugs = 100 - % unbound drugs

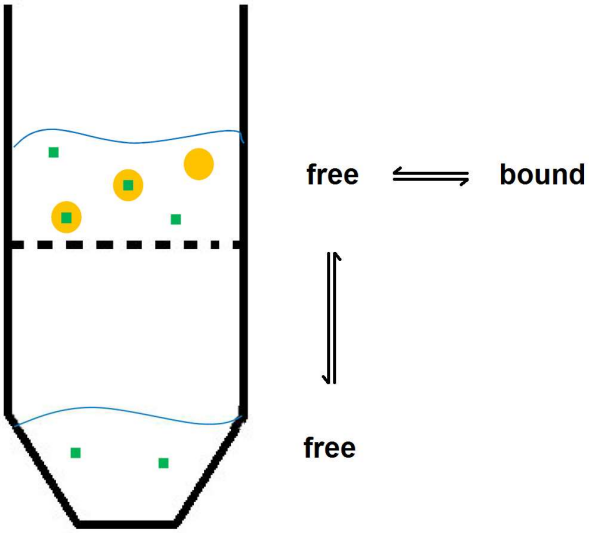


Figure 13: Principle of ultrafiltration.

The biggest disadvantage of UF is the high nonspecific adsorption to the UF cellulose membrane. Therefore only the unbound concentration can be determined via UF and a negative control with buffer cannot be compared with a biological fluid. More complex matrices as plasma show less adsorption due to protective effects from its components. (Nilsson, 2013) Lower protein fluids such as lymph or interstitial fluid could show much higher adsorption and may be not analyzable via UF.

Centrifugation speed has to be adjusted until 20-50 % of the sample has passed the membrane. Within this range the unbound fraction should be unaffected. (Borga, 1999)

1.5.4 Ultracentrifugation (UC)

Ultracentrifugation has been used successfully for plasma protein binding studies according to literature. (Boulton, 1998; Nakai, 2003) With centrifugation over 100,000 x g it is possible to separate a sample by its density and therefore the protein fraction can be separated from the unbound drug fraction. A centrifuged sample can be divided into fractions from top to bottom. The lower density fraction on top should be protein free and contain the unbound drug. As soon as proteins precipitate the protein fraction begins. Via this equation the percentage of unbound drugs can be determined:

$$\% \text{ unbound drugs} = [\text{conc unbound drugs}] / ([\text{conc unbound drugs}] + [\text{conc protein fractions}]) * 100$$

$$\% \text{ bound drugs} = 100\ \% - \text{unbound drugs}$$

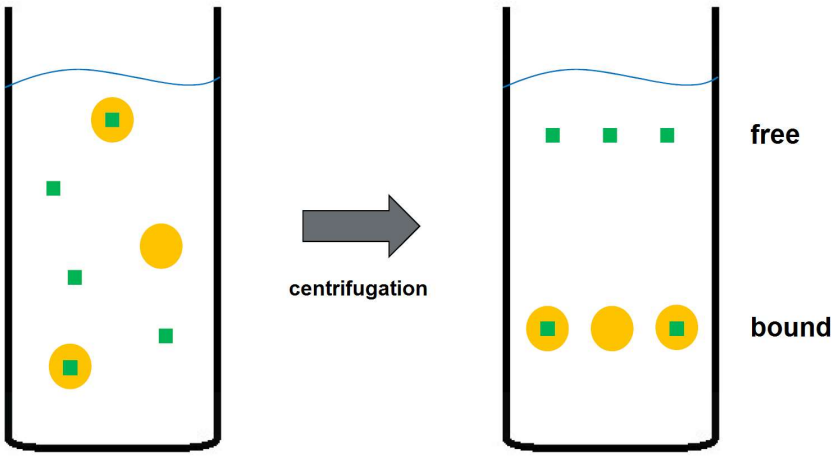


Figure 14: Principle of ultracentrifugation.

The advantage of this method is that it is not invasive as no dilutions are made and no solutions are added. In general the results of a density separation via ultracentrifugation can be improved by placing the sample on a prepared sucrose solution prior to centrifugation. The solutions contain increasing sucrose concentrations from top to bottom. Thus a density gradient is formed and the separation is more effective. This improved method cannot be done here as the dilution would significantly disturb the equilibrium and the results cannot be reasonably interpreted any more. (Chamouard, 1985)

1.5.5 Solid-phase microextraction (SPME)

In this approach a C18 coated SPME fiber extracts the free drugs from a sample which can be desorbed and analyzed thereon. The technique is based upon the adsorption mechanism of Fick's Law. There is a differential migration between unbound analytes and analytes that migrate into the fiber. The migration is dependent on the affinity of the analyte for the coat material. In order to reach a steady-state the user's manual suggests good agitation and at least 30 minutes of extraction.

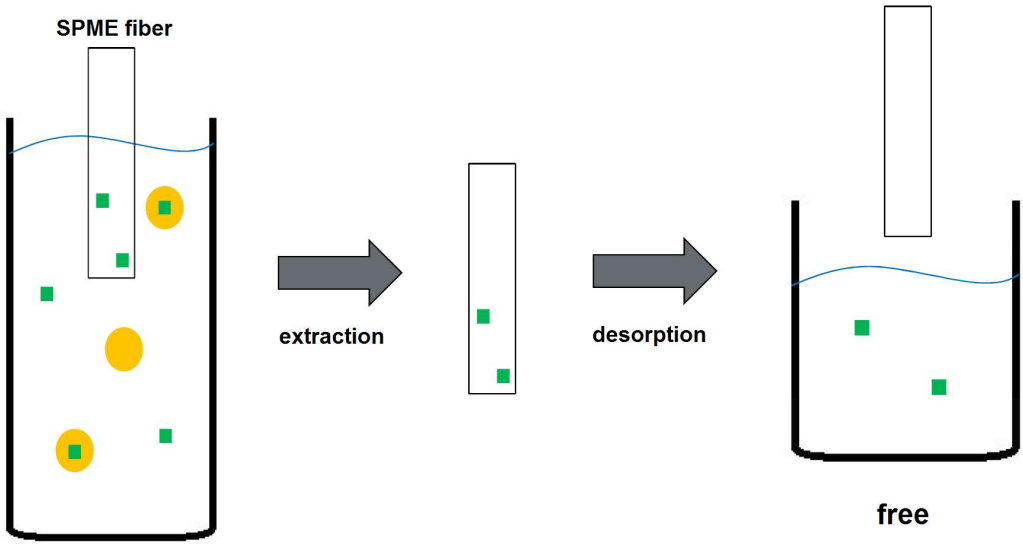


Figure 15: Principle of solid-phase microextraction.

As every analyte has its own affinity it is important to use a spiked buffer sample as well to get a 100 % unbound value for each analyte. Then the following equation can be used:

$$\% \text{ unbound drug} = [\text{conc sample}] / [\text{conc buffer}] * 100$$

$$\% \text{ bound drug} = 100 - \% \text{ unbound drug}$$

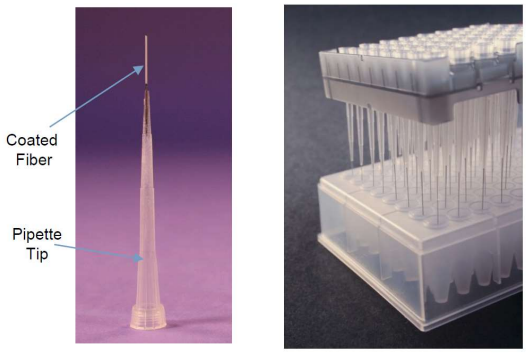


Figure 16: Single Use SPME LC-Tips with C18 Chemistry from Supelco.

1.5.6 Size Exclusion (SE)

Through the principle of size exclusion chromatography molecules can be separated according to their size. Different materials are available for the stationary phase, such as polyacrylamide or agarose. Larger molecules like proteins cannot enter the porous beads resulting in a smaller elution volume while smaller particles have to travel a longer way through the pores of the beads and thus elute later. This technique has not been found to be used for plasma protein binding studies so far. Nevertheless it should be suitable as in theory the protein-bound drug fraction could be separated from unbound drugs. The calculation of the unbound fraction would be the same like for ultracentrifugation:

% unbound drug = [conc unbound drug] / ([conc unbound drug] + [conc protein fractions]) *100

% bound drug = 100 - % unbound drug

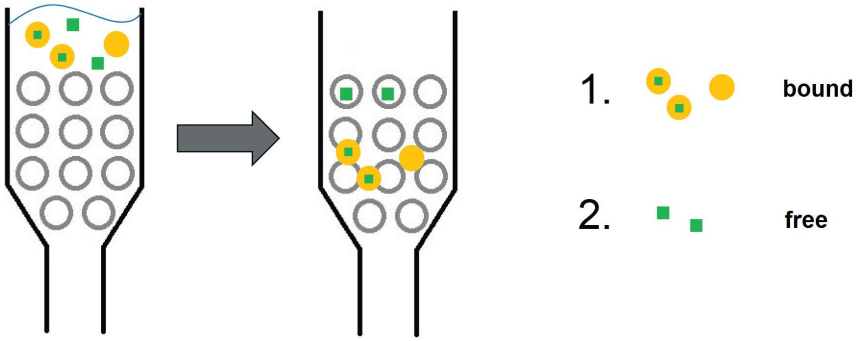


Figure 17: Principle of size exclusion.

1.5.7 Pig alpha-1-acid glycoprotein ELISA

To measure the concentration of pig alpha-1-acid glycoprotein (AGP) a highly sensitive two-site enzyme linked immunoassay (ELISA) was used. The samples react with anti-AGP antibodies on the surface of modified 96-well plates. Unbound proteins are washed away, while new anti-AGP antibodies, which are conjugated with horseradish peroxidase (HRP), bind again to AGP molecules. Next the chromogenic substance 3,3′,5,5′-tetramethylbenzidin (TMB) is added which gets yellow by the peroxidase. The absorbance of the colored product can be measured at 450 nm. As the quantity of bound enzyme is directly proportional to the concentration of AGP a standard curve can be drawn.

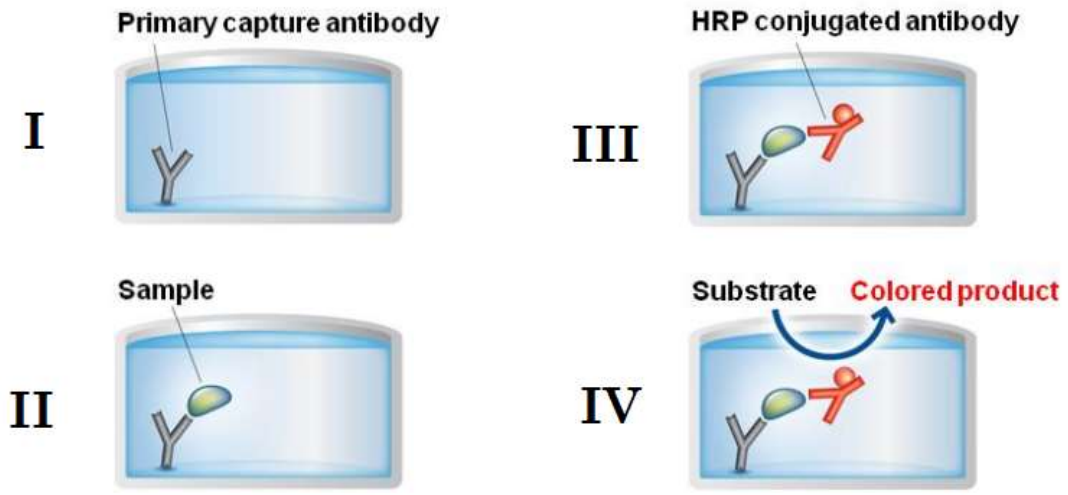


Figure 18: Principle of an ELISA. (I) Antibodies are captured at the surface, (II) then samples bind to the antibodies and (III) the HRP is conjugated. (IV) Finally the TMB becomes yellow by the peroxidase.

1.5.8 Additional Methods

There are few additional methods available to determine drug-protein binding. They were not used as some special equipment would have been needed, which was not available. Some are listed below:

- High-performance frontal analysis(Shibukawa, 1999)
- Charcoal adsorption (Yuan, 1995)
- Gel filtration (De Moor, 1962)
- High-performance affinity chromatography (Hage, 2011)

1.6 Aim of the thesis

Since now protein binding of drugs is mainly tested with plasma samples although it is usually not the site of action. As open flow microperfusion (OFM) seems to be a promising technique to sample interstitial fluid (ISF), the area where the therapeutic effect takes place, it would be interesting to know whether protein binding differs in the ISF in comparison to plasma. One aim of this thesis was to compare protein binding of drugs in different biological fluids. Also the interstitial fluid from OFM samples by recirculations and suction were evaluated and compared.

On the other hand all techniques for investigating protein binding have their own bottlenecks. The second aim was to examine and compare some of the most often used and most promising techniques for protein binding analysis with detection via HPLC-MS. The most suitable technique was used for further analysis to examine environmental influences like pH and temperature.

2 MATERIALS & METHODS

2.1 Standard laboratory instruments

Micropipettes were used from Eppendorf "Reference" series ranging from 10 µl to 5 ml (Eppendorf, Hamburg/Germany). For repetitive volume additions an Eppendorf Multipette M4 was used.

Centrifugations were performed in an Eppendorf Centrifuge 5415 D for volumes until 2 ml and bigger volumes in a Heraeus Multifuge X3R (Thermo Fisher Scientific, Bremen/Germany).

A Mettler Toledo pH meter FE20 was used for pH adjustments. The electrode was stored in a HI 70300 storage solution, while calibration was performed using HI 7004 pH 4.01 buffer solution, HI 7010 pH 10.01 buffer solution (Hanna Instruments, Graz/Austria) and Roth A518 pH 7.00 buffer solution (Roth, Karlsruhe/Germany).

As a heated shaker a Hamilton Microlab STARlet robot was used (Hamilton,

Bonaduz/Switzerland).

For absorbance measurements a Synergy HTX Multi-Mode Reader from BioTek Instruments, Vermont/USA, was used together with the supplied software Gen5. Standard curves, concentrations and accuracy were calculated by Gen5 and results were exported to MS Excel.

2.2 Reagents and standards

Human serum albumin (HSA) (A1887), human alpha-1-acid glycoprotein (AGP) (G9885), porcine serum albumin (PSA) (A1830) and bovine serum albumin (BSA) (A7030) were purchased from Sigma Aldrich, St. Louis/USA. Human pooled plasma with K3 EDTA as anticoagulant was obtained from Biotrend, Cologne/Germany. Plasma, lymph and interstitial fluid from pigs were self-gathered by a veterinary during open flow microperfusion (OFM) experiments at the Institute of Cytology at University Hospital Graz. The pigs were standard Austrian landrace. Plasma was obtained via EDTA anticoagulant addition and lymph was extracted from the thoracic duct. Interstitial fluid was gathered via OFM catheters and pumps, either by recirculation or by suction method.

Water was highly purified with a Milli-Q Water Purification System (Merck Millipore, Darmstadt/Germany).

Methanol (14262), acetonitrile (34851) and 2-propanol (34965) were purchased from Sigma Aldrich, St.Louis/USA, in HPLC gradient grade quality. Formic acid (27001) was bought from Sigma Aldrich as well. A Roti-Stock 10x PBS buffer pH 7.4 (1058) in BioScience grade was purchased from Roth, Karlsruhe/Germany.

The six analytes for protein binding experiments and their internal standards are listed here together with mass to charge ratio for positive mode (H^+):

Table 3: Analtyes and internal standards.

Analyte	m/z (H^+)	Product No.	Manufacturer
Amitriptyline:HCl	278.1884	CIL-ULM-8350	Cambridge Isotope Laboratories, Tewksbury/USA
Amitriptyline-d6:HCl	284.2294	DLM-3008-S	Cambridge Isotope Laboratories, Tewksbury/USA
Clobetasol Propionate	467.1995	C8037	Sigma Aldrich, St.Louis/USA
Clobetasol Propionate-d5	472.2266	C583502	Toronto Reseach Chemicals, Toronto/Canada
Diclofenac sodium salt	296.0240	M012380	Arch Pharmalabs, Mumbai/India
Diclofenac-(acetophenyl ring-13C6) sodium salt	302.0426	35361	Sigma Aldrich, St.Louis/USA
Hydrocortisone	363.2166	H0888	Sigma Aldrich, St.Louis/USA
Hydrocortisone-d4	367.2401	705594	Sigma Aldrich, St.Louis/USA
Lidocaine	235.1805	PHR1034	Sigma Aldrich, St.Louis/USA
Lidocaine-d10 hydrochloride	245.2413	L397802	Toronto Reseach Chemicals, Toronto/Canada
Metronidazole	172.0717	M3761	Sigma Aldrich, St.Louis/USA
Metronidazole-d4	176.0956	M338882	Toronto Reseach Chemicals, Toronto/Canada

For gel electrophoresis the following chemicals were bought from Roth, Karlsruhe/Germany: Coomassie Billiant Blue R 250 (3862.1), acetic acid (3738.5), TRIS HCl (4855.3), ethanol (9065.5) glycine (3790.2), sodium dodecyl sulfate (SDS) (0183.2), N,N,N',N'-tetramethylethylendiamin (TEMED) (2367.3) and glycerol (7530.4). Acrylamide (A9099) was purchased from Sigma Aldrich, St.Louis/USA and bromphenol blue (108122) from Merck, Darmstadt/Germany. Dithiothreitol (DTT) (27565-41-9) was ordered from Formedium, Hunstanton/United Kingdom, and ammonium persulfate (APS) (17874) from Thermo Fisher Scientific, Bremen/Germany.

2.3 Albumin measurement

The albumin concentration was measured with an ALB plus Kit (11970909) from Roche Diagnostics, Mannheim/Germany. The determination was performed as described in the supplied manual. The albumin concentration is directly proportional to a change of color when bound to bromcresol green. To determine the concentration a calibration curve was generated. The test was performed in duplicates and was validated via measuring three quality control samples. Absorbance measurements were performed in a BioTek Synergy HT microplate reader (BioTek, Bad Friedrichshall/Germany).

2.4 Pig alpha-1-acid glycoprotein ELISA

To measure the amount of alpha-1-acid glycoprotein (AGP) in biological fluids an ELISA kit (ab205068) was used from Abcam, Cambridge/United Kingdom. The kit was used as described in the supplied manual. For absorbance measurements a Synergy HTX Multi-Mode Reader from BioTek Instruments, Vermont/USA, was used together with the supplied software Gen5. Standard curves, concentrations and accuracy were calculated by Gen5 and results were exported to MS Excel.

2.5 Gel electrophoresis

Electrophoretic separation of proteins was carried out with one-dimensional SDS−polyacrylamide gel electrophoresis using the Bio-Rad Mini-Protean Tetra Handcast Systems and a PowerPac Basic Power Supply (Bio-Rad Laboratories, Richmond/USA).

Gels were 0.75 mm thick and 8.6 x 6.7 cm in size. They were prepared with 8 % and 12.5 % polyacrylamide - detailed ingredients are listed in table 4. After polymerization was started the solution was poured in the glass form and some 2-propanol was filled on top to create an ideal horizontal surface. When the separation gel became firm and the 2-propanol was washed away, the stacking gel was filled on top of the separation gel.

A Tris-glycine-SDS buffer was used as an electrophoretic buffer with concentrations of 0.025 M Tris, 0.192 M glycine and 0.1 % SDS at pH 8.5. 1 µl sample was diluted with 4 µl water and 5 µl sample buffer. The solution was incubated for 10 minutes at 95 °C and 500 rpm to fully denature the proteins and to reduce disulfide bonds. The sample buffer contained 0.5 M dithiothreitol, 50 % glycerol, 10 % SDS, 0.02 % bromphenol blue and 0.3

M Tris HCl (pH 6.8). 3 µl of PageRuler Prestained Protein Ladder (SM067) for 10 to 170 kDa (Thermo Fisher Scientific, Bremen/Germany) was used as a standard.

Electrophoresis was carried out with 17 mA per gel for 2 hours. The gel was then put into a staining solution at 30 rpm for 20 minutes and was destained overnight. The staining solution consisted of 0.25 % coomassie blue, 10 % acetic acid and 40 % ethanol, while the destaining solution was the same but without coomassie blue.

The gels were scanned and analyzed with the program ImageJ.

Table 4: Ingredients for SDS-PAGE gels.

Ingredient	**8 % gel**	**12.5 % gel**	**Stacking gel (2x)**
Water	2.06 ml	1.48 ml	3.69 ml
1 M Tris HCl	1.875 ml (pH 8.8)	1.875 ml (pH 8.8)	625 µl (pH 6.8)
30 % Acrylamid	1 ml	1.56 ml	563 µl
20 % SDS	25 µl	25 µl	25 µl
APS	24 µl	24 µl	35 µl
TEMED	5 µl	5 µl	10 µl

2.6 HPLC-MS analysis

The unbound drug concentrations were determined using a DIONEX Ultimate 3000 HPLC system (Dionex, Germering/Germany), which consisted of an UltiMate 3000 RS Autosampler, an UltiMate 3000 Pump and an UltiMate 3000 Flow Manager. Mass spectrometric detection was performed on a LTQ Orbitrap XL instrument (Thermo Fisher Scientific, Bremen/Germany) equipped with an HESI-II probe ion source in positive ion polarity mode.

The software Thermo Xcalibur 2.1 was used for data processing and quantification.

Internal standards were used for quantification and can be seen in Table 3. The stock solutions had 1 µg/ml.

The settings used for measuring the analytes are listed below:

Table 5: HPLC settings.

HPLC Device	Dionex UltiMate 3000
Analytical Column	Waters Atlantis T3, 150x2.1 mm, 3µm, 100 A
Column Temperature	25°C
Mobile Phases	A: MilliQ-Water + 0.1 % formic acid
	B: Acetonitrile + 0.1 % formic acid
Gradient	0 min: 5 % B
	1.5 min: 50 % B
	2 min: 90 % B
	4 min: 90 % B
	4.1 min: 5 % B
	7 min: 5 % B
Flow Rate	0.3 ml/min
Injection Volume	10 µl

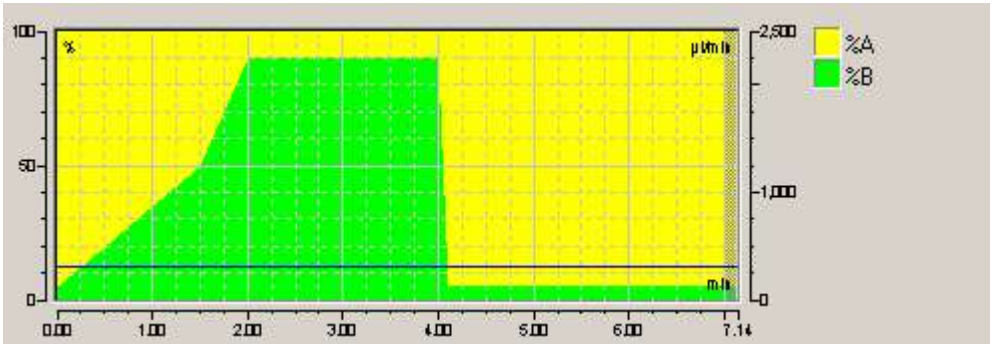


Figure 19: Gradient visualized.

Between 2.5 and 5.5 minutes the sample was transferred to the MS as the analyte retention times occur in between. The rest was sent to waste (figure 20).

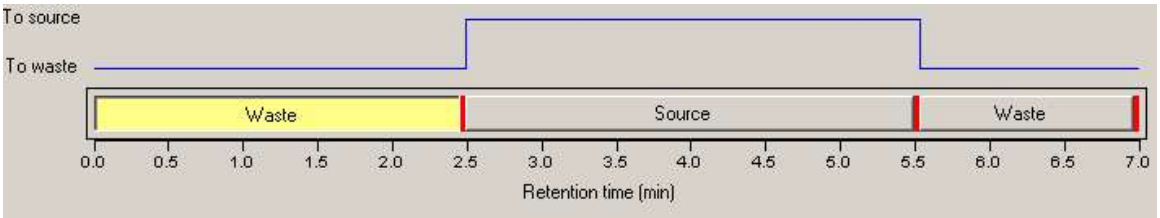


Figure 20: Source and waste. Because of possible protein residues the eluate of the first 2.5 min was discarded.

Table 6: MS and ionization settings.

Heater Temperature	300 °C
Sheath Gas	30arb
Aux Gas	5arb
Sweep Gas	0arb
Spray Voltage	3.50 kV
Capillary Temperature	275 °C
Capillary Voltage	9 V
Tube Lens	55 V
Ionization Mode	HESI+ Full Scan m/z 100-500

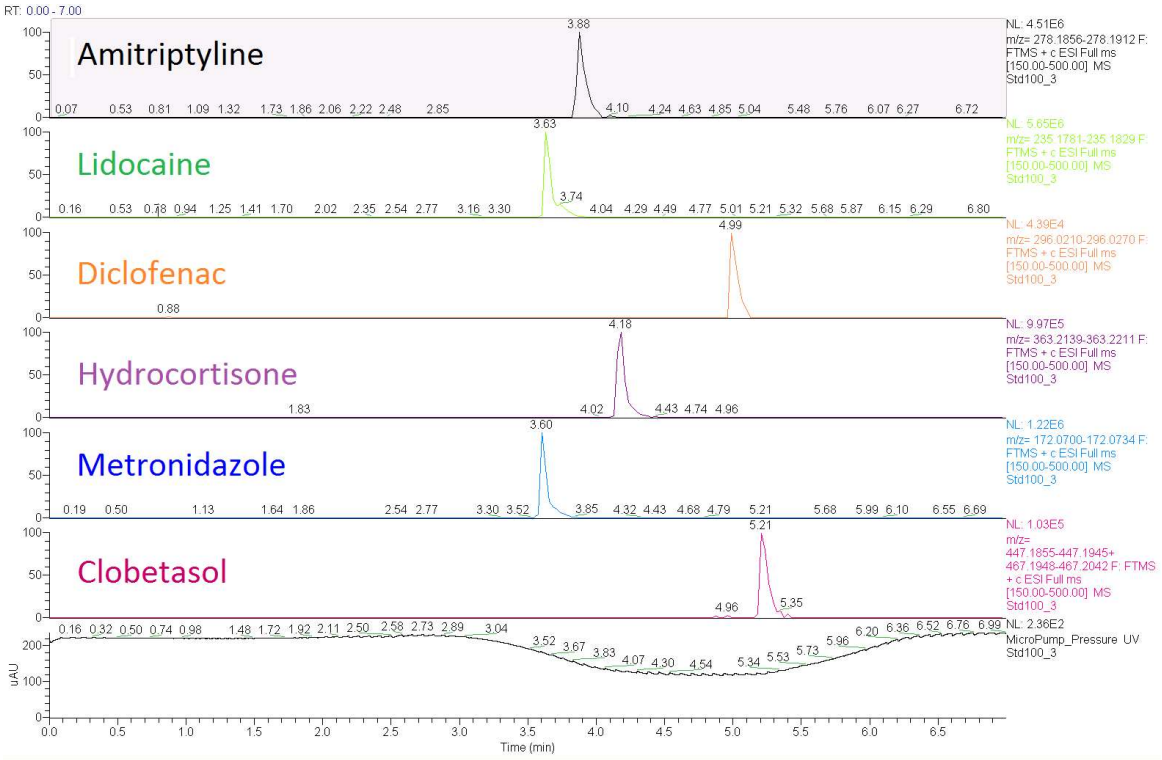


Figure 21: Example chromatogram of the six analytes at a concentration of 100 ng/ml.

2.7 Methods for drug-protein binding analysis

2.7.1 General Procedures

If not particularly mentioned all experiments were performed under physiological conditions of 37 °C and a pH of 7.4. Before any experiment the samples were equilibrated for 1 hour at 37 °C in order to facilitate proper drug-protein binding. A negative control

with spiked PBS was used in each experiment which was treated similar to the samples. If solutions contained proteins after an experiment these were precipitated prior to HPLC-MS analysis. Therefore acetonitrile with 1 % formic acid was added to the samples. Then the samples were gently inverted and centrifuged with 14,000 x g for 10 minutes. Afterwards the solvent of the supernatant was evaporated with compressed air and the residue redissolved in 5 % acetonitrile. The protein-free samples were then analyzed.

2.7.2 Rapid Equilibrium Dialysis (RED) experiments

The RED device for Rapid Equilibrium Dialysis (90006) was bought from Thermo Scientific Pierce, Rockford/USA. It is a 96 well-plate for 48 samples as each sample has a pair of two chambers. It is a single-use plate with 8K MWCO inserts. The RED method was performed as described in the owner's manual. During the experiments 50 µl of the samples containing 100 ng/ml of each analyte were placed into the sample chamber and 300 µl PBS buffer was placed into the buffer chamber. The plate was incubated at 37 °C while agitated with 300 rpm on an orbital shaker. After 4 hours 40 µl of each chamber were taken out and 10 µl of all internal standards stock solutions were added. Then proteins were precipitated and the analyte concentrations were measured with HPLC/MS.

2.7.3 Ulrtafiltration (UF) experiments

For ultrafiltration experiments Amicon Ultra 0.5 mL centrifugal filters (UFC501096) with 10K MWCO from Millipore, Carrigtwohill/Ireland, were used. The UF method was performed as described in the owner's manual. Thereby, 200 µl of sample were added to the filtration tube.

50 µl of filtrate and 50 µl of concentrate were mixed with 20 µl internal standard each. Then proteins were precipitated and the analyte concentrations were measured with HPLC/MS.

2.7.4 Ultracentrifugation (UC) experiments

Ultracentrifugation was performed in cooperation with the group of Frank Madeo at Karl-Franzens-University of Graz at the Institute of Biochemistry. Therefore a Beckmann Optima LE-80K Ultracentrifuge was used with a SW 41 Ti Rotor for 6 centrifuge tubes

with a minimal sample volume of 12 ml. Centrifugation was performed at 20 °C for 21.5 hours and 38,000 rpm - equal to 170,000 x g. Only spiked 1 %-HSA and PBS solutions were investigated due to the high amount of volume needed. After centrifugation fractions of 1 ml each were taken from the samples. 50 µl of each fraction was mixed with 10 µl internal standard and proteins were precipitated. The analyte concentrations were measured with HPLC/MS.

2.7.5 Solid-phase microextraction (SPME) experiments

Supelco SPME C18 LC Tips were bought from Sigma Aldrich, St. Louis/USA, as they claim to be particularly designed for drug-binding studies. Before an experiment the fibers had to be washed in methanol for 10 minutes and then equilibrated with desalted water for 10 minutes. It is important that the fibers do not dry out before the experiment, otherwise the extraction efficiency decreases. A volume of 800 µl of spiked samples was filled into Eppendorf Protein LoBind 96-Deepwell plates (Eppendorf, Hamburg/Germany). Spiked PBS buffer samples were added to the plate as well in order to have a 100 % unbound value as the buffer contains no proteins. The fibers were placed in the spiked samples (100 ng/ml) and the well plate was strongly agitated with approx. 1000 rpm for 1 hour. Then the extracted drugs were desorbed for 15 minutes in 200 µl acetonitrile which contained the internal standards. The acetonitrile was then evaporated with compressed air and 200µl of 5 % acetonitrile was added. The samples were then analyzed via HPLC-MS. A short overview of the workflow can be seen in figure 22.

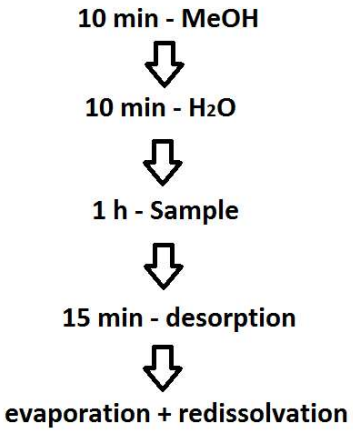


Figure 22: Overview of the workflow from the SPME experiment.

2.7.6 Size exclusion experiments

For size exclusion chromatography we tested different columns from Bio-Rad, based on polyacrylamide beads, and from Agarose Bead Technologies (ABT) with agarose beads. The Micro Bio-Spin Chromatography Columns P-30 Gel with Tris buffer (7326223) and SSC buffer (7326202) were purchased from Bio-Rad Laboratories, The crosslinked agarose bead solution 10 % BCL Agarose Bead (A-1101S), Empty Plastic Small Columns (CS-20) and Empty Mini Spin Columns (SP-25) were bought from Agarose Bead Technologies, Madrid/Spain.

The polyacrylamide catridges were supplied with different buffers - Tris and SSC. They were used with the delivered buffer and were also exchanged and equilibrated with PBS buffer according to the supplied manual. The samples were loaded onto the column and eluted by centrifugation. To elute the next fraction buffer was added and centrifuged again.

The agarose columns were filled by hand with certain amounts of agarose beads, and were equilibrated with PBS buffer. The samples were eluted by centrifugation but also by gravity to exclude the influences of centrifugal forces on protein binding.

3 RESULTS & DISCUSSION

3.1 Drug-protein binding studies and comparison of methods

In order to find a suitable method for drug-protein binding measurements different methods were tested and discussed here.

3.1.1 Ultracentrifugation

Ultracentrifugation takes place in areas of more than 100,000 x g. This could separate proteins from free drugs due to their smaller density. To investigate the separation principle, an ultracentrifuge was loaded with 12 ml sample solution. Only spiked 1 %-HSA and PBS solutions were used, because of the high volume needed. The samples were collected layer by layer and examined individually. Due to the strong centrifugation all proteins should have been sedimented while the free drugs should be in the upper

fractions.

No complete separation was obtained. A protein gradient over many layers had formed and even the lightest fraction on top of the centrifuge tube contained some protein. Therefore, no calculation for protein binding could have been done.

However, in principle the result was as expected as the most dense fraction from the bottom of the vial showed the highest amount of protein and the highest drug concentration in case of hydrocortisone and clobetasol – which are meant to be highly protein bound. It could be possible that a more intensive centrifugation could lead to a separation of proteins and free drug. But as the method should be suitable to investigate biological fluids the sample volume of 12 ml is too high. Therefore no further experiments were done with UC. In general there would be UC devices available that can perform 500,000 x g which could be used for 200 µl sample volume as well. (Naikai, 2004)

3.1.2 Size Exclusion

With the SSC and PBS buffer system the polyacrylamide beads led to a very good separation of proteins and free drugs. Unfortunately, the separation did not isolate free and bound drugs but apparently led to a full release of bound drugs, and therefore no analyte was detected in the protein fraction. This observation was reproducible even after variation of the buffers and centrifugation speeds. Possible reasons for breaking the protein binding could be an interaction between the protein and the column material. Another considerable reason could be a squeezing effect by high centrifugal forces during centrifugation. To rule out interactions with polyacrylamide, also columns with agarose beads were tested and the influence of centrifugal forces was examined as well.

However, the agarose bead experiments did not give any results as well. Here the main problem was an insufficient chromatographic separation which led to a continuous elution of the proteins over several fractions. It was not possible to separate the free drug from the protein, neither with centrifugation columns nor with the gravity columns.

Since none of the size exclusion chromatographic methods gave satisfying results the experiments were discontinued. The experiments with both bead types had the problem that there was no complete separation of proteins and the free drug.

3.1.3 Ultrafiltration

Many UF experiments were performed and did not give any positive result. The main reason found for that were high adsorption values. The initial experiments were all performed with spiked HSA and PBS solutions. In order to examine the adsorption different albumin concentrations were used and human plasma. The results are listed below in table 7:

Table 7: Adsorption in % measured for amitriptyline, hydrocortisone and clobetasol during UF experiments with different albumin concentrations (0.2 %, 1 %, 4 % and 8 %) in PBS buffer and human Plasma.

	Amitriptyline	Hydrocortisone	Clobetasol	Metronidazole
0.2 % Albumin	84	64	89	2
1 % Albumin	77	59	81	4
4 % Albumin	60	41	62	2
8 % Albumin	42	23	43	0
Plasma	14	15	15	6

Low-protein fluids usually have higher adsorptions as compounds that are not surrounded by a dense and protective matrix. This can be seen in the results as the adsorption decreases when the albumin content increases. As the complexity of the medium rises the smaller the adsorption gets.

In plasma the adsorption was 15 %, which clearly affects the binding results. Metronidazole had nearly no loss due to adsorption, most likely because of its hydrophilic properties. As most drugs are rather lipophilic, adsorption plays a major role in terms of low-protein drug-binding studies. Lidocaine and diclofenac showed the same effect, but not as drastic (not shown). Therefore, ultrafiltration is not in accordance with the demands of low-protein fluids for the majority of drugs. For the analysis of plasma UF should generally work.

3.1.4 Solid-phase microextraction

Initial problems concerning the reproducibility of the results could have been solved with a stronger agitation. Good mixing is an essential criterion to gain useful results.

Table 8: Values of bound drug (in percentage) from SPME results with n=3. Human plasma, pig plasma and pig lymph were investigated. Also 1:1 diluted human plasma with PBS was examined.

	Lidocaine	Metronidazole	Amitriptyline	Hydrocortisone	Diclofenac	Clobetasol
Human Plasma	**73**	**13**	**86**	**96**	**100**	**93**
St.dev.	±1.4	±9.2 (n=2)	±1.2	±0.2	±0	±0.4
Diluted human Plasma	**55**	**4**	**75**	**92**	**100**	**87**
St.dev.	±5.3	±0.8 (n=2)	±5.3	±0.7	±100	±5.8
Pig Plasma	**60**	**6**	**86**	**82**	**100**	**88**
St.dev.	±2.9	±2.4 (n=2)	±0.4	±0.7	±0	±0.8
Pig Lymph	**59**	**16**	**83**	**84**	**100**	**85**
St.dev.	±5.1	±1.3 (n=2)	±2.7	±0.9	±0	±1.9
Literature (human Plasma)	60-80	<5	96	>90	99,7	98

Results from human plasma were in accordance with the values found in literature. Amitriptyline and clobetasol were slightly lower, but are still in a ratio which is described for biological fluids and natural fluctuations. Interestingly there are only little differences when comparing human and pig plasma. Lidocaine and hydrocortisone are slightly less bound, but the other drugs show similar results. Also plasma and lymph from pig showed the same results, although lymph has only around 60 % of the protein amount of plasma. The dilution of plasma led to a general reduction of the binding values except of diclofenac, which seems to be extremely strong bound.

As SPME seems to show reliable results, also low-protein fluids were examined. Therefore different types of albumins were used in order to investigate again the differences between species. Human serum albumin (HSA) was used in two forms, one was the already solved Albunorm and one was pure solid albumin which was solved in PBS. Albunorm contains N-acetyl-DL-tryptophan and caprylic acid as stabilizers, which are known to be bound by proteins.

Table 9: Values of bound drug (in percentage) from SPME results with n=3. Different albumin solutions were investigated – bovine (BSA), porcine (PSA) and human (HSA). Also the addition of 0.1% AGP in 1% HSA was examined.

	Lidocaine	Metronidazole	Amitriptyline	Hydrocortisone	Diclofenac	Clobetasol
1% BSA	**44**	**5**	**63**	**66**	**100**	**64**
St.dev.	±8.8	±6.8	±9.6	±6.5	±0	±12.0
1% PSA	**42**	**-10**	**29**	**78**	**100**	**52**
St.dev.	±13.3	±18.9	±11.8	±5.3	±0	±9.2
1% HSA (Albunorm)	**46**	**3**	**28**	**71**	**100**	**54**
St.dev.	±4.2	±6.0	±6.9	±1.5	±0	±3.1
1% HSA	**41**	**9**	**49**	**70**	**100**	**60**
St.dev.	±3.9	±9.4	±7.2	±3.0	±0	±6.2
1% HSA + 0.1% AGP	**75**	**12**	**75**	**69**	**100**	**80**
St.dev.	±0.8	±12.4	±1.1	±0.7	±0	±4.2
Literature (human Plasma)	60-80	<5	96	>90	99,7	98

When comparing the albumins of the different species lidocaine, metronidazole, diclofenac and clobetasol seem to be equally bound by all albumins. Hydrocortisone is lower and amitriptyline slightly higher bound in bovine albumin in comparison to both other species. Additionally, hydrocortisone is also bound slightly higher in porcine albumin. HSA from solid source showed nearly no difference to HSA from Albunorm. Only the amitriptyline binding and faintly the one from clobetasol seems to be influenced by N-acetyl-DL-tryptophan and caprylic acid within Albunorm. It has been shown for warfarin and digitoxin that the stabilizers in Albunorm can lower the binding affinity in albumin solutions with PBS as well. (Olsen, 2004) But for all other drugs used here these stabilizers have no effect. When comparing HSA with the solution that contained HSA and AGP it can be seen that especially lidocaine is strongly bound by AGP and amitriptyline and clobetasol as well, but less strong. 1 % HSA with 0.1 % AGP is also the solution which is the closest to the plasma literature value. This verifies that albumin and AGP are the proteins with the main influence on drug binding in plasma besides of lipoproteins.

The SPME technique is a robust method with many advantages, as the experimental

procedure is very time effective, reproductive and simple to perform. Furthermore it can be easily used for high-throughput approaches. It also eliminates the need for protein precipitations and minimizes matrix interferences. Also results of the binding experiments in plasma are comparable with the reports in literature. The only disadvantage of SPME and its usage for drug-protein binding determination in low-protein fluids is the required sample volume of at least 800 µl. Using smaller volumes the results were not reproductive. Currently the sampling of interstitial fluid is slow with a few µl per hour. Additionally, there is the problem with extraction of hydrophilic substances, although the majority of drugs tends to be lipophilic. SPME has a great potential for in-vivo measurements. The fibers can be placed via a syringe directly into the tissues. However, this in-vivo method must still be properly investigated.

3.1.5 Rapid equilibrium dialysis (RED)

The experiments with RED gave very robust and reproducible results.

Table 10: Values of bound drug (in percentage) from RED results with n=3. Human plasma, pig plasma, pig lymph and OFM recirculation were investigated. Also 1:1 diluted pig and human plasma with PBS were examined.

	Lidocaine	Metronidazole	Amitriptyline	Hydrocortisone	Diclofenac	Clobetasol
Human Plasma	**55**	**-8**	**98**	**93**	**100**	**96**
St.dev.	±3.7	±7.7	±0.3	±0.6	±0	±0.2
Diluted human Plasma	**23**	**-5**	**87**	**86**	**100**	**91**
St.dev.	±4.1	±5.1	±2.3	±1.1	±0	±0.2
Pig Lymph	**59**	**3**	**100**	**78**	**100**	**91**
St.dev.	±4.2	±3.7	±0.6	±2.0	±0	±0.1
Pig Plasma	**66**	**-6**	**99**	**82**	**100**	**93**
St.dev.	±1.7	±10.4	±0.1	±0.9	±0	±0.7
Diluted Pig Plasma	**38**	**-1**	**95**	**68**	**100**	**86**
St.dev.	±5.8	±3.1	±1.7	±3.6	±0	±1.9
OFM recirculation	**23**	**-8**	**93**	**57**	**96**	**77**
St.dev.	±7.2	±6.7	±2.4	±1.3	±3.1	±3.2
Literature (human Plasma)	60-80	<5	96	>90	99,7	98

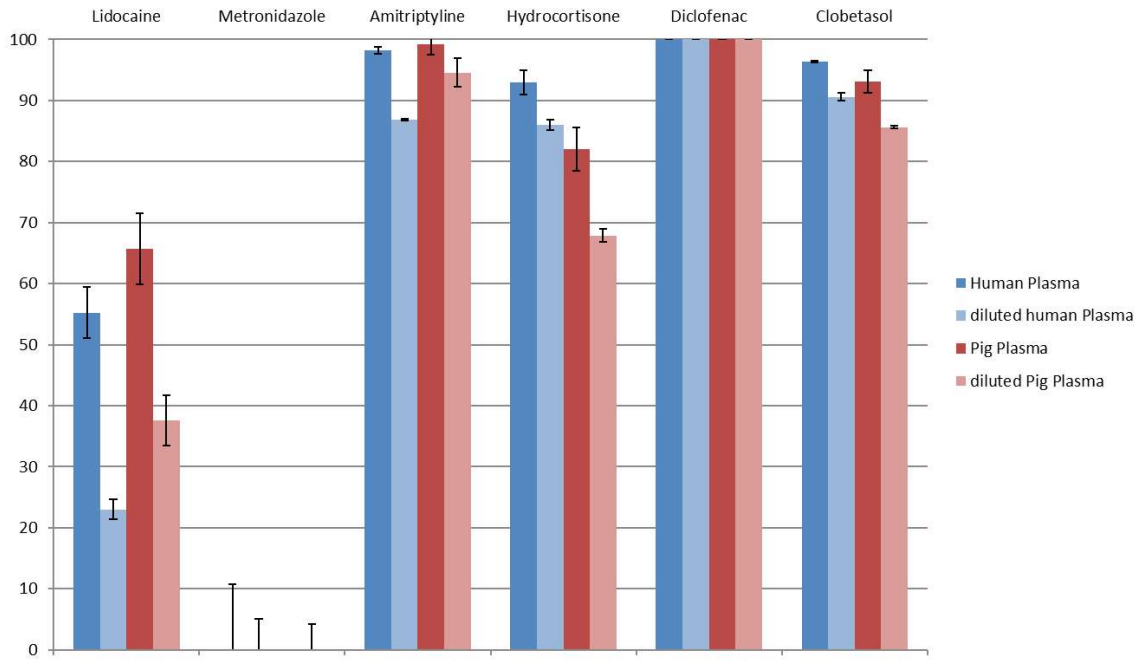


Figure 23: Visualization of the results shown in Table 10 to compare human and pig plasma.

The results of human plasma correlate with the literature values. Again, like in the results from SPME, human plasma showed the same binding behavior as porcine plasma, while only hydrocortisone is little bit less bound in pig plasma. Also lymph and plasma from pig showed the same binding values. For both species the dilution of plasma led to a slight reduction of the binding values as well. OFM recirculation samples originated from pig and should be comparable with the interstitial fluid. The results were different to those from all other pig fluids. In general the drugs in the OFM recirculation sample were bound to a lower extend. This could either mean that the ISF has different binding values than plasma or that the recirculation method did not deliver proper or diluted ISF.

Beside the OFM recirculation also OFM suction was used as method to sample ISF. As this method is still in development very little fluid could have been extracted from pig tissue. With the amount of fluid obtained only a single experiment could be carried out. The results are shown in a comparison with the OFM recirculation.

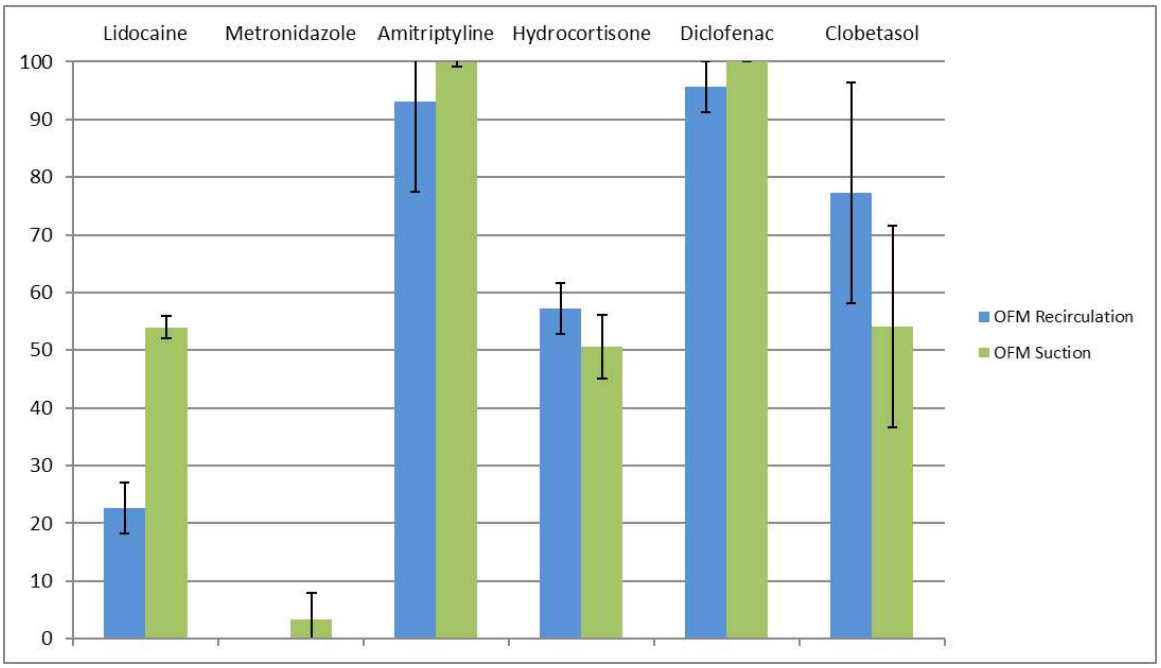


Figure 24: Visualization of the values of bound drug (in percentage) from OFM recirculation (n=3) and OFM suction (n=1).

Both OFM fluids have similar binding behaviors for metronidazole, amitriptyline, hydrocortisone and diclofenac. The values of clobetasol and lidocaine show in contrast very different binding values. In OFM recirculation lidocaine is less than 50 % compared to the experiment using OFM suction. For clobetasol the difference is not as drastic but still the standard deviation is very high. It is not possible to make a valid conclusion as only one sample of the OFM suction fluid was available and no statistical test could be performed. At this state it cannot be said if one method is better or if any method does even reflect ISF. To clarify this more fluid has to be gathered and many more experiments are necessary.

Also low-protein fluids were investigated with RED.

Table 11: Values of bound drug (in percentage) from RED results with n=3. Different albumin solutions were investigated – bovine (BSA), porcine (PSA) and human (HSA). Also the addition of 0.1 % AGP in 1 % HSA was examined.

	Lidocaine	Metronidazole	Amitriptyline	Hydrocortisone	Diclofenac	Clobetasol
1% HSA (Albunorm)	**9**	**9**	**43**	**28**	**100**	**76**
St.dev.	±8.3	±8.6	±15.1	±5.4	±0	±3.8
1% PSA	**7**	**3**	**59**	**45**	**100**	**66**
St.dev.	±2.6	±3.2	±3.1	±5.4	±0	±2.1
1% BSA	**10**	**-3**	**67**	**23**	**100**	**64**
St.dev.	±6.0	±5.4	±0.8	±3.1	±0	±2.5
1% HSA	**6**	**-3**	**51**	**29**	**100**	**85**
St.dev.	±4.9	±7.0	±4.8	±10.9	±0	±3.7
1% HSA + 0.1% AGP	**60**	**1**	**89**	**39**	**100**	**97**
St.dev.	±2.6	±1.7	±1.9	±5.1	±0	±2.3
Literature (human Plasma)	60-80	<5	96	>90	99,7	98

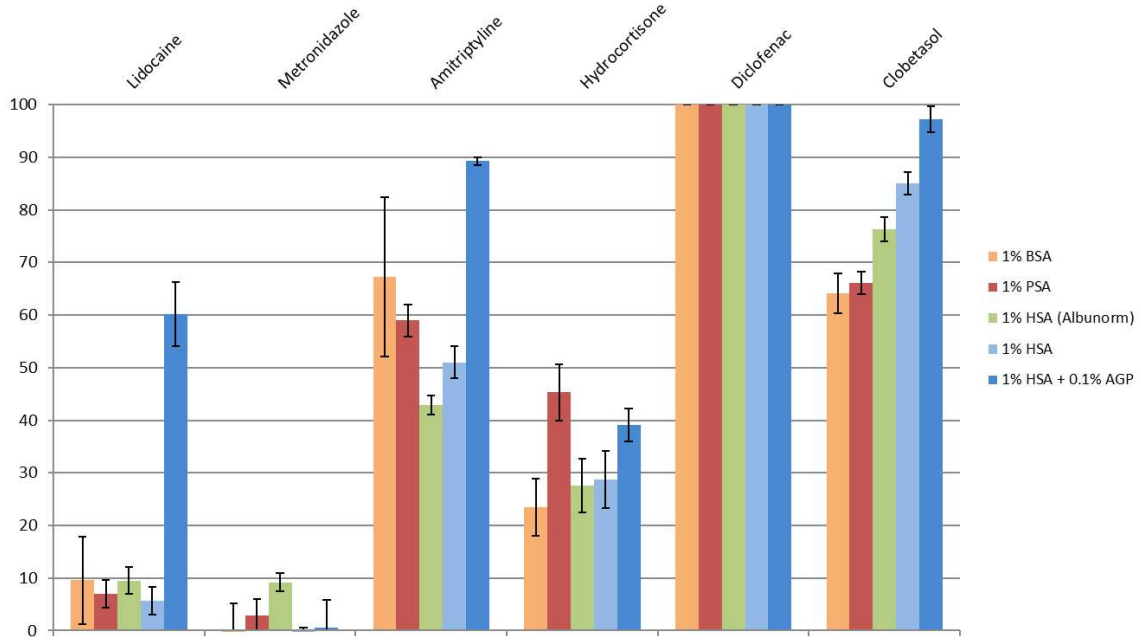


Figure 25: Visualization of the results shown in Table 11.

Similar to the low-protein fluid results from SPME all tested drugs seem to have similar albumin binding affinity. Amitriptyline is again slightly more and hydrocortisone less bound by BSA. Hydrocortisone is also higher bound in PSA. The only difference to the SPME results is that all lidocaine and hydrocortisone binding values are much lower here. The AGP addition has a crucial effect on the binding of lidocaine. Likewise amitriptyline and clobetasol, but here also hydrocortisone, are bound to AGP. Little negative effects on the binding by stabilizers in Albunorm can be seen for amitriptyline and clobetasol.

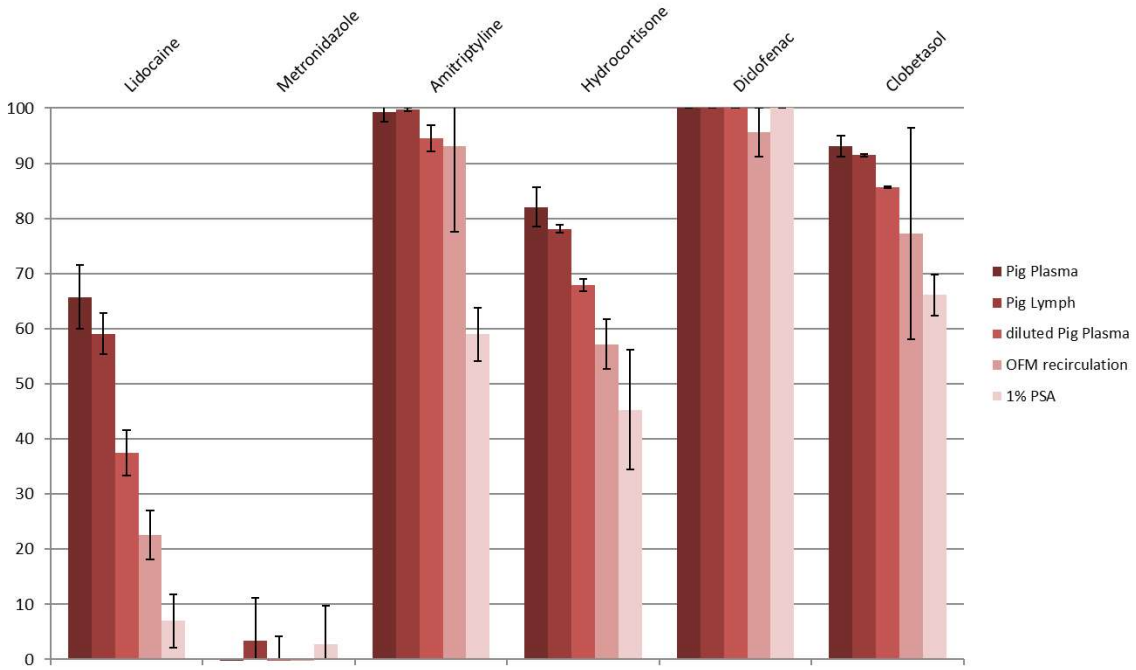


Figure 26: Visualization of the results from pig fluids shown in Table 10 and Table 11.

RED has also the same advantages as SPME. It is very robust, reproducible and simple to perform. The RED devices are also suitable for pipetting robots and high-throughput approaches. Furthermore, RED works - unlike SPME - with small volumes of 50 µl as well. Also adsorption has no effect on the binding results as a new equilibrium is formed always. All together RED would be the method of choice for good results in drug-protein binding experiments and is suitable for low-protein fluids.

3.2 Further investigations and validation of rapid equilibrium dialysis

Further experiments were carried out to validate the RED method. First it had to be verified that the different drugs do not interfere with each other in the binding experiments. Therefore the drug binding was examined for each single drug and in a mixture with all six drugs.

Table 12: Values of bound drug (in percentage) from RED results. All drugs were examined one by one in single drug experiments (n=2) and once all together in a mixture (n=3). The media was 1:2 diluted human plasma with PBS.

	Lidocaine	Metronidazole	Amitriptyline	Hydrocortisone	Diclofenac	Clobetasol
Single (n=2)	**23**	**-8**	**91**	**86**	**100**	**92**
St.dev.	±1.3	±1.1	±0.03	±0.1	±0	±1.6
Mixture (n=3)	**23**	**-5**	**87**	**86**	**100**	**91**
St.dev.	±4.1	±5.1	±2.3	±1.1	±0	±0.2
Difference	0	3	4	0	0	1

It can be seen that there is practically no influence on the binding result whether the drugs are used separately or simultaneously in a single protein solution. So for these six drugs it can be clearly said, that there is no interference via cross interactions.

Additionally, some environmental influences have been investigated as not much was found in the literature about the influence of due pH or temperature changes on drug-protein binding. (Abdel-Rehim, 2000) Therefore this had been examined with RED as well.

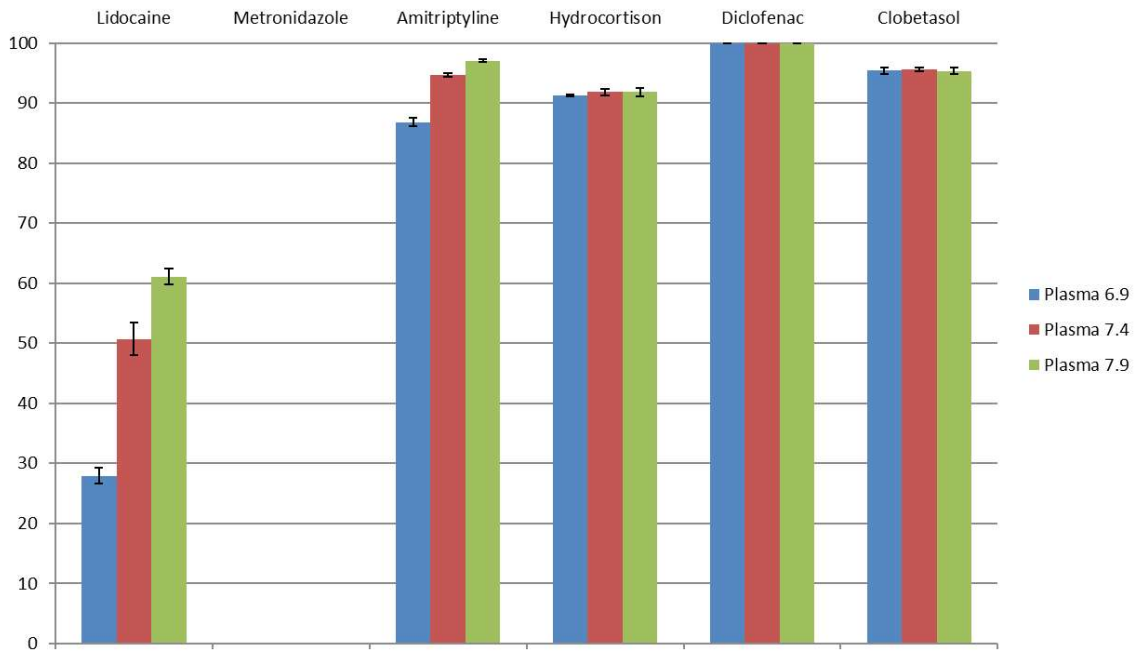


Figure 27: Visualization of the values from drugs bound (in percentage) in human plasma with different pH values of 6.9, 7.4 and 7.9.

For most of the drugs the pH changes do not have an influence on drug binding. The values for lidocaine differ strongly as the binding value is increased for a pH of 7.9 when the value of 7.4 is defined as the normal physiological state. Compared with the pH value of 6.9 the difference is even stronger as the binding is nearly only the half of the binding at 7.4. This very likely due to the pKa value of 7.9 from lidocaine. The deprotonated form of lidocaine at pH 6.9 seems to bind much less by plasma proteins. The effect is not as intensive for amitriptyline as it has a pKa value of 9.4, but still a small influence can be seen. The result shows that it would be safer to use drugs with a pKa value which is not near to the physiological pH of 7.4. It could be dangerous in a way of drug overdose for people who suffer for example under acidosis where the blood pH can be lowered. It has been shown that the blood pH values can vary between 6.8 and 7.8 in mammals. Outside this range it can lead to irreversible cell damage. (Needham, 2004)

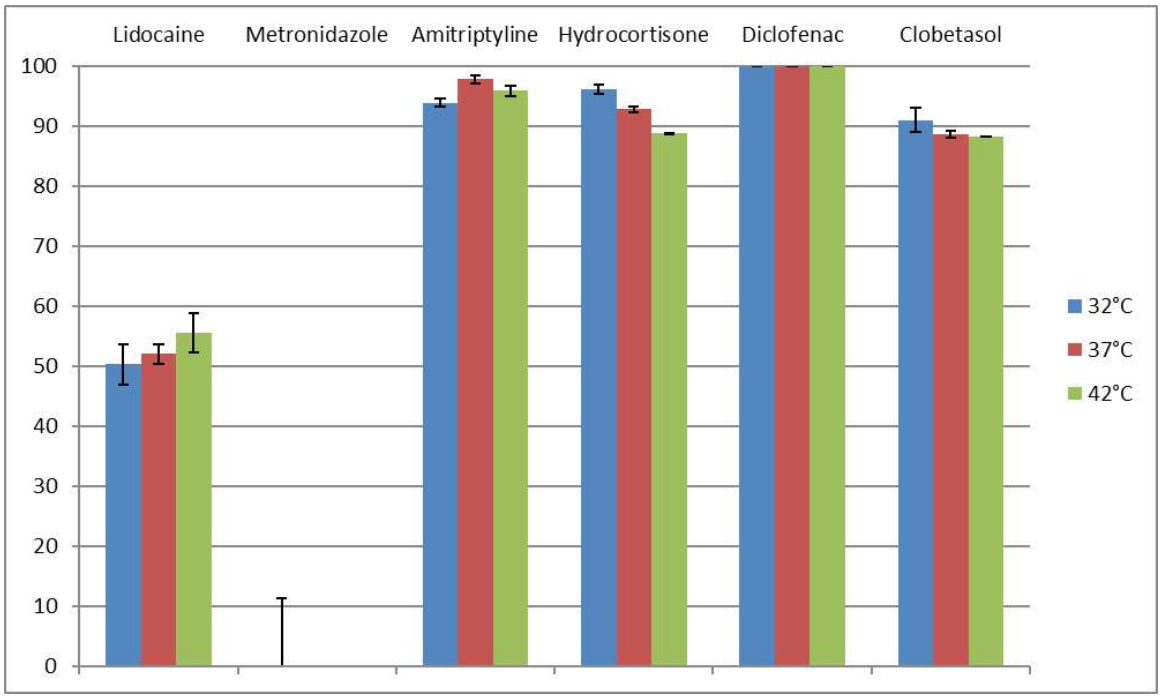


Figure 28: Visualization of the values from drugs bound (in percentage) in human plasma with different temperature values of 32°C, 37°C and 42°C.

Temperature changes between 32°C and 42°C do not seem to have an influence as all binding values vary minimally. It can be said that the drug-protein binding of those six drugs is not sensitive to temperature.

Besides the influence of pH and temperature it has been examined if different drug concentrations have an effect on the drug binding. Because of the more complex experimental setup amitriptyline was selected since this represents a highly bound drug.

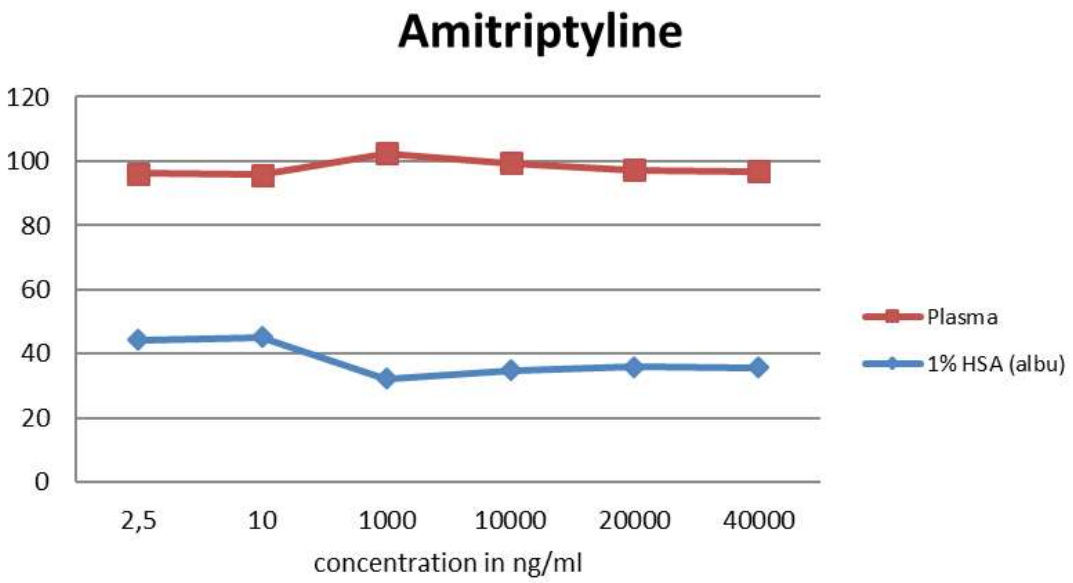


Figure 29: Visualization of the values from amitriptyline bound (in percentage) in plasma and in 1 % HSA. Amitripyline has been used in the concentrations 2.5 ng/ml (0.009 µmol), 10 ng/ml (0.036 µmol), 1000 ng/ml (3.6 µmol), 10000 ng/ml (36 µmol), 20000 ng/ml (72 µmol) and 40000 ng/ml (144 µmol).

The values for bound amitriptyline are relatively constant for each medium from low to high concentrations of the drug. In plasma it is highly bound, which is in line with the literature value of 96 % bound in plasma. In 1 % HSA amitriptyline is approximately 40 % bound in average. In earlier experiments using 1 % HSA it was bound to around 50 %, which is in an acceptable range. The result shows that the concentration of amitriptyline does not have an influence on the binding of drugs to proteins. It can only be assumed that once all proteins are fully loaded with amitriptyline, the bound value drops as no molecules can be bound anymore. As the commercial stock solution had the concentration of 40 µg/ml the maximum for the experimental setup had been reached.

3.3 Investigation of biological fluids

3.3.1 Comparison of protein compositions from biological fluids by SDS-PAGE

To get an additional insight in the whole protein spectrum of the used biological fluids some SDS-PAGE experiments had been performed. The resolution of a normal SDS-PAGE is not sufficient to get detailed information about all plasma proteins but at least

about the 14 most abundant which already count for 94 % of total protein content. All the other proteins are present in very small concentrations and may not have a reasonable effect on drug-protein binding. The protein identification would be better if a 2D-SDS Page would have been performed but no equipment was available for that. (Clement, 2012) In general every protein besides albumin, AGP, and lipoproteins could also bind a drug possibly. There is not much data available on the protein spectrum of interstitial fluid. Possibly the protein pattern of the biological fluids could give information about the different binding behaviors.

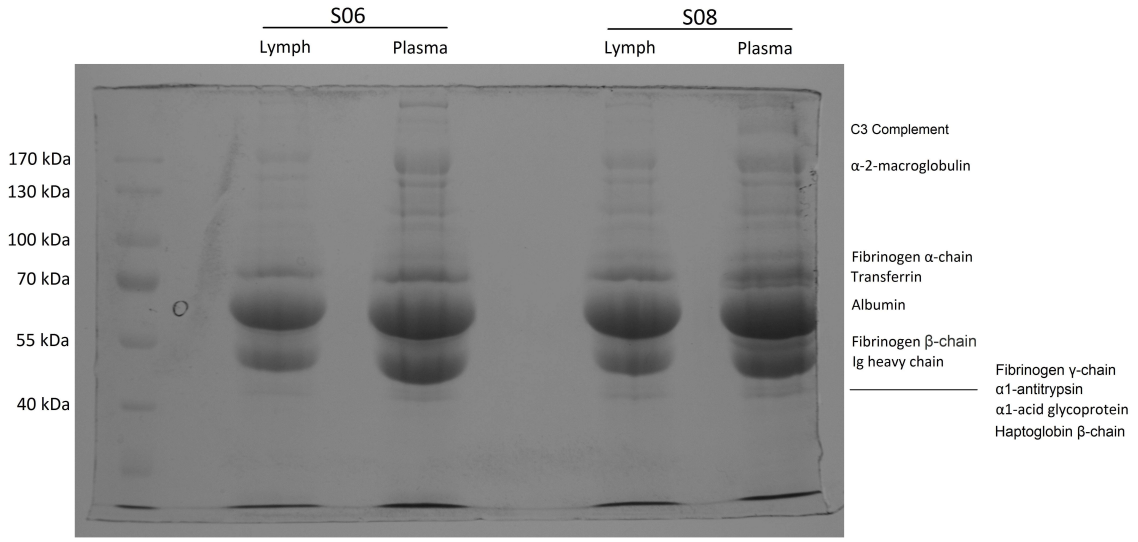


Figure 30: SDS-PAGE of porcine plasma and lymph samples. The standard can be seen in the first row.

On the first gel lymph and plasma samples from two pigs, subject 06 and 08, were loaded. The general pattern of protein bands from both subjects is similar for plasma and lymph, although lymph bands are slightly fainter. This result is in line with literature as lymph is said to contain only 50 % of the protein amount compared to plasma. (Anderson, 2002) To get a closer look the intensity of the samples on the gel were analyzed with the program ImageJ and compared.

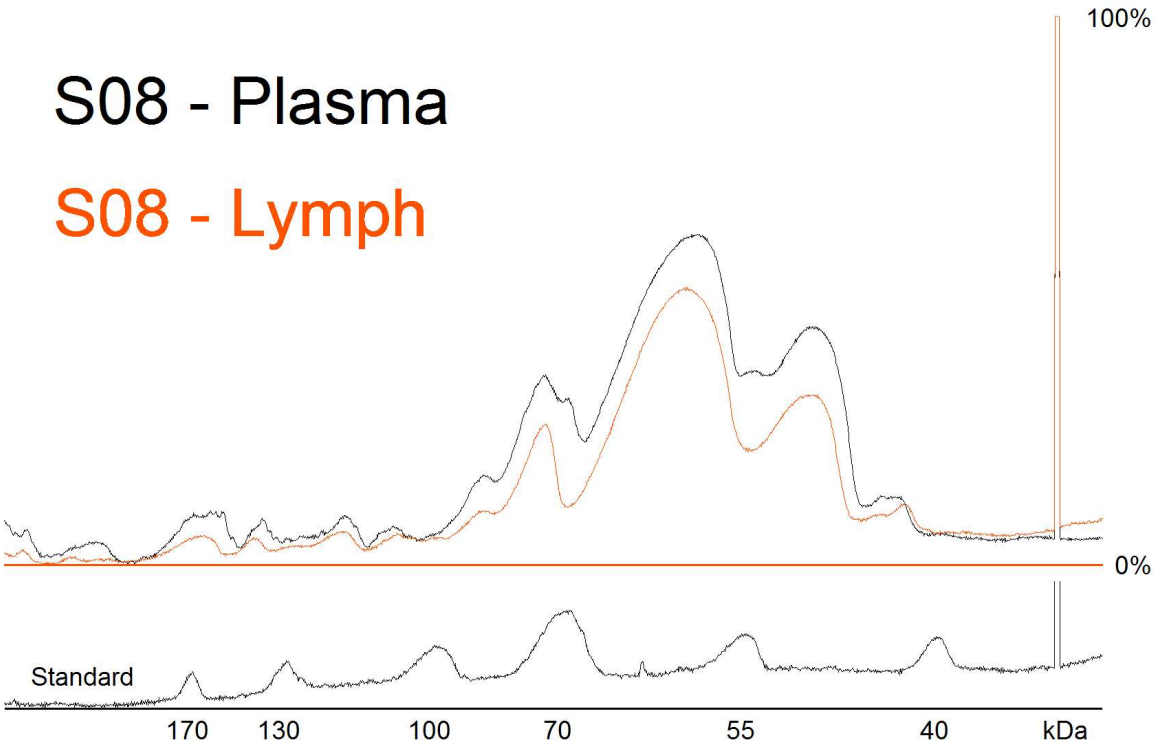


Figure 31: Comparison of the band intensities of plasma and lymph samples from a SDS-PAGE gel. Samples came from the pig No. 08. A 100 % value was added manually by adding a black line in order to get a reproducible proportion.

The areas under the curve (AUC) showed that the lymph from S08 is around 67.4 % of the AUC from plasma. Transferred to the comparison of protein content between plasma and lymph, two thirds of the amount in plasma is a reasonable value for lymph. Again it can be seen that the protein pattern of lymph and plasma is similar. This could be the reason why the binding results from chapter 3.1.5 were also very similar between plasma and lymph.

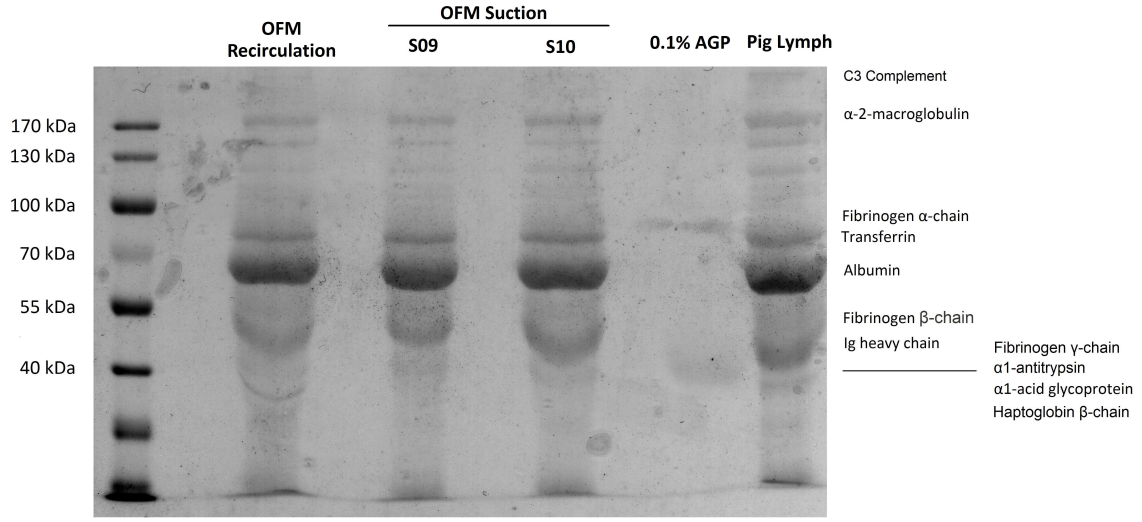


Figure 32: SDS-PAGE of porcine lymph, OFM recirculation and OFM suction samples and additionally 0.1 % AGP in PBS. The standard can be seen in the first row.

The second gel was loaded with a pooled OFM recirculation sample from four pig subjects, two OFM suction samples from pig No. 09 and 10, pig lymph and a 0.1 % AGP sample in PBS. The protein pattern from all OFM samples showed the same protein pattern within each other. Also compared to lymph and plasma the OFM samples are similar. OFM samples are fainter to the lymph, which was expected. The binding results from chapter 3.1.5 showed that OFM samples have lower binding values in comparison to lymph and plasma. The results between OFM recirculation and suction were slightly different, but they are not really comparable as the suction binding results come from a single sample only. One reason for the high similarity of the protein pattern to pig plasma could be that the OFM samples were not properly sampled interstitial fluid but diluted plasma. But what can be said clearly is that the low-protein content either diluted plasma or interstitial fluid, shows lower binding values although the patterns are similar. This means that the pattern of proteins does not give information about the binding behavior. Maybe a more precise technique of protein quantification, like for example 2D-SDS-PAGE or MALDI-TOF, could give information about the binding affinity of each biological fluid.

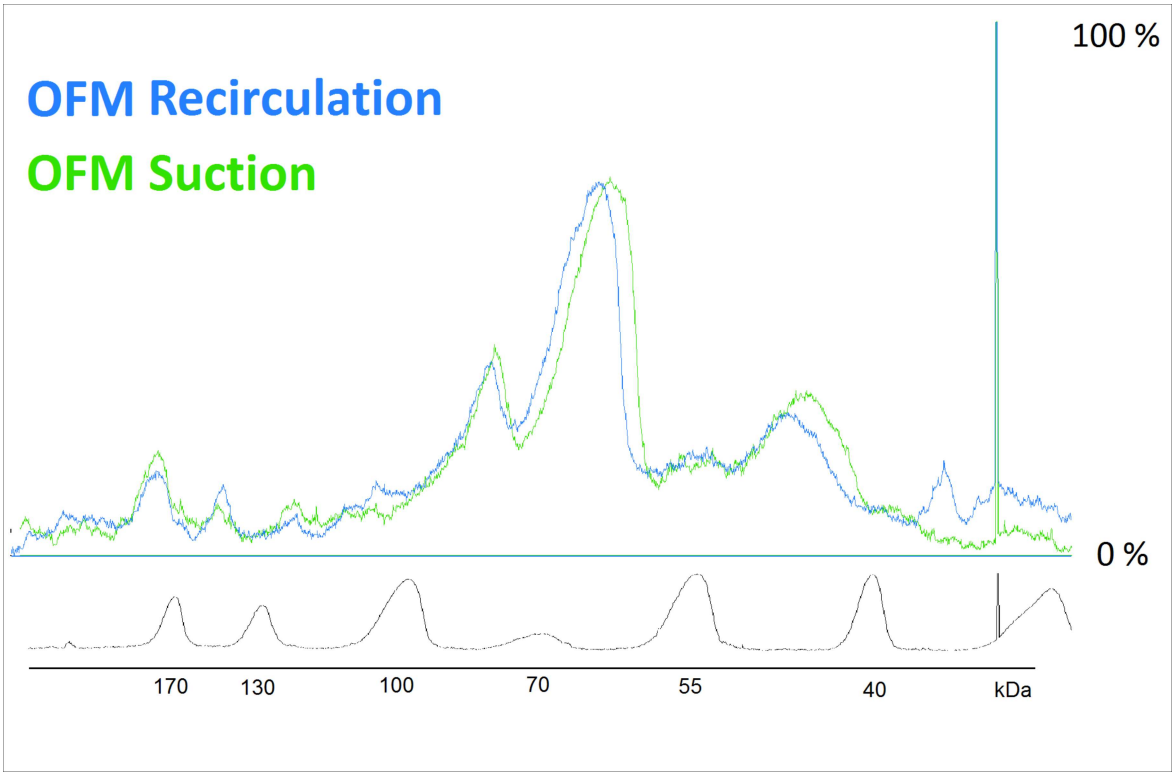


Figure 33: Comparison of the band intensities of OFM recirculation and suction samples from a SDS-PAGE gel. The result from the suction sample came from pig No. 09. A 100 % value was added manually by adding a black line in order to get a reproducible proportion.

The patterns between OFM samples and lymph were compared as well and showed again a high similarity (data not shown).

3.3.2 Albumin content

The results of the albumin concentration determinations of used biological fluids through the ALB plus Kit are listed below:

Table 13: Albumin concentrations for pooled pig plasma, lymph and OFM recirculation samples and pooled human plasma. Values are listed in g/l and % in the fluid. Only human plasma was bought. Gender distribution was tried to be as equal as possible.

Biological Fluid	Source	Concentration	Percentage
Pooled Human Plasma	100 individuals	40.93 g/l	4.1 %
Pooled Pig Plasma	7 individuals	26.95 g/l	2.7 %
Pooled Pig Lymph	7 individuals	17.25 g/l	1.7 %
Pooled Pig OFM recirculation	4 individuals	10.41 g/l	1.0 %
Pig OFM Suction	1 individual (S09)	11.23 g/l	1.1 %

The albumin content in lymph is nearly the half of plasma which is in line with literature. (Anderson, 2002) Also the human plasma value reflects what is found in literature as the albumin level in plasma should be around 35-50 g/l. (Nilsson, 2013) The OFM sample shows a lower albumin content than lymph. This is maybe because the lymph fluid is collected from lymphatic ducts. Probably albumin is more gathered in the lymphatic ducts. The pig plasma has in comparison to human plasma lower albumin content. The difference between recirculation and suction cannot be discussed as only one individual for suction sampling was available.

3.3.3 Alpha-1-Acid Glycoportein content

The results from the ELISA for the same biological fluids but with regard to the concentration of AGP are listed below:

Table 14: AGP concentrations for pooled pig plasma, lymph and OFM recirculation samples. Values are listed in g/l and % in the fluid. Only human plasma was bought. Gender distribution was tried to be as equal as possible.

Biological Fluid	Source	Concentration	Percentage
Pooled Pig Plasma	7 individuals	0.573 g/l	0.057 %
Pooled Pig Lymph	7 individuals	0.403 g/l	0.04 %
Pooled Pig OFM recirculation	4 individuals	0.208 g/l	0.021 %
Pig OFM Suction	1 individual	0.163 g/l	0.016 %

Human plasma could not be examined as the ELISA is selective only for porcine AGP. But human AGP levels are usually expected between 0.5-1.4 g/l. (Fournier, 2000) The AGP value for pig plasma is with 0.573 g/l at the lower range of the value assumed for humans. For albumin pig plasma also showed a slightly lower amount than found in human plasma. The same behavior as with albumin can be seen as plasma has the highest amount of AGP, lymph less and the OFM samples have the lowest amount. The difference between recirculation and suction cannot be discussed since only one individual sample for suction sampling was available.

4 PROSPECTS

To get a better insight into the differences of drug binding behavior between plasma and interstitial fluid the sampling method still must be optimized. Another very promising approach would be the SPME method as suitable fibers are also available for direct sampling within a tissue. This method was tried twice for the pig experiments. The syringe was placed slightly below the skin for one hour, while the pigs had infusions with lidocaine, amitriptyline, diclofenac and clobetasol. The fibers were then analyzed via HPLC-MS and the drugs could have been detected. This was the first step of verifying that this method could deliver promising results.

There are also approaches for predicting the drug-protein binding, but still this is very difficult to handle as the binding is influenced by too many factors. (Kratochwil, 2002)

A new and yet unused method for measuring drug-protein binding could be via an alkyl diol silica (ADS) pre-column for HPLC-MS. It has been shown once in literature that during measurements via ADS anthocyanins were bound by plasma proteins. (Murkovic, 2000) The ADS pre-column has a surface with hydroxy groups and if a sample is sent through the pre-column, proteins and drugs that are bound to proteins do not interact with the column and are washed off. But the column also has C18 modified pores where free drugs can enter and are retained. By changing the mobile phase, the unbound drugs can be eluted from the ADS prec-olumn onto the column of the HPLC-MS.

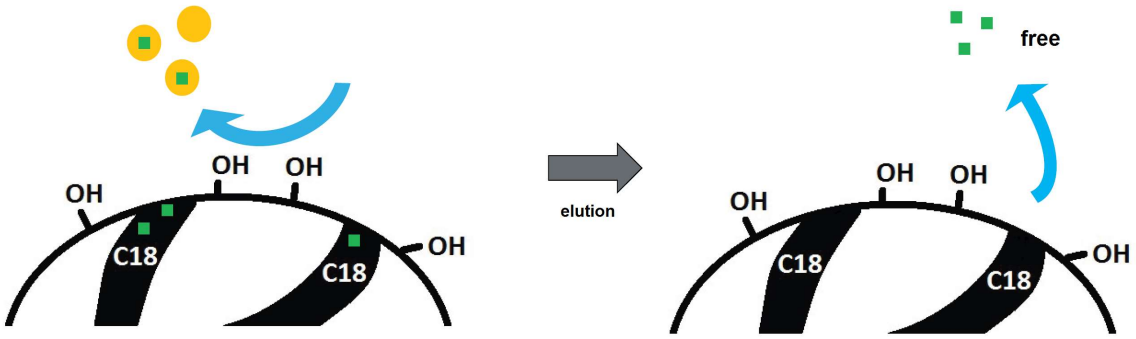


Figure 34: Principle of free and protein-bound drug separation by ADS.

During initial tests it could be seen that the six drugs are bound on the ADS precolumn, but difficulties occurred during the elution. Very high flow rates where needed which diluted our samples that the concentrations fell below the limit of detection. In principle the method could deliver good results but much effort must be invested.

5 REFERENCES

Abdel-rehim, M., Carlsson, G., Bielenstein, M., Arvidsson, T., & Blomberg, L. G. (2000). Evaluation of solid-phase microextraction for the study of protein binding in human plasma samples, Journal of Chromatographic Science, vol. 38, no. 10, 458–464.

Adkins, J. N., Varnum, S. M., Auberry, K. J., Moore, R. J., Angell, N. H., Smith, R. D., … Pounds, J. G. (2002). Toward a Human Blood Serum Proteome, Molecular & Cellular Proteomics, 1, 947-955.

Anderson, N. L. (2002). The Human Plasma Proteome: History, Character, and Diagnostic Prospects. Molecular & Cellular Proteomics, 1, 845–867.

Anguizola, J., Matsuda, R., Barnaby, O. S., Joseph, K. S., Wa, C., Koke, M., & Hage, D. S. (2014). Review : Glycation of human serum albumin, Clinica Chimica Acta Volume 425, 64–76.

Borga, O., & Borga, B. (1997). Serum Protein Binding of Nonsteroidal Antiinflammatory Drugs : A Comparative Study, B. J Pharmacokinet Pharmacodyn, 25: 63.

Chamouard, J. M., & Tlllement, J. P. (1985). Equilibrium Dialysis , Ultrafiltration , and Ultracentrifugation Compared for Determining the Plasma-Protein-Binding Characteristics of ValproicAcid, Clinical Chemistry Jan 1985, 31 (1) 60-64.

Clement, C. C., Aphkhazava, D., Nieves, E., Callaway, M., Olszewski, W., Rotzschke, O., & Santambrogio, L. (2012). Protein expression profiles of human lymph and plasma mapped by 2D-DIGE and 1D SDS – PAGE coupled with nanoLC – ESI – MS / MS bottom-up proteomics, J Proteomics; 78: 172–187.

De Moor, P., K. Heirwegh, J. F. Heremans, and M. Declerk-Raskin. (1962). Protein binding of corticoids studied by gel filtration. J. Clin. Invest. 41: 816-827.

Desborough, J. P. (2000). The stress response to trauma and surgery, Br J Anaesth; 85 (1): 109-117.

Dufaux, B., Assmann, G., & Hollmann, W. (1982). Plasma Lipoproteins and Physical Activity : A Review, Article in International Journal of Sports Medicine 3(3):123-36.

DrugBank database (2016), Available from: http://www.drugbank.ca/ Accessed 19 Apr 2016

Fasano, M., Curry, S., Terreno, E., Galliano, M., Fanali, G., Narciso, P., ... & Ascenzi, P. (2005). The extraordinary ligand binding properties of human serum albumin. IUBMB life, 57(12), 787-796.

Feingold KR, Grunfeld C. (2000) Introduction to Lipids and Lipoproteins. [Updated 2015 Jun 10]. In: De Groot LJ, Beck-Peccoz P, Chrousos G, et al., editors. Endotext [Internet]. South Dartmouth (MA): MDText.com, Inc., Available from: http://www.ncbi.nlm.nih.gov/books/NBK305896/ Accessed 19 May 2016

Fournier, T., Medjoubi-N, N., & Porquet, D. (2000). Alpha-1-acid glycoprotein. Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology, 1482(1), 157-171.

Hage, D. S., Anguizola, J., Barnaby, O., Jackson, A., Yoo, M. J., Papastavros, E., Tong, Z. (2011). Characterization of drug interactions with serum proteins by using high-performance affinity chromatography. Current Drug Metabolism, 12(4), 313–328.

Hein, K. L., Kragh-Hansen, U., Morth, J. P., Jeppesen, M. D., Otzen, D., Møller, J. V., & Nissen, P. (2010). Crystallographic analysis reveals a unique lidocaine binding site on human serum albumin. Journal of Structural Biology, 171(3), 353–360.

Kratochwil, N. A., Huber, W., Müller, F., Kansy, M., & Gerber, P. R. (2002). Predicting plasma protein binding of drugs: a new approach. Biochemical pharmacology, 64(9), 1355-1374..

Kim, M. R., & Kim, C. W. (2007). Human blood plasma preparation for two-dimensional gel electrophoresis. Journal of Chromatography B, 849(1), 203-210.

Kommmu, Radhika; Inv, I. J., & Sci, P. (2013). Detection of proteins in body fluids and plasma protein binding of various drugs. International journal of inventions in pharmaceutical sciences, 1(1), 53–58.

Kool, J., Reubsaet, L., Wesseldijk, F., Maravilha, R. T., Pinkse, M. W., D'Santos, C. S. & Heck, A. J. (2007). Suction blister fluid as potential body fluid for biomarker proteins. Proteomics, 7(20), 3638-3650.

Kosa, T., Maruyama, T., & Otagiri, M. (1997). Species differences of serum albumins: I. Drug binding sites. Pharmaceutical research, 14(11), 1607-1612.

Lee B, Bergan J, Rockson SG (eds) (2011); Lymphedema: a concise compendium of theory and practice, 1st edn., Springer, London, 66-68

Margarson, M. P., & Soni, N. (1998). Serum albumin: touchstone or totem?. Anaesthesia, 53(8), 789-803.

Murkovic, M., Adam, U., & Pfannhauser, W. (2000). Analysis of anthocyane glycosides in human serum. Fresenius' journal of analytical chemistry, 366(4), 379-381.

Nakai, D., Kumamoto, K., Sakikawa, C., Kosaka, T., & Tokui, T. (2004). Evaluation of the Protein Binding Ratio of Drugs by A Micro-Scale Ultracentrifugation Method, 93(4), 1–2.

Needham, A. 2004. Comparative and Environmental Physiology. Acidosis and Alkalosis.

New Zealand Medicines and Medical Devices Safety Authority (2016), Available from: http://www.medsafe.govt.nz/ Accessed 19 Apr 2016

Nilsson, L. B. (2013). The bioanalytical challenge of determining unbound concentration and protein binding for drugs. Bioanalysis, 5(24), 3033-3050.

Nilsson, L. B., & Schmidt, S. (2001). Simultaneous determination of total and free drug plasma concentrations combined with batch-wise pH-adjustment for the free concentration determinations. Journal of pharmaceutical and biomedical analysis, 24(5), 921-927.

Olsen, H., Andersen, A., Nordbø, A., Kongsgaard, U. E., & Børmer, O. P. (2004). Pharmaceutical-grade albumin: impaired drug-binding capacity in vitro. BMC Clinical Pharmacology, 4, 4.

Patton KT, Thibodeau GA; Anatomy & Physiology - 9th edition. 2015, St. Louis; Mosby/Elsevier; page 61.

Pieber, T., Birngruber, T., Bodenlenz, M., Höfferer, C., Mautner, S., Tiffner, K., & Sinner, F. (2013). Open flow microperfusion: an alternative method to microdialysis?. In Microdialysis in Drug Development, Springer New York, 283-302.

Routes, A.E., 2004: Drug absorption, distribution and elimination. Pharmacokinetics. http://www.columbia.edu/itc/gsas/g9600/2004/GrazianoReadings/Drugabs.pdf, Accessed 13 Apr 2016

Sadava, D., Heller, H. C., Orians, G. H., Purves, W. K., & Hillis, D. M. (2013). Life: The Science of Biology. 10th Edition; Gordonsville, W. H. Freeman; 816.

Shen, Q., Wang, L., Zhou, H., Jiang, H., Yu, L., & Zeng, S. (2013). Stereoselective binding of chiral drugs to plasma proteins. Nature Publishing Group, 34(8), 998–1006.

Shibukawa, A., Kuroda, Y., & Nakagawa, T. (1999). High-performance frontal analysis for drug–protein binding study. Journal of pharmaceutical and biomedical analysis, 18(6), 1047-1055.

Sloop, C. H., Dory, L., & Roheim, P. S. (1987). Interstitial fluid lipoproteins. Journal of lipid research, 28(3), 225-237.

Wang, H. P., Lian, J. F., & Wang, C. L. (2011). System Building for Safe Medication. In Risk Management Trends. InTech.

Wasan, K. M., Brocks, D. R., Lee, S. D., Sachs-Barrable, K., & Thornton, S. J. (2008). Impact of lipoproteins on the biological activity and disposition of hydrophobic drugs: implications for drug discovery. Nature Reviews Drug Discovery, 7(1), 84-99.

Wiig, H., & Swartz, M. A. (2012). Interstitial fluid and lymph formation and transport: physiological regulation and roles in inflammation and cancer. Physiological reviews, 92(3), 1005-1060.

Yuan, J., Yang, D. C., Birkmeier, J., & Stolzenbach, J. (1995). Determination of protein binding byin vitro charcoal adsorption. Journal of Pharmacokinetics and Pharmacodynamics, 23(1), 41-55.