Measuring pharmacokinetic properties of established and potential PET-tracers.

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List of abbreviations and symbols

Fp	Free fraction
k'	Retention factor
t_r	Retention time
ß$^+$	Positron
ACN	Acetonitrile
ADME	Administration, Distribution, Metabolism and Excretion
BBB	Blood brain barrier
CNS	Central Nervous System
CPM	Counts per minute
Da	Daltons, atmoic mass unit, 1 Da= $1.66 \cdot 10^{-27}$kg
DPM	Decays per minute
ED	Equilibrium dialysis
FoP	Freshness of plasma
GIT	Gastrointestinal tract
HDPE	High density polyethylene
HPLC	High performance liquid chromatography
IIV	Interindividual variance
kDa	Kilodaltons, Da$\cdot 10^3$

LSC	Liquid scintillation counting
mAU	milli absorbance units
MeOH	Methanol
mM	milli molar
MW	Molecular weight
MWCO	Molecular weight cut-off
ng	nanogram = g·10^{-9}
ODP	Octadecyl-poly(vinyl alcohol)
PET	Positron emission tomography
PMT	Photo multiplier tube
rcf	Relative centrifugal force
RCP	Radiochemical purity
RP-HPLC	Reversed phase high performance liquid chromatography
RT	Room temperature
SD	Standard deviation
SSC	Solid scintillation counting
$T_{\frac{1}{2}}$	Half-life
UC	Ultracentrifugation

Abstract

Measuring pharmacokinetic properties is notoriously difficult. The *in vitro/ex vivo* laboratory experiments seek to mimic the physiological responses of the body to the drug, but it rarely gives a good estimate of what happens *in vivo*.

Lipophilicity is traditionally measured by the shake-flask method, in which the distribution of the analyte between two immiscible phases (one organic, one aqueous) is investigated. It is a tedious process, and faster and less laborious methods are desirable. Calibration of a column/eluent-system on HPLC has been proposed, but the chromatographic process does not mimic the distribution process perfectly, and the results need to be related to each other.

In the research group, plasma protein binding is traditionally measured by equilibrium dialysis, a method that relies on passive diffusion. This may take a long time (+24 hours), and ultracentrifugation is proposed as a new and much faster method (app. 1 hour).

In this project, new methods for measurement of lipophilicity and plasma protein binding are developed and evaluated. The group of compounds measured by the methods are all established or potential PET-tracers.

LogD-values obtained by shake-flask and HPLC was not in accordance, especially not for logD-values in the low range. For plasma protein binding, accordance between the methods was found in the range 0-10 % free fraction, but for higher free fractions accordance was only found for some compounds.

Keywords: Lipophilicity, plasma protein binding, shake-flask, HPLC, equilibrium dialysis, ultracentrifugation.

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1 Introduction

When a drug enters the body, a lot of processes take place. If the drug is administered orally, it firstly has to withstand enzymes in the saliva trying to break it down. Upon entering the GIT (Gastrointestinal tract), the drug will arrive in the stomach. Harsh conditions are found there, and the drug needs to withstand pH=1 before it enters the intestines. Here, it is supposed to diffuse through the intestinal wall and into the blood stream. The lipophilicity of the drug plays a great role here – a drug with too high or low lipophilicity will pass through the GIT without interacting with the body. If the drug is able to cross the intestinal wall, it may still be directly excreted in the kidneys if the lipophilicity is too low, or stored in fatty tissue if the lipophilicity is too high.

Carried by the blood stream, the drug is now travelling towards its target where it will make its effect – but this can only happen if the drug is not bound to proteins or other macro-molecules in the blood. A large component of the blood is the group of proteins called albumin, and should the drug have higher affinity for albumin than for its target, it may not be effective.

Travelling through the liver, the drug has to withstand metabolism – if metabolized, it may be rendered useless because of the structural change.

Pharmacokinetics is a concept regarding what happens to a drug when administered to a person. The concept is closely related to pharmacodynamics, which regards the drug's interaction with its target. Pharmacokinetics is often described as ADME (Administration, distribution, metabolism and excretion), but also includes plasma protein binding, lipophilicity, biological half-life etc. Lipophilicity may not fall directly under the definition of pharmacokinetics, but is an important and usefeul property in drug design.

1.1 The Drug Development Process

Developing drugs *de novo*[1] is a complicated process. New drug candidates undergo a lot of testing, usually first *in silico*[2] and later on *in vitro* and *in vivo*. At any step in the process, the drug candidate may show unwanted properties. These may be remedied by structural changes of the drug, or the drug candidate may be dropped. For instance, a drug that is supposed to affect the CNS (Central nervous system) but is not capable of crossing the BBB (Blood brain barrier), will be dropped quite quickly.

The purpose of *in silico* and *in vitro* testing is to assess the properties of the drug candidates before exposing humans to them, thereby minimizing the risk of unwanted side-effects.

Often development of new drugs is based on already existing drugs, that the developers want to improve, and of which the mechanism wants to be mimiced. An example is the very basic "Lipinski's Rule of Five", that states some guidelines a drug should fulfill to be orally active. Orally active drugs are easy to administer for the patients, and will take advantage in a market, where competing drugs are administered in less convenient ways like intravenously or as suppositories. Examining drugs already available in the market, Christopher A. Lipinski found in 1997, that for a drug to be orally active, four simple rules needed to be fulfilled:

1. Maximum 5 hydrogen donors.
2. Maximum 10 hydrogen bond acceptors.
3. A molecular weight of maximum 500 Da. (Daltons).
4. A logP not greater than 5.

[1] From the beginning.

[2] Testing by computer calculations.

Drugs, that do not fulfill these criteria may be excluded from further investigations in the drug development plan.

1.2 Pharmacokinetic analyses

Measuring pharmacokinetic properties *in silico* or *in vitro* in the lab is an attempt to mimic the physiological response of the body to the drug. Metabolism can be investigated in a vial using microsomes extracted from a liver, distribution in the body can be estimated with the help of lipophilicity and the plasma protein binding can be examined by different methods. Common to them all is, that they are not absolute. Even though the metabolism of a drug happens in one way with extracted liver microsomes, it may happen differently *in vivo*.

Common to them all is, that detection techniques are required to somehow measure the analyte in question.

1.2.1 Using radio-labeled compounds in pharmacokinetic analyses

Performing pharmacokinetic analyses on radiolabeled compounds provides possibilities that are not present with unlabeled compounds. Radiodetectors are often sensitive, and, provided only one compound is labeled, highly selective – a single analyte can be distinguished from a very complex biological matrix very easily. Using a $ß^+$-emitter can eliminate the need for extraction procedures, as the sample matrix has no effect on the measured activity. Analysis by chromatography will however require some sort of extraction procedure.

Using the radiolabeled compounds however poses the question of radiation protection. Consideration should go into the issues of dosage to personnel, monitoring thereof and similar matters.

1.3 PET-scanning

PET is an acronym for Positron Emission Tomography, and it is a functional scan making use of a radio-labeled compound, the PET-tracer[3] , intravenously injected into the animal/person to be scanned. The information obtained from the scan depends on the nature of the PET-tracer, and choosing the right PET-tracer will facilitate applications in oncology, neuroimaging etc. As PET-scans make use of positron emission, all PET-tracer must be labeled with an isotope that exhibits this decay.

The PET-scanner has an amount of scintillation detectors positioned in a circle, all facing the center of a scanning tunnel, see figure 1 on page 12. Because of this setup, and the properties of the ß$^+$-decay, the exact position[4] of each decay can be calculated, thereby facilitating imaging.

1.3.1 The ß$^+$-decay

The positron is the antiparticle of the electron. As electrons are abundant, the positron will quickly meet an electron. Upon this meeting, the two particles will annihilate, giving rise to two 511 keV-photons with direction anti-parallel to each other. The photons are always antiparallel to each other, and this explains the PET-scanners ability to calculate the origin of the decay: Two scintillation detectors s must each detect a photon within a short time-frame, and a line is drawn between them. Based on all the lines obtained in the scan, an image is reconstructed.

Should a pair of oppositely placed PMT's (Photo multiplier tube) detect a single photon, it is disregarded as not originating from a ß$^+$-decay – this is the principle of coincidence measurement. See figure 1 on page 12 for a

[3] Also known as radioligand, radiotracer, PET-ligand, tracer etc.

[4] ± 2.7 mm [1]

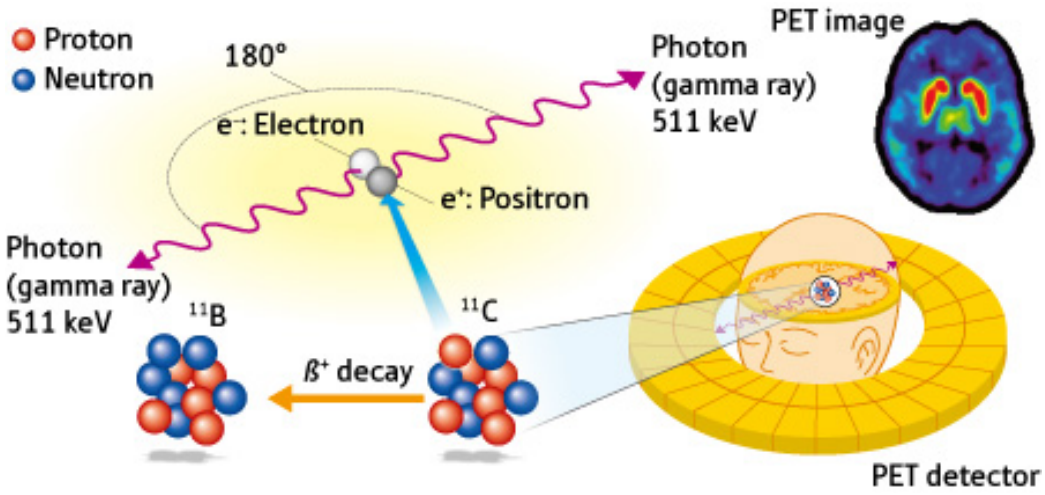


Figure 1: A schematic of the ß$^+$-decay and the PET-scan. Picture courtesy of RIKEN Research, Japan.

schematic of the decay and PET-scanner.

1.3.2 Choosing a PET-isotope

Numerous factors need to be considered when choosing an isotope for labeling of a PET-tracer, these include mode of decay, decay energy, half-life, biocompatibility, chemical behaviour, radiation protection and so on.

There is a balance (or dilemma) between the half-life of an isotope and the scan-time. Choosing an isotope that decays slowly will give few counts and increase scan-time, and thereby increase inconvenience for the test-person or patient, and also allow fewer people to be scanned pr. time-unit. Slow decay may mean having to inject a larger amount of activity in order to have enough counts to create a good image.

Choosing an isotope that decays quickly decreases scan-time and allows more people to be scanned. It may give the possibility of injection of small doses, as all of it will decay within a short period. A short-lived isotope

requires production of the PET-tracer to take place nearby the scanner, as transportation over long distances will take too much time.

To achieve short scan-times, small injection doses and minimization of inconvenience to the test-person/patient, a short-lived isotope should be chosen.

In analytical chemistry, it is desirable to have as much time as needed to perform a certain analysis, as this will increase certainty of the result. Using radiodetection on samples from a PET-scan with a short-lived PET-tracer, this cannot be achieved. Increasing scan-time or dosage to the test-person/patient is not desirable, so the analytical methods making use of radiodetection should be fast. To illustrate these considerations, a project triangle has been designed, see figure 2 on page 14. The project triangle stipulates three things of which, only two can be achieved:

Low activity and short-lived isotope It is very desirable to use low activity, to decrease radio-exposure for the test-person/patient and the personell. It is also desirable to use a short-lived isotope ($T_{\frac{1}{2}}$ in the range of minutes or hours), as the fast decay will decrease scan-time. With these combined, an elaborate metabolism study cannot be performed using radiodetection, as the low activity will be difficult to detect.

Low activity and time consuming analysis To have these two will exclude the possibility of using a short-lived isotope. Thus using a longer half-life isotope would lead to increased scan time.

Short-lived isotope and time consuming analysis In order to perform a scan with a short-lived isotope and elaborate metabolism studies, a large dose of PET-tracer will have to be injected, leading to high

radio-exposure to the test-person/patient.

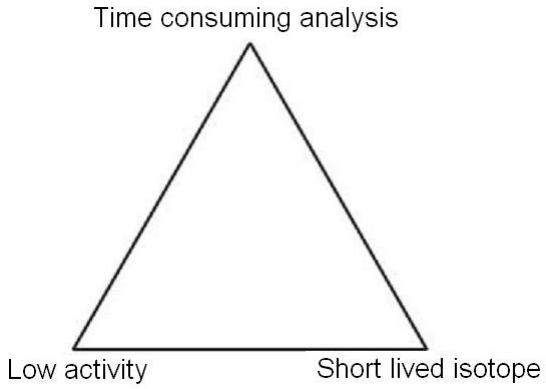


Figure 2: The project triangle describing the considerations in choosing a radio-/PET-isotope. A project triangle stipulates three things, that are impossible to have all at once – maximum two can be had at once.

1.4 Lipophilicity

1.4.1 Definition

Lipophilicity literally means "love of fat", and is use synonymously with *hydrophobicity*, which means "fear of water". The lipophilicity of a compound describes the property of how "fat-like" or "water-like" it is. A lipophilic molecule will preferentially solubilize in oily/fatty-phases, and hydrophilic molecules will solubilize in aqueous phases. Given the possibility, a lipohpilic compound will distribute towards the more lipophilic phase in a mixture of two phases and vice versa.

The word "lipophilicity" is actually misleading, as there is greater affinity between a hydrocarbon and a water molecule than between two hydrocar-

bons, but the attractive forces between water molecules are so much stronger, that the hydrocarbons are squeezed out.[2]

The lipophilicity is expressed as a distribution coefficient, P, between two phases, and for convenience, the common base 10 logarithm is used, logP. For compounds that are ionizable, the distribution depends on pH of the aqueous phase, and the distribution coefficient, D, is used instead, to point out the non-standard circumstances.

For unionizable compounds, one equilibrium exists, namely the distribution between the two phases:

$$A_{Water} \rightleftharpoons A_{Octanol} \tag{1}$$

$$P = \frac{[A]_{Octanol}}{[A]_{Water}} \tag{2}$$

$$logP = log\left(\frac{[A]_{Octanol}}{[A]_{Water}}\right) \tag{3}$$

where A designates an unionizable analyte and P is the distribution coefficient. For analytes, that are ionizable, two equlibria are present:

$$BH^{+}_{Buffer} \rightleftharpoons B_{Buffer} + H^{+} \tag{4}$$

$$B_{Buffer} \rightleftharpoons B_{Octanol} \tag{5}$$

where B designates a basic analyte. As ionized compounds are energetically unfavourable in organic phases, only the neutral analyte, for all practical purposes, is in equilibrium with the organic phase:

$$BH^+_{Buffer} \rightleftharpoons H^+ + B_{Buffer} \rightleftharpoons B_{Octanol} \tag{6}$$

$$D = \frac{[B]_{octanol}}{[B]_{Buffer} + [BH^+]_{Buffer}} \tag{7}$$

$$logD = log\left(\frac{[B]_{octanol}}{[B]_{Buffer} + [BH^+]_{Buffer}}\right) \tag{8}$$

where D is the distribution coefficient.

The same example works for an acidic analyte. This shows, that the logD greatly depends on the degree of ionization of the analyte. As a consequence of this, when measuring logD of ionizable analytes, it is of great importance to control the pH using a buffer. Acetate-buffer should be used for pH app. 3, borate-buffer for pH app. 10 and phosphate-buffer for intermediate pHs. [2]

1.4.2 Methods

Measuring lipophilicity experimentally is notoriously difficult, and a simple literature search may give several, and very different, results for the logP of one particular compound. Given that logP relies on the experimental conditions, a value from the literature that matches the experimental conditions in question may not be available.

The partitioning method is the original and correct way to measure lipophilicity. Other methods are available, each having their advantages and disadvantages.

Partitioning Traditionally, the lipophilicity of a compound is expressed as a distribution coefficient between to immiscible liquids, where one should be water or water-like. The other liquid is traditionally 1-octanol, but could be CS_2, ether or other liquids. [2] The method is empirical, and relies on laboratory experiments.

The phases are pre-saturated with each other, as even small transfers of one phase the other may bias the analysis significantly. The analyte is solvated in one of the saturated phases, where the solubility is expected to be the lesser of the two phases, and the other saturated phase is added. Thorough mixing is performed, allowing the equilibrium (Eq. 1 or eq. 6 on page 15) to occur.

Proper separation between the phases is assured, and microemulsions may be removed by centrifugation. The phases are separated, and their respective concentration of analyte is quantified. If both phases are quantified, two calibration curves are needed in order to exclude influences from the matrix on the detector response. Some shortcuts to the result can be made:

- The analyte concentration is quantified in the first phase before and after addition of the second phase. The concentration in the second phase can the be calculated by the extracted amount of analyte from the first phase, and only one calibration curve is needed.

- Using radiodetection may render calibration unnecessary. The amount of analyte can be quantified in count-rates per. volume or mass and used in the calculation instead of concentration. This applies as long as there are no matrix effects in the radioactivity quantification assay. It is necessary, that the detector response is linear in the measured range.

Partitioning is tedious and laborious. It, however, provides the most correct values.

When using radiolabeled compounds in the partitioning method, small radiochemical impurities may seriously bias the analysis. A "double-shake" can remove the impurities by a liquid-liquid extraction.[3–6]

Calculation by fragments Another approach to estimating logD is using computer calculations. A lot of experimental data from different compounds is collected, and by comparing their structures with the data, estimates of the pK_a's of the molecule can be made by adding contributions of each fragment of the molecule. For instance, para-substituted phenol may provide information on the identically substitued toluene or aniline.

The pK_a is then used to calculate the logD at a given pH. This *in silico* approach is widely used, but it may fail, as all calculations are estimates, and only experiments can provide the *true* value.

Chromatographic methods High performance liquid chromatography (HPLC) is used to separate and detect different compounds from each other in a sample matrix. A continuous flow of liquid, the eluent, flows, under high pressure, through a compartment with a certain material in it, the column. The sample is introduced into the stream, and when flowing throguh the column, its different constituents will be retained more or less by the column material, thereby being separated from each other. At the end of the line, there is a detector, and a response vs. time-plot can be created, the chromatogram.

The separation of the constituents depends on several factors, but primarily the combination of eluent and column. For separation of organic compounds in an aqueous matrix, a column with octadecyl-chains bound to silica particles can be used with an eluent composed of water/buffer and an organic solvent. The compounds in the sample have different affinities for the column material, and will wash off the column in a certain order.

If the eluent constitution is the same throughout the chromatogram, it is called isocratic. If the eluent changes in the course of the chromatogram,

(ie. from a low to a high organic content) it is called gradient. When using a primarily aqueous eluent and a primarily lipohpilic column, it is called reversed phase HPLC, RP-HPLC.[5]

Attempts have been made to calibrate an HPLC-system to measure lipophilicity.[7–15] Different eluent/column systems have been used and both isocratic and gradient. The system will be specific to a certain compound class, and it should be changed or modified before measuring compounds of another class.

The HPLC-methods are quite repeatable and reproducible, but an eluent/column-system that mimics the octanol/water partitioning-system perfectly does not yet exist. The results are estimates, and should be evaluated thoroughly.

1.5 Plasma protein binding

A drug's performance may be related to its non-specific binding to proteins and other macromolecules in the blood stream. If the drug is "occupied" being bound to a protein, it will not be available to interact with its target, hence the concept of free fraction. The binding is reversible, and the free drug is in equilibrium with the drug-protein complex:

$$\text{Drug} + \text{Protein} \rightleftharpoons \text{Drug}\cdot\text{Protein} \tag{9}$$

If the drug binds to either its target or unspecifically to other components, the drug concentration decreases, and the equilibrium is shifted to the left.

The terminology of plasma protein binding can be ambiguous. It has to be made very evident, if the value in question designates the **bound** or

[5] *Reversed* because the first chromatographic systems had lipophilic eluents and hydrophilic columns.

the **free** fraction. *Fp* designates the free fraction, and is usually given in fraction. Throughout this text, only the **free** fraction will be used, and it will be expressed in percent.

1.5.1 Equilibrium dialysis method

The gold standard of measuring plasma protein binding is the equilibrium dialysis method, in which the free drug is supposed to diffuse through a membrane only permeable to compounds of a certain maximum molecular weight. A sketch can be seen in figure 3 on page 20.

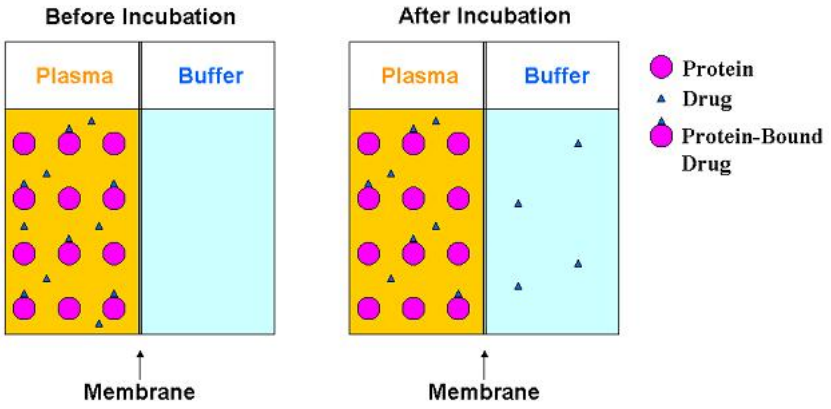


Figure 3: Schematic of equilibrium dialysis. Picture courtesy of Drumetix Laboratories. The free drug is able to diffuse through the membrane to the buffer, but the drug bound to proteins will be retained in the plasma.

On one side of the membrane is the plasma with the drug[6], and on the other side is a buffer, preferentially a buffer of the same pH as plasma, containing the same salts as plasma in the same concentrations, to mimic physiological

[6]The PET-tracer may be added *in vivo* or *in vitro*, but this may give rise to different results

conditions. After incubation, an equilibrium (Eq. 11 on page 21) between the two phases has arised with free drug and drug-protein complex in the plasma, but only free drug in the buffer. The concentration of free drug is equal in the two phases, and the free fraction can be calculated as the ratio of free drug in buffer and total drug in plasma.

$$\text{Drug}\cdot\text{Protein}_{\text{Plasma}} + \text{Drug}_{\text{Plasma}} \rightleftharpoons \text{Drug}_{\text{Buffer}} \tag{10}$$

$$Fp = \left(\frac{\text{Drug}_{\text{Buffer}}}{\text{Drug}\cdot\text{Protein}_{\text{Plasma}} + \text{Drug}_{\text{Plasma}}} \right) \tag{11}$$

The diffusion of the free drug from the plasma-side to the buffer side is caused by a concentration gradient, and will end, when the concentration gradient no longer is present, ie. when the concentration of free drug is equal in the two phases.) Diffusion is a complex phenomenon, and it is not the objective of this thesis to deduce the exact diffusion equation for the method in question. However, the rate of diffusion will depend on factors such as temperature, agitation, viscosity of the liquids, density of the liquids and the compound in question.

The primary object of considering diffusion in relation to equilibrium dialysis of radiolabeled compounds is to make sure the euqilibrium has been reached within a reasonable time period compared to the half-life of the isotope. As passive diffusion relies on thermal energy, it is evident, that the rate of diffusion can be increased by increasing temperature. Increased temperature may move the equilibrium (Equation 9 on page 19) in either direction, and the temperature must be kept at 37 °C to mimic physiological conditions.

1.5.2 Ultracentrifugation

Another approach for measurement of plasma protein binding is ultracentrifugation (also known as ultrafiltration). The method does not rely on passive diffusion as equilibrium dialysis does, and is therefore a lot faster.

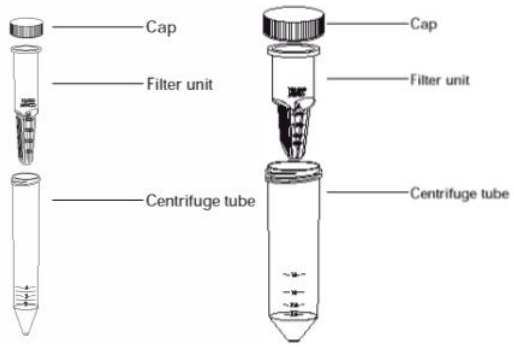


Figure 4: Schematic of an ultracentrifugation unit

The ultracentrifugation unit generally consists of four parts: A collection vial, a membrane with a certain MWCO (Molecular weight cut-off), a membrane vial and a cap. The membrane is fixated in the membrane vial, either horizontally or at an angle. The membrane vial is placed on top of or in the collection vial and fitted with a cap. An example of an ultracentrifugation unit can be seen in figure 4 on page 22.

The sample, of which the free fraction is to be measured, is placed in the membrane vial. This sample can be any biological fluid, most often plasma. The whole unit is centrifuged. During centrifugation, the centrifugal force applied on the sample will cause some of it to penetrate the membrane. The free drug will be able to cross the membrane and end up in the filtrate, but the bound drug will not. The filtrate will have the same concentration of the free drug as the sample in the membrane vial. After centrifugation, the total concentration of drug in both the original sample and in the filtrate are

determined, and the free fraction in percent can be calculated by equation 12 on page 23.

$$Fp = \frac{[Analyte]_{Filtrate}}{[Analyte]_{Reference}} \cdot 100 \tag{12}$$

2 Aims

Lipophilicity

- Develop and validate an HPLC-method for the measurement of logD of established and potential non-labeled PET-tracers.
- Measure a number of PET-tracers and compare the methods' precision, reproducibility and results to the shake-flask method.

Plasma protein binding

- Develop and validate an ultracentrifugation assay for the measurement of the free fraction, Fp.
- Measure a number of PET-tracers and compare the methods' precision, reproducibility and results to the equilibrium dialysis.

3 PET-tracers

All the structures of the PET-tracers used in this project are shown in figure 6 on page 26. In general, the structures are heterocyclic compounds with few acidic hydrogens, many nitrogens and a few halogens.. A lot of methoxy-groups are present, this prevents metabolism taking place on mildly acidic hydrogens. Many of the rings are highly substituted. There is enzymatic esterase-activity in the blood, and both SB207145 (Fig. 6p) and flumazenil (Fig. 6g) have ester-functionality. Flumazenil has proved to be stable in plasma in spite of being a target for the esterase enzymes, but this is not the case for SB207145 is not. To prevent metabolism of the compound, dichlorvos is added to the plasma prior to SB207145.

Dichlorvos Dichlorvos is an esterase inhibitor used in dilution: Dichlorvos B = 71 ng (nanogrammes, g·10^{-9}) dichlorvos/ml in 0.35 % ACN (acetonitrile) in H_2O.

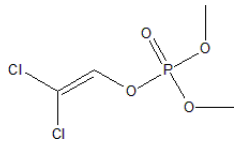
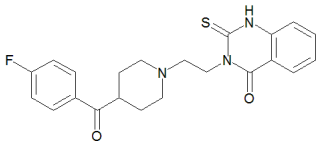
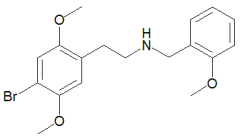


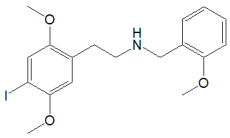
Figure 5: The structure of dichlorvos, the esterase inhibitor used to protect SB207145 from esterases in plasma



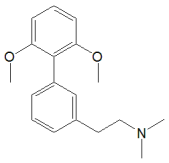
(a)
Altanserin



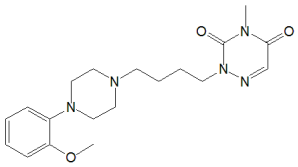
(b)
CIMBI36



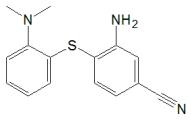
(c)
CIMBI5



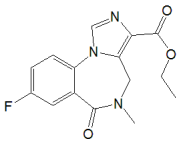
(d)
CIMBI806



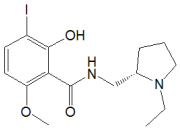
(e)
Cumi101



(f)
DASB



(g)
Flumazenil



(h)
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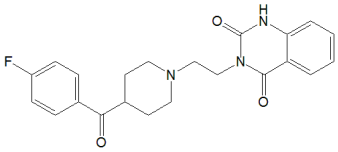
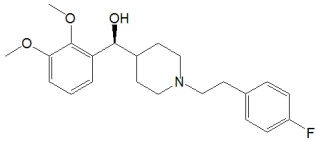
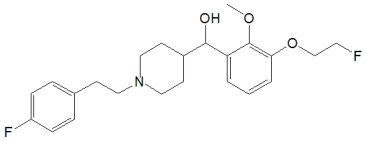


(i) Ketanserin



(j)
MDL100907



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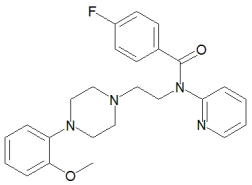

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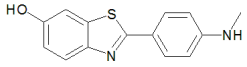

(m)
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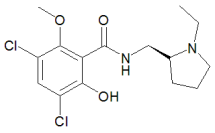
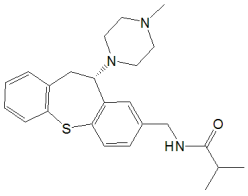
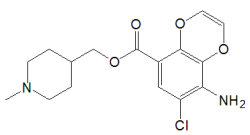


(n)
Raclopride



(o) S35



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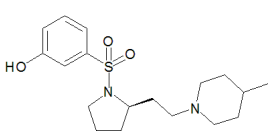
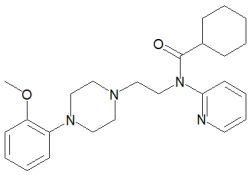


(q)
SB269970



(r)
WAY100635

Figure 6: Structures of the PET-tracers.

4 Common materials and methods

This project consists of two separate parts, one investigating lipohpilicity measurements, and one investigating plasma protein binding measurements. These two parts will be treated individually (Materials, methods, results and discussion), but some of the materials and methods are common to both parts, and will be described here.

4.1 Centrifuges

Three different centrifuges were used.

1. Eppendorf Centrifuge 5804 R. Fixed angle.
2. Eppendorf miniSpin plus. Fixed angle.
3. Heraeus Megafuge 1.0 R. Swinging buckets.

4.2 Radioactivity quantification assays

4.2.1 Materials and methods

Different radio-isotopes were used in the project, and their half-life and type of decay can be seen in table 1. Samples not run on HPLC were measured by scintillation counting. [^{3}H]-samples were counted on the Tri-Carb liquid scintillation analyzer, the rest were counted on the Cobra solid scintillation counter.

For LSC (Liquid scintillation counting), opaque 4 ml Pico Pro vials made from HDPE (High density polyethylene) from PerkinElmer was used, and for SSC (Solid scintillation counting), transparent 5 ml polypropylene vials from Sarstedt were used.

Scintillation liquid The scintillation liquid was Ultima Gold™ from PerkinElmer.

Scintillation counters The liquid scintillation counter was a Tri-Carb 2900TR liquid scintillation analyzer with QuantaSmart software (v. 1.31), both from Packard. The solid scintillation counter was a Cobra™ II D5003 Auto-Gamma® from Packard with integrated software.

Table 1: The radioisotopes used in the project and their main properties and parameters of analysis.

	Properties	
Isotope	Half-life	Decay
^{3}H	12.32 yr	ß(0-18.6 keV)
^{11}C	20.33 min	ß$^{+}$ (γ (511 keV))
^{18}F	109.77 min	ß$^{+}$ (γ (511 keV))
^{123}I	13.22 hr	γ (159 keV)

	Analysis parameters			
Isotope	Window	Decay correction	Scintillation liquid	Counting time
^{3}H	0-18.6 keV	Start of assay	Ultima Gold (2 ml)	5 min
^{11}C	400-1200 keV	Ref. time	None	2 min
^{18}F	400-1200 keV	Ref. time	None	2 min
^{123}I	143-175 keV	Ref. time	None	2 min

4.2.2 Discussion

CPM vs. DPM For both LSC and SSC, the CPM-value (Counts per minute) has been used, as opposed to the DPM-value (Decays per minute) thus not taking counting efficiency into account. This should not be a problem with the high-energy isotopes counted by SSC, but it will be discussed further for ^{3}H in section 4.2.2.

Solid scintillation counting For the SSC, the most apparent issue is counting statistics with high activity samples being underestimated because of the dead-time[7] of the instrument. For counts less than 200,000 CPM, this should not be a problem.

The ß$^{+}$-emitters give rise to a very well defined γ-decay of 511 keV — so why not measure in a window of 511±5 keV instead of the chosen 400-1,200 keV? There are two reasons for this:

Geometry The ß$^{+}$ particle will within a very short travelling distance meet its anti-particle, the electron, and annihilate with it. This gives rise to two fotons, each with the energy of 511 keV, going in opposite directions (180 deg). The detector of the instrument may detect only one or both fotons, giving rise to a count of 511 keV or 1,022 keV respectively, see figure 7 on page 30. The peak at 1,022 keV would have been missed if a more narrow energy window had been used.

Custom practice The second reason is custom practice. The protocol already defined in the instrument was used, and had been used for a long time, so in case data from this project is to be compared to older data, it is prefereable, that samples are measured on the same protocol.

[7]The dead-time is the period of time there has to be between two decays for the instrument to be able to distinguish them as two separate decays.

The protocol is adequate for the measurements of this project.

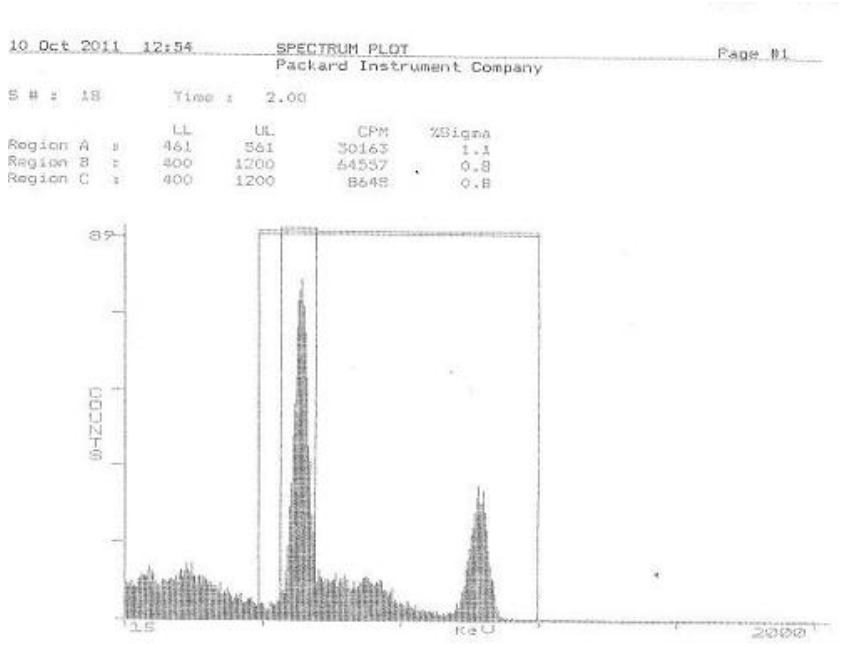


Figure 7: A spectrum of a [^{11}C]-SB207145 blood sample with the photon energy on the x-axis and the number of counts on the y-axis, showing the 511 keV- and 1,022 keV-peaks clearly. The energy windows are shown with vertical lines connected horizontally.

Liquid scintillation counting For LSC, more consideration goes into chosing the right equipment, namely the counting vials and scintillation liquid. Vials are generally made from either glass or some kind of plastic. Glass vials are not suitable for low-level LSC, as glass contains naturally occuring ^{40}K (Decays by ß (89.28 %) and ß$^{+}$ (10.72 %)[16]) which will bias the analysis. Therefore, plastic vials are preferred.

The 4 ml plastic vials used for LSC were opaque, which quenches the sample compared to transparent plastic vials. However, this error is systematic, and therefore not of importance in assays where the ratio is calculated,

provided the counts are high relative to the background.

A real source of error occurs when mixing plasma with the scintillation liquid. For optimal measurements, the sample has to be transparent with no precipitate. Assessing whether a liquid sample is transparent in an opaque vial is not easy.

When comparing counts from octanol-phases and buffer-phases there may be differences in counting efficiency, leading to incomparable CPM-values. Instead, CPM-values should be corrected by a factor to produce comparable DPM-values. The factor can be determined experimentally by measuring a known standard in the given matrix, but this has not been performed.

It was assumed, that the counting efficiencies of the octanol- and buffer-samples are equal, thus allowing comparison of CPM-values.

Choice of scintillation liquid A few different scintillation liquids were available in the laboratory. Using PerkinElmer's "Cocktail & Vial Guides"[8], Ultima Gold is recommended for biological samples with direct addition.

It was later learned from a PerkinElmer engineer, TJ McCully, that Ultima Gold can accommodate $\leq$1.0 ml plasma pr. 10.0 ml of scintillation liquid. The plasma samples produced were most often made from 200 µl of plasma with addition of 2.0 ml of Ultima Gold, keeping the ratio within the recommended range and thus avoiding colour quenching.

As PET-tracers are ß$^+$-emitters, there is no need for scintillation liquid, and the analysis is not biased by matrix-differences.

[8] http://www.perkinelmer.com/dk/tools/CocktailsAndGuides.aspx

4.3 Statistics

All plots and statistical calculations were performed using GraphPad Prism v. 5.04 for Windows, GraphPad Software Inc. For comparison of methods, a paired two-tailed t-test with 95 % confidence interval was used. For correlation tests, a Pearson's two-tailed correlation test with 95 % confidence interval was used. If the statistical test conditions were different, it will be noted in the text.

The Pearson's correlation test is parametric. The result is an r-value, which designates to which degree a correlation is present, and a p-value, which states the probability of a concluding a correlation is present, when it in fact is not. The p-value should, if a correlation is present, be $p < 0.05$. The r-value is interpreted as follows:

$\boldsymbol{r = 1.0}$: Perfect correlation

$\boldsymbol{r = 0 \text{ to } 1}$: The two variables tend to increase or decrease together.

$\boldsymbol{r = 0}$ The two variables do not vary together at all.

$\boldsymbol{r = -1 \text{ to } 0}$ One variable increases as the other decreases.

$\boldsymbol{r = -1.0}$ Perfect negative or inverse correlation.

4.3.1 Discussion

Student's t-test is a statistical, parametric test, that compares measurements to each other or to a specified value. A paired t-test can compare results measured on the same samples with different methods. In both cases, a normal distribution of the data is assumed. In the case of few measurements (n$\leq$5), a non-parametric test, like Wilcoxon signed-rank test, should be used. Throughout this project the t-test is used to make conclusion for two reasons:

1. The parametric and non-parametric tests gave the same results for most of the comparisons.

2. The data is assumed to follow a normal distribution if n increases.

The 95 % confidence interval is generally accepted in analytical chemistry.

5 Lipophilicity measurements

An HPLC-method for determining LogD of the potential PET-tracers was developed to replace the tedious and chemical consuming 1-octanol/water shake-flask method. In order to validate the new method, it was compared to the shake-flask method and predicted values.

5.1 Materials and methods

Chemicals 1-Octanol and methanol were HPLC-grade from Sigma-Aldrich, Missouri, USA. The water was technical grade with a conductivity of less than 12 $S \cdot m^{-1}$ provided by the hospital's pharmacy.

Calibration standards The compounds used for calibration and their literature lipophilicities can be seen in table 2 on page 35, their structures can be seen in figure 8 on page 36.

5.1.1 HPLC system and methods

HPLC-equipment The HPLC system was a semi-preparative Dionex Ultimate 3000 equipped with two 3-channel pumps, degasser, autosampler, column-oven, UV/VIS-detector, radio-detector and fraction collector. The column was an Agilent Zorbax SB-C8 column, 5 µm, 250 x 4.6 mm.

HPLC samples The standards and unknowns used were, as far as possible, dissolved in 50:50 (v/v) H_2O:MeOH (Methanol). Did this prove insufficient, steps were taken to dissolve the compound in the following order, after which all samples were completely dissolved.

1. Heat the sample to about 60 °C.

Table 2: Lipophilicites of reference compounds. As all compounds are not ionizable, their logP equals their logD. Values were found in the OECD guideline.[17]

Compound	LogP
Phenol	1.5
Acetophenone	1.7
p-Cresol	1.9
Benzene	2.1
Toluene	2.7
Chlorobenzene	2.8
Benzophenone	3.2
Naphtalene	3.6
Diphenyl	4.0
Phenanthrene	4.5

2. Addition of more MeOH.

3. Addition of a few drops of CH_2Cl_2. (Dichloromethane)

Calibration The calibration curve was created making a logD vs. t_r-plot and using linear regression by the method of least squares.

HPLC-method 1 The HPLC-method was based on the previously described method by the OECD[17] and adjusted to fit the available equipment. See the appendix for an exhaustive description of the development process. The eluent was 50:50 (v/v) phosphate buffer (25 mM (milli molar), pH 7.4) and MeOH. The flow-rate was 1.5 ml/min, column-oven temperature was

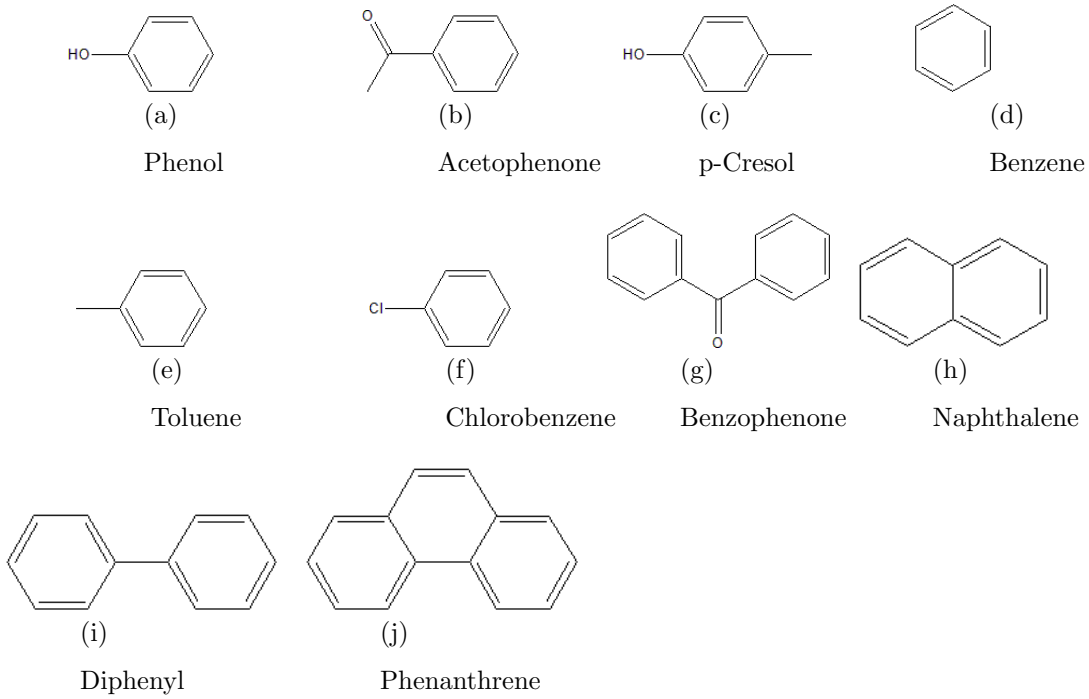


Figure 8: Structures of the calibration standards

37 ± 0.5 °C and UV-detection was at 254 nm. Injection volumes varied from 50-100 µl.

HPLC-method 2 The eluent was 50:50 (v/v) phosphate buffer (25 mM, pH 7.4) and MeOH. The flow-rate was 2.0 ml/min, column-oven temperature was 50 ± 0.5 °C and UV-detection was at 254 nm. Injection volumes varied from 50-100 µl.

HPLC validation experiment The most important parameter in this part of the project was the retention time, t_r. Therefore, the repeatability and reproducibility of the retention time were tested by injecting a mix solution of six compunds (LogD's ranging from 1.5 to 3.6) five times on three different

days. On one of those three days, a new batch of buffer was used.[9]

5.1.2 The shake-flask method

In the course of the project, different methods were used. The reason for this is discussed in section 5.3.1. Some validation experiments were performed as well.

Shake-flask method 1 The method was based on the guideline from the OECD[18] and modified to fit this project. Equal amounts of 1-octanol and phosphate buffer (10 mM, pH 7.4) were saturated with each other by mixing in a separation funnel, and allowed to separate over night. The phases were separated into bluecap bottles, discarding the boarder layer.

2,000 µl of the pre-saturated buffer was transferred to a glass vial, and 1 µl of the radiolabeled compound in solution was added. 2,000 µl of the pre-saturated octanol was then added. The vial was vortexed for 60±5 sec. The vial was centrifuged at 3,500 rcf (relative centrifugal force) for 10 min. 500 µl of the octanol phase was transferred to a counting vial. The rest of the octanol was removed by pipette and discarded. 500 µl of the buffer phase was transferred to a counting vial. The samples were counted in according to table 1 on page 28. The logD was calculated by equation 8 on page 16.

Shake-flask method 2 The temperature of all solutions and the centrifuge was controlled to be 37 °C. After vortexing, the samples were left to separate for at least 5 minutes. 500 µl of the octanol phase was transferred to a counting vial. The rest of the octanol phase was removed and discarded by pipette. The vials, now containing almost only buffer, were centrifuged at

[9] New buffer was produced when the previous batch was finished.

3,500 rcf, for 10 min. 500 µl of the buffer phase was transferred to a counting vial. Except for this the methods were identical.

Shake-flask validation experiment 1 – Has the equilibrium been reached? To investigate whether the equilibrium (Equation 9 on page 19) was reached within the 60±5 sec vortex, a validation experiment was set up, see section 5.1.2 on page 37. App. 30 ml of the pre-saturated octanol was transferred to a vial. App. 10 ml of the pre-saturated buffer and 12 µl of [^{3}H]-SB207145-solution was added. The vial was securely capped and hand-shaken for 60 sec.[10] The vial was centrifuged at 3,500 rcf, RT (Room temperature) for 10 min.

Meanwhile, to each of 12 vials was added 2,000 µl of the pre-saturated buffer. 2,000 µl of the centrifuged octanol phase was added to each. The vials were vortexed for 60±5 sec. Samples 1 and 2 were extracted immediately: Left to separate for at least 5 min. 500 µl of the octanol was transferred to a counting vial. The remainder of the octanol phase was discarded. The sample was centrifuged at 3,500 rcf, RT for 10 min, and 500 µl of the buffer-phase was transferred to a counting vial.

Samples 3-12 were placed on a shaking table, and extracted in duplicate at time points t=[1,2,6,24,47] hr measured from the time of extraction of samples 1 and 2. All samples were counted in according to table 1 on page 28.

Shake-flask validation experiment 2 – the double-shake To exclude contamination of samples with hydrophilic impurities/radiolytes, a "double shake" was performed. The method is similar to shake-flask method 1 and shake-flask method 2, but with a "liquid-liquid extraction" to remove

[10]The vortex mixer could not supply enough force to shake the large vial thoroughly.

hydrophilic impurities/radiolytes. Samples were run in triplicate on both shake-flask method 2 and the hereunder described method for comparison:

2,000 µl of the pre-saturated buffer was transferred to a vial, and 3 µl of the PET-tracer solution was added. 7,000 µl of the pre-saturated octanol was added, and the vial was vortexed for 60±5 sec. The sample was centrifuged for 10 min at 22 °C at 3,500 rcf. Meanwhile, 2,000 µl of the pre-saturated buffer was transferred to three vials. 2,000 µl of the octanol-phase from the previous centrifuged sample was transferred to each, and the samples were vortexed for 60±5 sec.

The different phases were extracted in according to shake-flask method 2, and counted according to table 1 on page 28.

5.1.3 Predicted values

A trial version of Pallas 3.7 by CompuDrug Chemistry Ltd. was used to predict logD at pH=7.40 for the PET-tracers in order to compare with values obtained both by HPLC and shake-flask.

5.2 Results

The logD-values by shake-flask for all [^{11}C]-, [^{18}F]- and [^{123}I]-labeled PET-tracers were obtained during PET-scans in the course of the project. The logD by shake-flask for the [^{3}H]-labeled PET-tracers were obtained independently of PET-scans. The results can be seen in table 3 on page 40.

The two HPLC-methods are compared to each other, to assess whether the methods measure the same. Comparisons of data include HPLC-values vs. shake-flask, predicted values vs. shake-flask and predicted values vs. HPLC-values.

Table 3: The results of the logD-measurements with SD (n=3) (Standard deviation of three repeated measurements)

Compound	LogD by HPLC-method 1	LogD by HPLC-method 2	LogD by single-shake	LogD by double-shake	LogD by Pallas	Literature-value
Altanserin	4.58	4.52±0.02	1.87±0.03	-	2.89	2.15 [19]
Cimbi36	5.15	5.61±0.03	1.95±0.02	-	3.44	-
Cimbi5	5.40	3.47±0.03	-	-	3.36	-
Cimbi806	-	4.78±0.02	1.03±0.01	-	2.29	-
Cumi101	3.23	3.47±0.03	0.94±0.11	-	-0.42	-
DASB	4.36	-	1.71±0.03	-	3.12	3.03 [a]
Flumazenil	-	-	1.71±0.03	-	1.87	1.07 [4]
IBZM	-	-	1.30±0.03	-	0.66	1.78 [20] [b]
Ketanserin	4.05	4.10±0.02	-	-	3.18	3.05 [c]
MDL100907	4.69	4.90±0.03	0.59±0.07	1.31±0.02	2.64	
MH.MZ	4.51	4.89±0.05	1.73±0.01	-	2.94	-
MPPF	4.35	4.63±0.05	0.87±0.05	-	3.02	
PIB	2.79	2.80±0.00	2.01±0.01	-	3.57	1.23 [21] [d]
Raclopride	2.42	2.57±0.00	0.36±0.03	0.85±0.03	0.83	1.18 [4]
S35	5.49	-	2.31±0.34	-	3.79	-
SB207145	0.70	4.70±0.02	0.07±0.03	0.33±0.02	-0.54	
SB269970	3.01	3.38±0.04	0.82±0.02	1.01±0.00	-1.17	
WAY100635	5.15	5.57±0.04	1.57±0.05	-	3.27	3.10 [4]

[a] http://pomper.sairp.rad.jhmi.edu/Private/Tracers/DASB/DASB.html, 09-12-2011

[b] LogP

[c] http://www.lookchem.com/Ketanserin/, 09-12-2011

[d] LogP

5.2.1 Comparison of data

LogD-values of 11 PET-tracers measured by both HPLC-methods are compared in figure 9 on page 41.

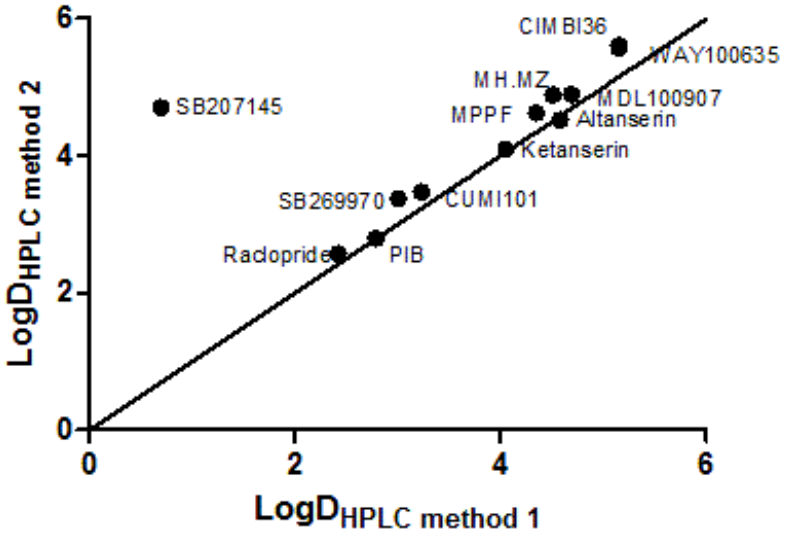


Figure 9: Identity plot comparing the two HPLC-methods. An outlier is evident, SB207145. N=1 for method 1, and n=3 for method 2. Method 2 is plotted with SD error bars. Linear regression excluding SB207145 gives $R^2 = 0.9755$.

As the HPLC-methods measure the same, only method 2 is compared to shake-flask values. An identity plot can be seen in figure 10 on page 42. Predicted values are compared to measured values, and an identity plot can be seen in figure 11 on page 43.

5.2.2 HPLC validation

The reproducibility and repeatability were tested with HPLC-method 1 with the compounds phenol, acetophenone, benzene, toluene, benzophenone and

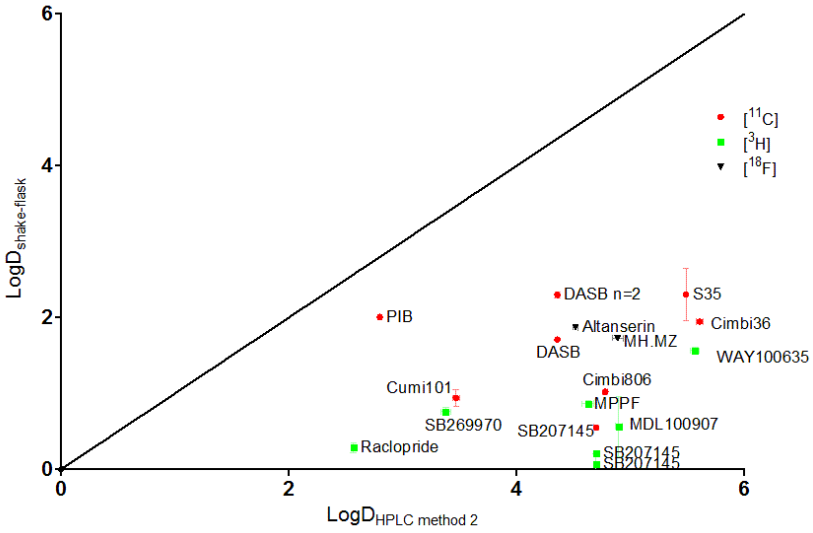


Figure 10: Identity plot of the logD-values obtained by the shake-flask method and by HPLC method 2 (Except for S35 and DASB, for which the HPLC method 1 values are used). The symbols indicate which isotope was used for the shake-flask method. Plotted with SD, n=3, unless otherwise mentioned in the label.

naphthalene. The results are presented in table 4 on page 44 and 5 on page 44.

The HPLC-calibration showed linearity in the range logD=[1.5;4.5], figure 12 on page 43. After the standards had been identified in the chromatograms, and the retention times had shown low relative standard deviations (Table 4 on page 44), a mix solution of the standard compounds was produced and used for calibration in a single injection.

5.2.3 Shake-flask method validation experiments

Has the equilibrium been reached? The 60±5 sec vortex was evaluated by leaving a vortexed sample on a shaking table for up to 47 hours, figure 13

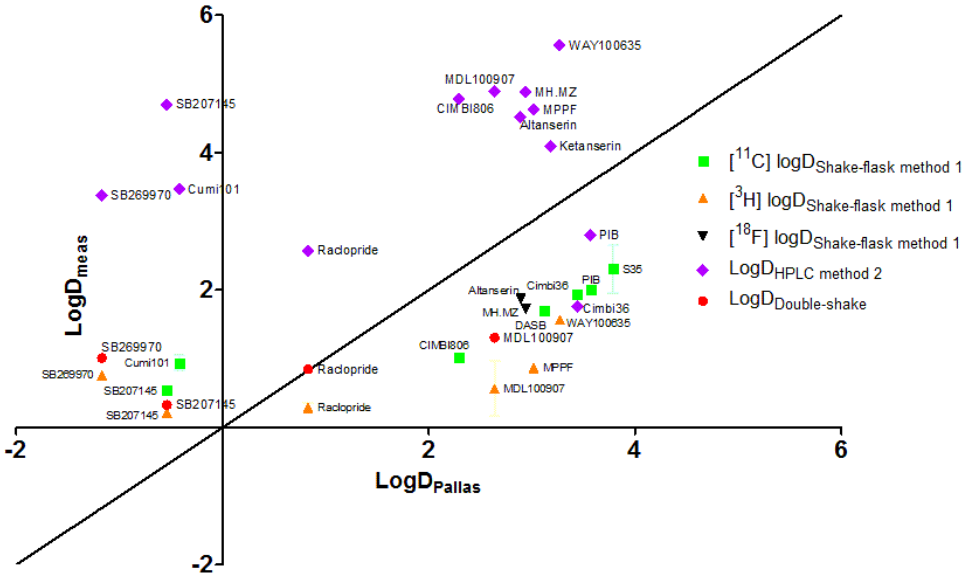


Figure 11: Identity plot of logD-values obtained by prediction (Pallas) and by experiments (Both HPLC and shake-flask). The experimental method is given in the legend.

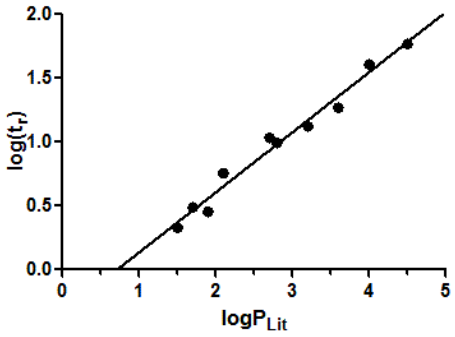


Figure 12: An example of a calibration curve run on HPLC-method 1. Linear regression by least squares gives a correlation coefficient of $R^2 = 0.977$.

on page 45. 47 hours exceed the incubation time of any PET-tracer used in the research project, and the 47 hours are assumed to be more than enough

Table 4: Reproducibility and repeatablity of the retention times. Values given with their respective relative standard deviations (n=5).

	t_r (min)		
Compound	Day 1	Day 2	Day 3
Phenol	2.899 ± 0.38 %	2.904 ± 0.13 %	2.906 ± 0.11 %
Acetophenone	4.209 ± 0.51 %	4.219 ± 0.07 %	4.221 ± 0.07 %
Benzene	8.110 ± 0.72 %	8.127 ± 0.14 %	8.131 ± 0.09 %
Toluene	16.111 ± 1.10 %	16.179 ± 0.11 %	16.192 ± 0.06 %
Benzophenone	20.699 ± 1.29 %	20.866 ± 0.11 %	20.881 ± 0.08 %
Naphtalene	29.677 ± 0.96 %	29.911 ± 0.15 %	29.943 ± 0.07 %

Table 5: The areas of each compound with their respective relative standard deviations (n=5). The SD's of benzene and toluene are much higher than for the other compounds. (mAU=milli absorbance units)

Compound	Area (mAU*min)
Phenol	5.166 ± 2.85 %
Acetophenone	12.402 ± 1.80 %
Benzene	3.530 ± 89.37 %
Toluene	5.735 ± 78.67 %
Benzophenone	16.846 ± 3.04 %
Naphtalene	23.288 ± 1.02 %

time to reach equilibrium.

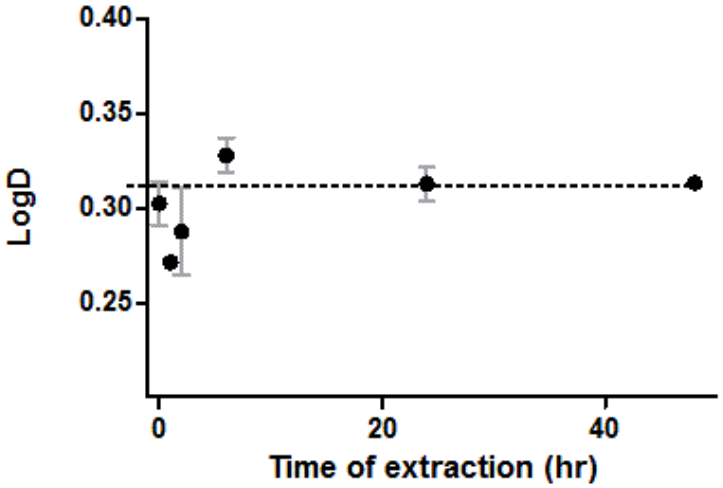


Figure 13: A plot of the data obtained from the first shake-flask validation experiment. The logD was measured at different time points from extraction of the first sample to investigate whether equilibrium had occured. Measured with [^{3}H]-SB207145. Plotted with SD, n=2.

The double-shake The double-shake values for the [^{3}H]-labeled compounds were significantly higher than the single-shake values (One-sided t-test, $p<0.05$), figure 14 on page 46. The four [^{3}H]-compounds were each measured in triplicate by both shake-flask method 2 and the double-shake method.

5.3 Discussion

5.3.1 Comparison of methods

Advantages and disadvantages of methods The shake-flask method is the gold standard for measuring logP and logD. Every other attempt of developing a new method of measuring lipophilicity is compared to the results of the shake-flask method.

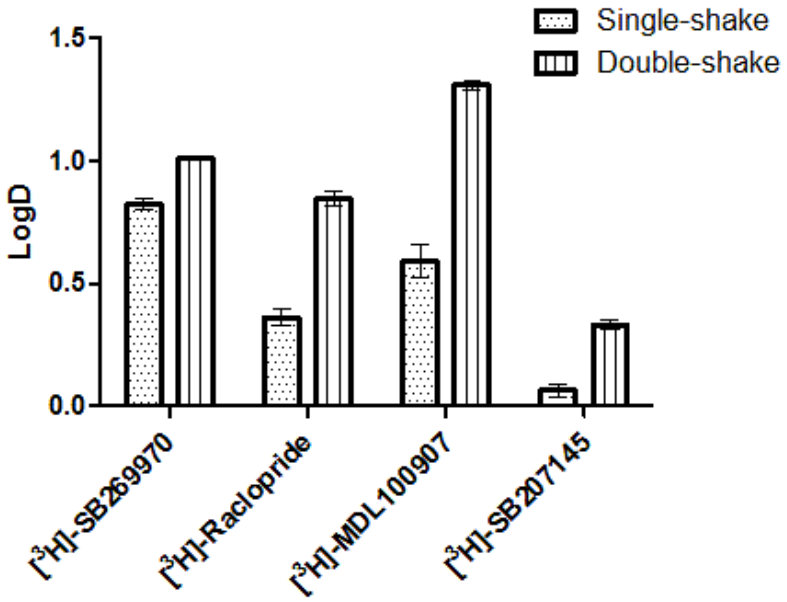


Figure 14: LogD values of four [^{3}H]-compounds measured by both shake-flask method 2 and the double-shake method. The double-shake removes hydrophilic radiochemical impurities, so that only the analyte – and possible lipophilic radiochemical impurities – are left.The results of the double-shake are significantly higher than the results of the shake-flask method 2 (one-sided t-test, $p<0.05$).

If an actual shake-flask is used (opposed to an automated system), the method is very cheap, but involves a lot of manual work. Measuring 10+ compounds at a time becomes a hassle. There is a lot of steps where random and systematic error can occur; volumes added, volumes extracted, separation of phases, quantification of analyte etc., and as it is shown in section 5.3.4, the purity of the analytes plays a great role depending in quantification methods.

The shake-flask method calls for quantification of analyte(s). The quantification of one of the phases can be avoided, measuring the phase

with the analyte before and after addition of the second phase, calculating the analyte extracted from the first phase.

If an HPLC is purchased for the sole purpose of measuring lipophilicity, it is, in comparison, a very expensive method. It also calls for calibration, for which standard compounds are needed. These compounds should preferably be like the analyte(s).

On the other hand, no quantification is needed, as the calculation is based on t_r. HPLC leaves little room for random error: Not much sample handling is necessary prior to injection. The system is highly automated, and may be left to measure without supervision.

Biological relevance Using the term "gold standard" for the shake-flask method for the measurement of lipophilicity applies to laboratory bench work. Whether the result of the shake-flask method or the HPLC-method is more correct is not relevant when measuring lipophilicity for the use in biological models. The better method is the one that mimics the property of the drug in the body most adequately. Assessing if the distribution process or the chromatographic process is the better method is not feasible, as no value of lipophilicity-in-the-body is available.

Comparison of data If two different analytical methods measure similar values of the same samples, the points of an identity plot should be on the line described by x=y. In the case of the identity plot of logD-values obtained by shake-flask and HPLC (Figure 10 on page 42), all points are below the line of identity. This result can be interpreted in three ways:

1. The shake-flask method underestimates the logD.

2. The HPLC-method overestimates logD.

3. A combination of 1 and 2.

Furthermore, there does not seem to be any trend in the data. There may, if each radioisotope is isolated in the plot, be slight linear trends with slopes lower than the slope of the line of identity.

In figure 11 on page 43, all the experimentally determined logD-values are compared to predicted values.

The data has been labeled to separate HPLC- from shake-flask-values and the individual radioisotopes from each other. The points are approximately evenly distributed around the line of identity. Information can be deduced from this plot:

1. The logD by HPLC values are overestimated compared to predicted values.

2. The shake-flask method underestimates logD compared to predicted values.

3. There may be trends in the data, but it depends on how the radioisotopes are viewed; as a cohort of radioisotopes or as individual isotopes. As a whole, there seems to be no trend, but when separated (by colour), the different isotopes seem to show linear correlations, increasing measured logD with increasing predicted logD. No regression line can however include all the points with their SDs.

Neither of the methods measures the same. Possible explanations for this will be discussed further in separate sections below.

Relating the results Even though the methods do not give the same results, it could be possible to calibrate the HPLC-values with shake-flask-values, thereby obtaining "shake-flask-values" with HPLC and interpolation.

A simple linear regression by least squares could be sufficient. A plot of the logD by HPLC method 2 vs logD by shake-flask method 1 with linear regression can be seen in figure 15 on page 49. There is no correlation and the calibration cannot be used. Another type of regression will presumably not give better results – the data is too inconsistent. The rank-order is wrong and the Pearson r-value is -0.1161 (indicating no correlation) with a p-value of 0.7912.

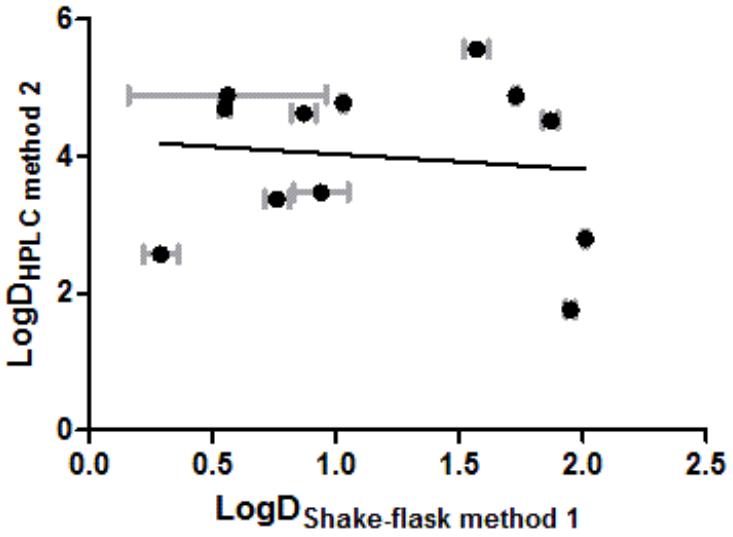


Figure 15: A linear regression calibration correlating the logD by HPLC method 2-values to the logD by shake-flask values. Plotted with SD, n=3. The linear regression is $y = -0.2209 \cdot x + 4.257$ with a $R^2 = 0.013$.

5.3.2 Shake-flask validation experiments

As the first shake-flask results from the scans, and from [^{3}H]-labeled compounds especially, had somewhat unexpected values, the method was investi-

gated. The first step taken was to change the method of extraction, see page 37 for method descriptions. The difference between the method regards only the extraction of the two phases, and was implemented to avoid contamination of the buffer-phase with the octanol-phase. In cases of high logDs, contamination of the buffer phase may be a real issue, causing low measured values. However, the [^{3}H]-labeled samples were all run with method 2, and all the results are peculiarly low, when comparing to literature values or predicted values.

Has the equilibrium been reached? The next step was to investigate whether the equilibrium (Equation 9 on page 19) was reached within the 60±5 sec vortex mixing. If not, it would explain the low shake-flask values, as too much analyte would still be left in the buffer. A protocol was set up to clarify this by measuring logD of [^{3}H]-SB207145 in a time-dependency. There is no obvious trend, and the value measured immediately after the vortex mixing is statstically the same as the value measured after 47 hours of incubation on the shaking table (figure 13 on page 45). This indicates, that the 60±5 sec vortex is sufficient to reach equilibrium of the radio-labeled compound between the phases. The point measured after 2 hours of incubation may seem like an outlier, but according to Grubb's outlier test, it is not (Confidence interval = 95 %). This experiment was only performed for one radio-labeled compound, but assumed to be valid for the cohort.

The double-shake Hydrophilic radio-labeled impurities will bias the analysis, and produce results significantly lower than the true value, if the actual logD$\geq$ 1. To make sure no hydrophilic radioactive impurities/radiolytes biased the analysis, the double-shake was tested and compared to the ordinary shake-flask method 2. If hydrophilic radioactive impurities/radiolytes were

present in the radio-labeled compound solution, these should be extracted into the first aliquot of buffer used – it can be seen as a sort of purifying liquid-liquid extraction. This will not remove lipophilic radioactive impurities/radiolytes, but as these pose a very small impact on measuring logD≥ 1 compared to hydrophilic radioactive impurities/radiolytes, it is disregarded.

For the four [^{3}H]-compounds tested, the measured logD-values were larger, and a t-test yields significant difference between the results. Based on this, it is concluded, that the radiochemical impurities present in the radio-labeled compound solutions have biased the analyses, and this may very well also be the case for the cohort of [^{11}C]-, [^{18}F]- and [^{123}I]-PET-tracers measured earlier with the single-shake.

Some of the [^{3}H]-compounds are affected more than others. This is probably due to difference in age of the compound solutions and constitution thereof. If the solutions are aqueous, or contain H_2O, spontaneous hydrolysis can occur, and the compounds are differently prone to that. The three most affected compounds (MDL100907, raclopride and SB207145) all contain methoxy- or ester-groups, that are susceptible to hydrolysis. The least affected compound, SB269970, does not.

In single-shake methods, the radio-labeled compound is added to the buffer, and has to move to the octanol during the shake. In double-shake methods, the radio-labeled compound has to move from the octanol to the buffer during the shake. As the logD>0, it could be suspected, that the latter process is slower than the first, or at least is more sensitive to this, causing higher measured logD than actual logD. This is not considered significant, but should be investigated in further studies.

An identity-plot of double-shake values with predicted values can be seen in figure 16 on page 52. If the double-shake is more correct than the single

shake, the data should fit the line of identity better. Unfortunately, only four values were obtained with the double-shake method (as it was just a validation experiment), impairing statistical analysis. More values were desirable, but only PET-scans with [^{11}C]-SB207145 were planned for the remainder of the project. Comparison with figure 14 on page 46 reveals, that both SB-compounds deviate more from the line of identity than with the single-shake. Raclopride and MDL100907 have both moved closer to the line of identity.

It seems, that the liquid-liquid extraction (i.e. the double-shake), may increase the lipophilic-radiochemical-impurities to compound-ratio for compounds with logD<1. Perhaps an inverted double-shake for compounds with logD<1 should be performed to remove lipophilic radiochemical impurities.

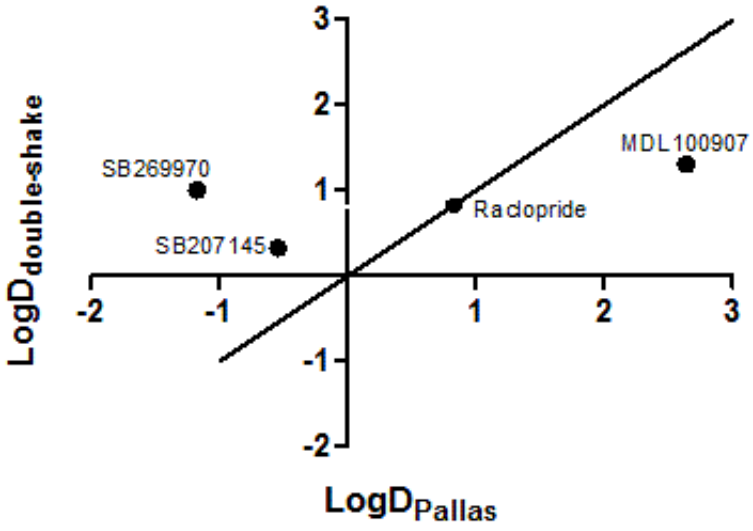


Figure 16: An identity plot comparing predicted values to double-shake values. Measured with [^{3}H]-labeled compounds. Plotted with SD, n=3.

5.3.3 Choice of shake-flask method

Different versions of the shake-flask method exist, but they all resemble the same basic setup: Two immiscible liquids, in which the analyte is solubilized. The phases are thoroughly mixed, separated and the amount of analyte is quantified in each.

The vessel used may be chosen by convenience – a 10 ml vial can be used, as well as a 500 ml separation funnel. The separation funnel offers easier separation of the phases, but with care, it can be done in vials as well. The material is only of importance, if only one of the phases is quantified, as the loss of compound due to non-specific binding to the vial walls will affect the one phase to the same extent before and after the shake.

Quantification can be done on both phases, or on one phase before and after addition of the second phase. Calculating the extracted amount provides information on concentration in each phase. This is very convenient, as there is only need for one quantification assay instead of two.

Equal volumes of the phases make the following calculations very easy, but using different volumes and volume-correction may yield a better result as the error of the analytical quantification methods will be similar. In cases of high logD, the aqueous phase may be hard to quantify due to issues with detection limit. If the expected logD-value is known, volumes of the phases should be chosen to ensure the analyte is distributed 50 %:50 % between them.[2]

For convenience, it was chosen to work with vials, and not shake-flasks. The vials were single use and are suitable for centrifugation. This facilitated quick separation, as is needed when working with a short-lived isotope. Equal volumes were used, leaving no need for volume correction. Both phases were analyzed, as the radioquantification assay is quite straightforward. Both

plastic and glass vials were used, and no effects due to non-specific binding to the vial walls were observed.

It is mentioned by Hansch and Leo[2] p. 101, that

> *(...)we put very little confidence in shake-flask values measured by radiotracer techniques. For reasons not entirely evident, these values (most often for very lipohpilic solutes) are very frequently as much as 3.0 log units below those values measured by other methods. (...) Because both phases were measured, adsorption to the walls of the container should not have been a factor.(...)*

This presumption is not discussed or investigated further in the text, but if it is true, it would render all the logD by shake-flask-values in this project questionable. A problem using radiolabeled analytes, and radiodetection, that Hansch and Leo may not have considered, is discussed in section 5.3.4.

The buffer was prepared to be strong enough to accommodate the amount of added PET-tracer without changes in pH. At 10 mM, this is accomplished. It was troublesome to separate and extract the phases in the vial, this was the reason to change from method 1 to method 2. Leaving the phases to separate by themselves seem to be adequate, and the octanol-phase can be extracted without problems. Should there be any aqueous droplets in the octanol-phase, the will have very little significance when measuring compounds of logD$\geq$1.0.

The repeatability of the shake-flask method is very satisfactory. The SD of repeated measurements (n=3) is maximum 0.11 logD-units, and as low as less than 0.00 logD-units. (Table 3 on page 40.)

5.3.4 Purity of measured compounds

The purity of a given compound may have a significant effect on the logD measured by the shake-flask method[4]. Because the [^{11}C]-, [^{18}F]- and [^{123}I]-labeled PET-tracers are meant for internal administration, produced on demand, stored in a capped vial and used within a short time, they are of high purity, and the radiochemical purity (RCP) is always $\geq$95 % – if not, the injection and subsequent scan are cancelled.

The PET-tracers produced on-site undergo purification and quality control procedures to ensure high quality of injected solutions. The potential impurities in the injection solution includes precursor, radiolytes and the labeling agent itself, which may be [^{11}C]CH_3OH, [^{11}C]CH_3I, [^{11}C]CO_2, $^{18}F^-$ etc.

The [^{3}H]-labeled compounds are meant for laboratory bench work, and may be stored for prolonged periods. Risk of contamination of the solutions is present when numerous aliquots are extracted from the vial. Should bacterial growth occur in the solution, it is very possible it will produce [^{3}H]-H_2O, and sponatenous hydrolysis will produce small hydrophilic [^{3}H]-impurities.

Effect of a small radiochemical impurity on the shake-flask method
Since the logD by shake-flask is measured using radiodetection, it is of importance, that only the PET-tracer in question is radiolabeled. To estimate and illustrate the effect of an radiochemical impurity in the PET-tracer solution, a thought example was calculated. For four compounds of actual logD = [1,2,3,4], the measured logD was calculated, assuming they each had an RCP of [100 %, 99 %, 95 %], and that the impurity would be distributed only to the buffer phase. The plot can be seen in figure 17 on page 56.
The plot shows the measured logD of the four imaginary compounds with

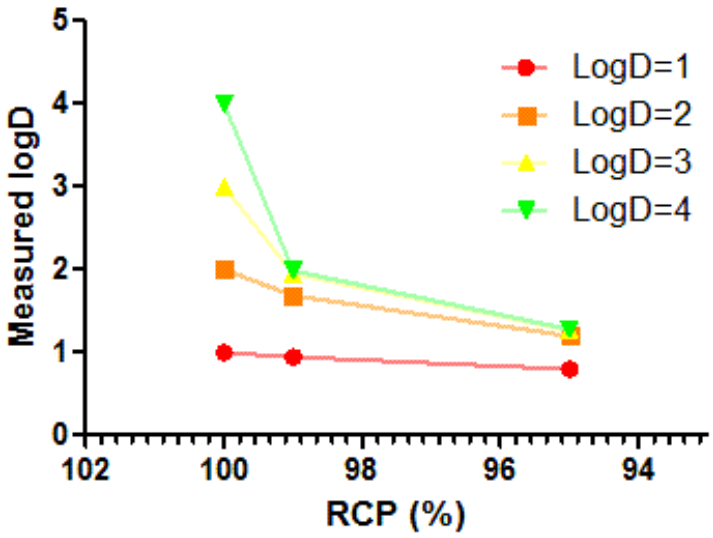


Figure 17: Radiochemical impurity effect on apparent logD using radiodetection. Four imaginary compounds with actual logD noted in the legend, each with 3 levels of a 100 % hydrophilic imaginary impurity. A similiar, and more exhaustive, study has been performed by Wilson et al.[4]

their actual logDs noted in the legend. For all four, the measured logD decreases quickly with RCP, giving rise to poor measured values.

The plot clearly shows, under the conditions assumed, that even a small radiochemical impurity changes the measured logD drastically – the effect is directly correlated to actual logD. This stresses the point, that measuring logD by shake-flask demands very pure compounds (or a double- og triple-shake), and renders the data collected in this project questionable: The RCPs of the PET-tracers is, as mentioned, never less than 95 %, but a 5 % impurity could severely bias the collected data.

Small impurities are always an issue with radio-labeled compounds, and

another method for measuring logD less influenced by these than the shake-flask method is desirable.

5.3.5 HPLC methods

Methods using gradient elution have been proposed earlier[7,9], but isocratic methods were developed in this project by advice from the supervisor.

The isocratic method gives good linearity, and no column equilibration is needed after a run. A gradient method could elute the highly retained analytes quicker than the isocratic method, but the calibration would most likely not be linear. Which method is best suited also depends on the column.

The only difference between HPLC method 1 and 2 is the column oven temperature, which is changed from 37 °C to 50 °C. The first temperature was chosen to mimic physological conditions. The temperature however, should not be of importance, as the system is calibrated with standards. To decrease analysis time and solvent consumption, the temperature was changed to 50 °C keeping within the temperature range recommendations of the column. As seen in figure 9 on page 41, the methods were concordant, and choosing method 2 was in order. There is an evident outlier in the plot, SB207145. This is explained by wrong identification of the peak in the chromatogram in HPLC-method 1.

The column used was very long. This caused high back pressure (sometimes around 300 bar), which caused leaks in the system and pumps, and this gave poor chromatograms. The dimensions of the column were necessary to separate compounds of logD=[1.0;1.5] with the eluent used, but perhaps the calibration range should have been changed to cover logD=[2.0;6.0] with a higher MeOH-content in the eluent. Also, the column was not chosen on the basis of the purpose, but on the availability. An ODP-column (Octadecyl

poly(vinyl alcohol)) was desired, as it showed very good linearity in the study by Donovan and Pescatore[7]. In the study, columns of different lengths (150 mm, 20 mm and 10 mm) were tested, and the 20 mm was found best. It could have been interesting to develop more than one HPLC column/eluent-system for comparison, especially to compare the column-performance.

5.3.6 Reproducibility and repeatability of the HPLC system

When testing the reproducibility and repeatability on the standards, the retention times did not differ between days on a 99 % confidence interval (n=5). There was no change in t_r in spite of a new batch of buffer, which may have had a slightly different pH than the previous batch.[11]

With no exceptions, t_r increases and SD decreases from day 1 to day 3 of the reproducibility and repeatability experiment. The maximum relative change in t_r from day 1 to day 3 is 0.89 % (Naphthalene). This is a slight drift, and it is considered insignificant, as new calibration curves were made on a day to day basis.

When inspecting the chromatograms visually, rather large differences were observed in peak areas for some of the standards between runs. This manifests itself in the peak area relative standard deviations for benzene and toluene in table 5 on page 44. In the five consecutive runs (each 35 minutes long), the signals drop significantly, see figure 18 on page 59. As the other compounds' peak areas do not change significantly, this effect is assigned to evaporation of benzene and toluene from the injection solution. However, as only the retention time is of interest, no measures were taken to improve the peak area, as long as the peaks were easily identifiable. Whenever toluene or

[11]This does not affect the standard compounds, as they are not ionizable. Compounds with pK_a around 7.4 are more susceptible to small changes in the buffer pH.

benzene could no longer be identified in the chromatograms, a new standard mix solution was produced.

If deviations in retention time of standards were observed, the other recorded signals were investigated, primarily the pump pressure. Often, a pressure drop was co-observed, perhaps caused by a small air bubble or a leak in the system. In this case, the chromatograms were discarded, the system inspected and tuned to keep the pump pressure constant. If the deviation could not be explained by a pressure drop, the sample was re-run.

Both standards and unknowns were preferentially dissolved in 50:50 MeOH:H_2O (v/v), but sometimes this was not the case. As mentioned in section 5.1.1, samples with more MeOH and/or some CH_2Cl_2 were run as well. This could potentially change the retention time, but it is considered of no significance, as the injection volume is very low compared to the flow. Should the retention time, against better judgement, be perturbed by the constitution of the injection solution, it is probably not more than ±2 min, and this has almost no effect on the calculated logD.

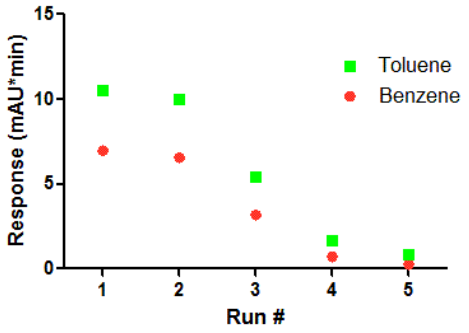


Figure 18: A plot of the peak areas of benzene and toluene from five consecutive runs.

5.3.7 HPLC calibration

In normal-scale calibration plots, it is desirable to have a linear calibration curve slope of 1 to increase robustness of the method. In this case, both axes are logarithmic, so a slope of 1 is still desirable.

The slope of the linear calibration line for method 1 (37 °C) is 0.46, and for method 2 (50 °C) it is 0.33 (Not shown). If this was the only factor to consider when chosing calibration conditions, method 1 is the obvious choice. (Or a third method with an even higher slope.) Given the great reproducibility of the HPLC-system, the time and solvents saved and the fact that the methods are concordant, method 2 is the obvious choice of the two.

As seen in figure 12 on page 43, the linear calibration curve shows a good fit with the data with no apparent outliers. The correlation coefficient of $R^2 = 0.977$ is satisfactory. A discussion of the validity of the calibration is however necessary.

Choice of calibration parameter Usually, the retention factor, k', is used when calibrating an HPLC-system. This was considered, but using t_r as the calibration parameter gave better correlation coefficients, and it was chosen to use t_r.

Choice of calibration method Chosing method 2 over method 1 was a matter of using less solvent and time in the analysis. In method 1, the column oven temperature (37 °C) was chosen to mimic physiological conditions, and in method 2, the column oven temperature (50 °C) was chosen to speed up the analysis and keep within column recommendations. A difference in column oven temperature may shift the equilibrium (Equation 6 on page 16), but using calibration, this should

not be an issue.

Choice of calibration standards The standards are all present in the list in the OECD guideline for testing of chemicals[17], and were chosen by their availability in chemical libraries available to this project. There may be an issue with structural insimilarity. The difference in t_r for different compounds is, this far, only attributed to difference in logD. This is only the case, if the column/eluent system mimics the partitioning system perfectly. This is hardly the case. There may be differences in t_r caused by other factors like molecular weight, column-specific interactions with heteroatoms or functional groups, aromaticity and so on. This distinguishing effect is in fact the chromatographic process.

A comparison of the calibration standards (Figure 8 on page 36) and the PET-tracers (Figure 6 on page 26) quickly reveals great differences in structural class. In general: The standards consist only of C, H and O. The PET-tracers consist of C, H, O, N, S and a few halogens. The mean MW (Molecular weight) of the standards is app. 125 Da, and for the PET-tracers, it is app. 360 Da.

Especially the –NH and $–NH_2$-groups of the PET-tracers may have column specific interactions with "naked" silanol-groups. This effect should be suppressed by adding an amino-salt to the eluent. This was not performed.

It is expected, that the measured values for the PET-tracers are biased by the lack of chemical similarity between standards and unknowns. So – is it valid to calibrate with one class of compounds, and compare that calibration to another class of compounds? The answer may be a corrected calibration curve. As long as the standards and unknowns

all belong to their individually same class of compounds, it should be possible to correct for the above mentioned factors mathematically.

A suggestion is, that the PET-tracers will adhere more to the column material than the calibration standards because of their size. Even though this may not be the only chromatographic effect causing higher retention of the PET-tracers, a $\log D_{\text{HPLC method 2}}$ vs. MW-plot was constructed (not shown). No correlation was found using Pearson's correlation test The t_r and logD by HPLC method 2 were also related to number of nitrogens in the molecule, primary, secondary and tertiary amines, oxygens, phenyls and carbonyls respectively. No correlations were found,[12] and the higher retention of the PET-tracers cannot be attributed to a single of the mentioned properties, but rather a combination of them.

Interpolation of unknowns Quite a lot of the measured compounds have a logD higher than 4 measured by HPLC. The calibration is in the range logD=[1.5;4.0], so strictly it is not valid to extrapolate the values from the calibration curve. The residual plot of the calibration is quite satisfactory with points randomly dispersed and no residual being numerically larger than 0.104 (not shown), and as the maximum logD-value exceeds the calibration by app. 1.7 logD-units, it is considered acceptable.

[12]This almost constitues a principal component analysis. Unfortunately, no available software was awailable to perform a full one.

5.4 Conclusion

A method of measuring logD by HPLC was developed and validated. The shake-flask method was replicated, investigated and validated. Concordance was neither found between logD obtained by HPLC and shake-flask, nor between experimental data and predicted data. The shake-flask method is the most correct method of measuring lipophilicity, and the HPLC-method could not emulate it. The results of the HPLC-method showed no statistically significant correlation to the data obtained by the shake-flask method.

The HPLC-methods showed excellent reproducibility, repeatability and robustness, even though the pK_a of some of the compounds was close to the buffer pH. As an example; one of the pK_as of ketanserin (Figure 6i on page 26) is 7.60 (Pallas prediction) and the SD of HPLC method 2 was only 0.02 logD-units.

The HPLC-method overestimates the lipophilicity compared to the other methods, indicating that the column/eluent-system does not mimic the partition-experiments properly. This may very well be caused by structural insimilarity between standards and unknowns, as mentioned in section 5.3.7.

Using radio-labeled compounds in the shake-flask method makes detection of the phases much faster and easier, but there are pitfalls, i.e. presence of other radio-labeled compounds than the analyte. The purity of the compound should be known or investigated prior to using radio-detection for the shake-flask method, or purification procedures, as the double-shake, should be implemented.

Measuring lipophilicity by HPLC has proven itself to not be as easy as desired. The developed HPLC-methods are easily executed, but the equipment may not work properly, (which has also been the case for this project), and a lot of time can be spent on getting the system up and running.

6 Plasma protein binding measurements

The plasma protein binding was measured with one ED-assay (equilibrium dialysis) and two UC-assays (ultracentrifugation). The ED-assay protocol was adopted from the research project's analysis routine, as the method has been validated earlier. To investigate the validity of the new UC-assays, a few validation experiments were conducted to assess whether the equilibrium dialysis can be substituted with ultracentrifugation in a routine analysis setup.

6.1 Materials and methods

Plasma In the case of a PET-scan being performed, a blood sample is harvested from the test-person in a heparinized plastic vial. The sample was centrifuged for 7 min at 3,500 rcf at a temperature between 4–28 °C. The plasma was used for both the ED and UC assay.

For validation experiments, both fresh and frozen plasma was used. In cases where the plasma needed to be fresh (freshness of plasma-experiments, routine analysis during PET-scans), it was harvested in Vacuette® tubes with K_3EDTA as anticoagulant and centrifuged for 7 min at 3,500 rcf at a temperature between 4-28 °C. In cases where frozen plasma was acceptable (Validation experiments except FoP), two bags of frozen plasma (app. 270 ml each), with citrate as the anticoagulant, were aquired from the blood distribution facility of the hospital. The frozen plasma was thawed once, divided into app. 40 ml aliquots in plastic vials, stored at –20 °C and thawed prior to use.

Dialysis buffer The buffer used in ED-assays was prepared according to table 6 on page 66 and the pH was adjusted to 7.40 with NaOH(aq) and/or

NH_3(aq). The buffer was stored in vials at –20 °C to prevent bacterial growth, and thawed prior to use.

Centrifuges The centrifuges used were the same as in the lipophilicity section, see section 4.1 on page 27.

Dialysis chambers The dialysis chambers, seen in figure 19 on page 65, were DIALYZER from Harvard Apparatus (Massachusetts, USA) with a chamber volume of 500 µl. The membranes used were Regenerated Cellulose Membranes with a MWCO of 10 kDa. Incubation took place in a Medax water bath at 37 °C.

Figure 19: Dialysis chambers. One assembled and one disassembled. The chambers shown differ in size from the ones used in this project. Picture courtesy of Harvard Apparatus.

Ultracentrifugation units The Centrifree® units were *Centrifree® Ultrafiltration Device with Ultracel YM-T membrane*, and had a 1,000 µl sample reservoir and a MWCO of 30 kDa. The membrane was made from Ultracel YM-T regenerated cellulose.

The Amicon Ultra units were *Amicon Ultra 0.5 ml centrifugal filters for protein purification and concentration* with a 500 µl sample reservoir and a MWCO of 10 kDa. The membrane was made from Millipore Ultracell

Table 6: Dialysis buffer

Compund	Concentration
NaCl	135 mM
KCl	3.0 mM
$CaCl_2$	1.2 mM
$MgCl_2$	1.0 mM
KH_2PO_4	2.0 mM

low-binding regenerated cellulose. Both units were from Millipore (Massachusetts, USA), and can be seen in figure 20 on page 67.

6.1.1 Equilibrium dialysis

Equilibrium dialysis method The general method for equilibrium dialysis was as follows: The number of dialysis chambers needed was assembled, and the different parts were chosen to secure a good fit. (Not all parts fit perfectly with each other.) A dialysis membrane was placed in the unit, making sure that a single membrane was used in each chamber, and that it did not crease.

After assembly, 500 µl of the dialysis buffer was transferred to the unit, and the first lid was fitted into place. 500 µl of the plasma, was placed on the other side of the membrane, and the second lid was fitted into place. After making sure the unit was properly assembled, it was incubated in a water bath at 37 °C for a specified incubation time.

After incubation, the units were retrieved from the water bath, and quickly dried with tissue paper. Firstly, the buffer-side was opened, and a volume was transferred to a pre-weighed counting vial. Secondly, the other

side was opened, and the liquid was transferred to a pre-weighed counting vial as well. Usually, 400 µl were retrieved from each side. The chamber was disassembled and the membrane was removed and disposed of. The parts were washed in lukewarm water with dish soap, and left to dry overnight.

6.1.2 Ultracentrifugation

(a) Amicon Ultra (b) Centrifree®

Figure 20: The ultracentrifugation units used in this project. Pictures courtesy of Millipore.

Ultracentrifugation method 1 The plasma was filtered through a 0.45 µm syringe filter into a vial. An appropriate amount of radio-labeled compound solution was added.[13] The plasma was thoroughly mixed by either

[13]Too little gives poor counting statistics. An aliquot of the radio-labeled compound solution was counted prior to the experiment to make sure the activity was suitable. The addition was of tracer dose.

inverting the sample at least 10 times, by vortexing or by pipetting up and down. An aliquot of the plasma was transferred to the UC unit and centrifuged in according to table 7 on page 68.

Table 7: The standard ultracentrifugation parameters. For centrifuge specifications, see section 4.1 on page 27.

Unit	Applied volume	Spin-time	Spin-force	Temp.	Centrifuge
Centrifree®	1,000 µl	20 min	2,000 rcf	22 °C	1
Amicon Ultra	500 µl	20 min	14,100 rcf	RT	2

Deviations from the standard parameters will be noted in the text. Parameters that can be varied in ultracentrifugation methods besides changing the ultracentriguation unit itself, include spin-force, spin-time, spin-temperature, applied volume, pre-treatment of sample and dilution factor.

UC validation experiment 1: Spin-time and -force variation The standard ultracentrifugation parameters are specified by the manufacturer in the product specification sheet. It was investigated whether the analysis would be biased by varying the spin-time and spin-force. The experiment was performed with both [^{3}H]-SB207145 and [^{3}H]-MPPF.

A vial of frozen plasma was thawed and filtered through a 0.45 µm syringe filter. 5.0 µl of the radio-labeled compound solution was added, and the plasma was mixed by inverting the sample 10+ times. A reference sample was produced by transferring 200 µl of the plasma to a pre-weighed counting vial. The rest of the plasma was distributed to ultracentrifugation units and centrifugated in duplicate in according to table 8 on page 69. Note that sample 5, 6, 23, and 24 can be used for both spin-time and -force variation curves.

Table 8: The setup for validation of spin-time and -force.

Unit	Sample #	Spin-time	Spin-force
Centrifree®	1,2	5 min	2,000 rcf
	3,4	10 min	2,000 rcf
	5,6	20 min	2,000 rcf
	7,8	40 min	2,000 rcf
	9,10	60 min	2,000 rcf
	11,12	20 min	500 rcf
	13,14	20 min	1,000 rcf
	15,16	20 min	3,000 rcf
	17,18	20 min	6,000 rcf
Amicon Ultra	19,20	5 min	14,100 rcf
	21,22	10 min	14,100 rcf
	23,24	20 min	14,100 rcf
	25,26	40 min	14,100 rcf
	27,28	60 min	14,100 rcf
	29,30	20 min	2,000 rcf
	31,32	20 min	5,000 rcf
	33,34	20 min	10,000 rcf

The filtrates were transferred to pre-weighed counting vials, weighed back, and counted according to table 1 on page 28.

UC validation experiment 2: Pre-treatment – freshness of plasma (FoP) The pre-treatment of the plasma may be filtration, incubation, cooling or freezing, and a few tests were conducted to clarify the effects of this. A blood sample was drawn from a volunteer test-person in Vacuette® tubes with K_3EDTA as anticoagulant. The sample was immediately centrifuged at

3,500 rcf for 7 min. at RT. The plasma (app. 7 ml) was filtered through a 0.45 µm syringe filter and 300 µl of dichlorvos B was added. The plasma was divided into three portions of app. 2 ml. One was used right away, one was stored at 5 °C and one was stored at –20 °C, both for app. 20 hours.

The portions were added 1 µl [^{3}H]-SB207145-solution each and mixed by pipetting up and down, and reference samples were produced with 200 µl of the plasma. Each sample was ultracentrifuged in triplicate in Amicon Ultra units, see table 7 on page 68. The filtrates were transferred to pre-weighed counting vials, weighed back, and counted according to table 1 on page 28.

After this, it was decided to furthermore investigate a time dependency in the first 6 hours of cooling at 5 °C. The experiment was performed in the same manner as the first with two exceptions:

1. The addition of dichlorvos B was done just before addition of radio-labeled compound (as opposed to right after collecting the plasma from the blood sample), as this was more in accordance with how it has been done in earlier research.

2. A data point made with ED was added to the experiment. Measured in triplicate opposite dialysis buffer with incubation time of 2 hours 20 min[14] and incubation temperature of 37 °C.

As these two experiments did not clarify the effects of temperature, a third experiment was conducted to verify the results of the first experiment. The first and third experiment were identical except in two things:

1. The dichlorvos B solution was added immediately before addition of radio-labeled compound, not, as in the first experiment, after filtration of the plasma.

[14]Standard incubation time for SB207145

2. Stricter temperature control prior to centrifugation was employed. I.e. the refrigerated and frozen samples were incubated to RT in a water bath before addition of both dichlorvos B and radio-labeled compound to exclude any differences in Fp due to difference in temperature of the plasma sample.

The fourth FoP was performed with addition of 6.0 µl of [^{3}H]-MDL100907 to each sample, a non-ester leaving no need for dichlorvos, to exclude any possible biases caused by addition thereof, in Centrifree® units in according to table 7 on page 68. All FoP-samples were counted according to table 1 on page 28.

UC validation experiment 3: Interindividual variance To assess whether the variance in different test-persons' Fp depends on their varying content of proteins in the plasma, a validation experiment was conducted. The same plasma pool from UC validation experiment 1 was utilized. (App. 40 ml filtered plasma with 2.0 ml dichlorvos B and 5 µl of [^{3}H]-SB207145.) Five dilutions were produced, using dialysis buffer as the dilutor, yielding dilutions with a plasma content of 100 %, 75 %, 50 %, 25 % and 1 %. A reference sample was produced from each dilution by transferring 200 µl to a pre-weighed counting vial. The dilutions were ultracentrifugated in triplicate in Amicon Ultra units according to table 7 on page 68. The filtrates were transferred to pre-weighed counting vials, and all samples were weighed back and counted according to table 1 on page 28.

To substantiate the experiment, previously recorded data was collected: Fp from earlier scan-dates measured by ED, and the related albumin-content, which is measured at the hospital's clinical biochemical laboratory as a standard for all included test-persons.

6.2 Results

6.2.1 ED vs UC

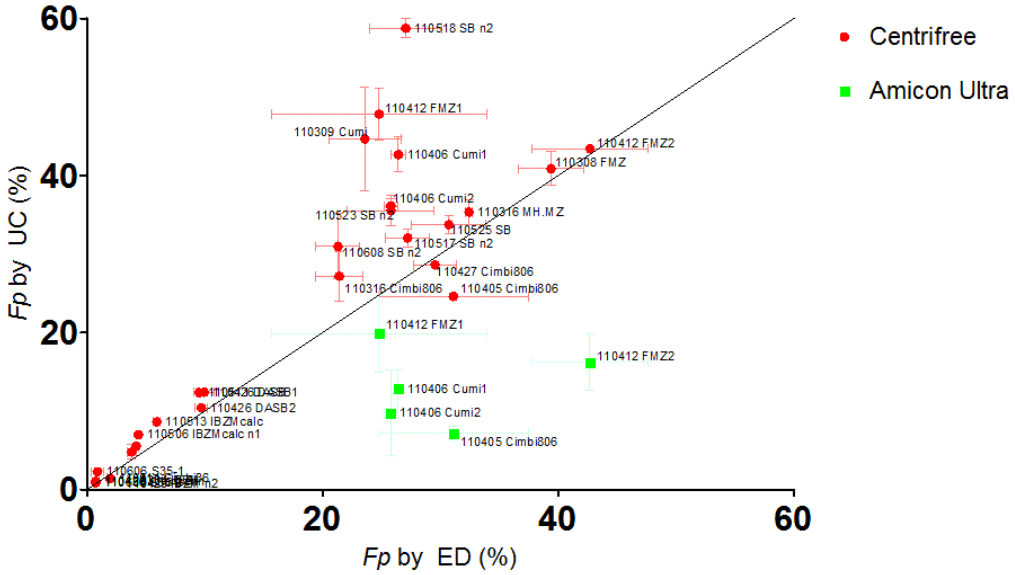
In the course of the project, the *Fp* was measured for different test-persons by both UC method 1 and ED method 1 in order to compare the two methods. The measurement was conducted on the same plasma for each test-person. A plot of the results can be seen in figure 21 on page 73.

6.2.2 UC

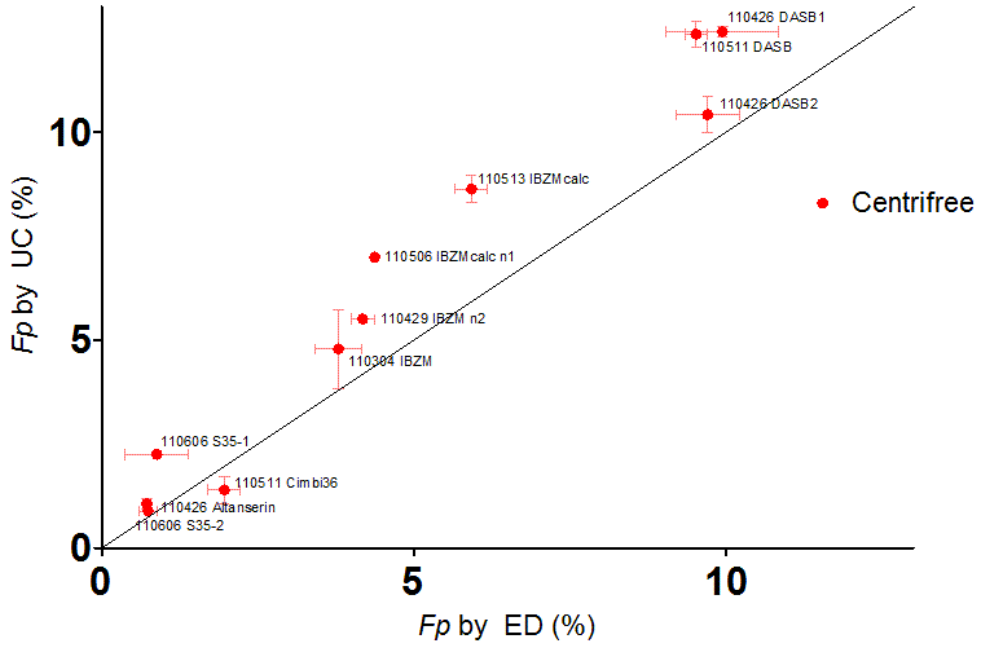
UC validation experiment 1: Spin-time and -force variation Plots of spin-time and -force variations measured with both Centrifree® and Amicon Ultra units on both [^{3}H]-SB207145 and [^{3}H]-MPPF can be seen in figure 22 on page 74.

UC validation experiment 2: Pretreatment – freshness of plasma It is evident, that the results of the FoP-experiments differ significantly from each other, especially the refrigerated sample gives a high value compared to the others (figure 23a on page 76.) This is also confirmed by a Grubb's outlier-test, $p<0.05$. It was decided to investigate whether a free fraction time-dependency was present in the first 6 hours after the sample was refrigerated, but no tendency seemed to be evident, and furthermore, the ED-result differed significantly from the UC-results. (Figure 23b.)

For the third FoP-experiment, of which the experimental circumstances are essentially identical to the first, the data is still inconsistent. No outlier is detected using Grubb's test, but there is an evident downwards trend (figure 23c). As the data was still inconclusive, a fourth attempt was made, using [^{3}H]-MDL100907 (non-ester) to avoid interactions with dichlorvos, and the results improved, giving the same result of *Fp* for all three samples (figure

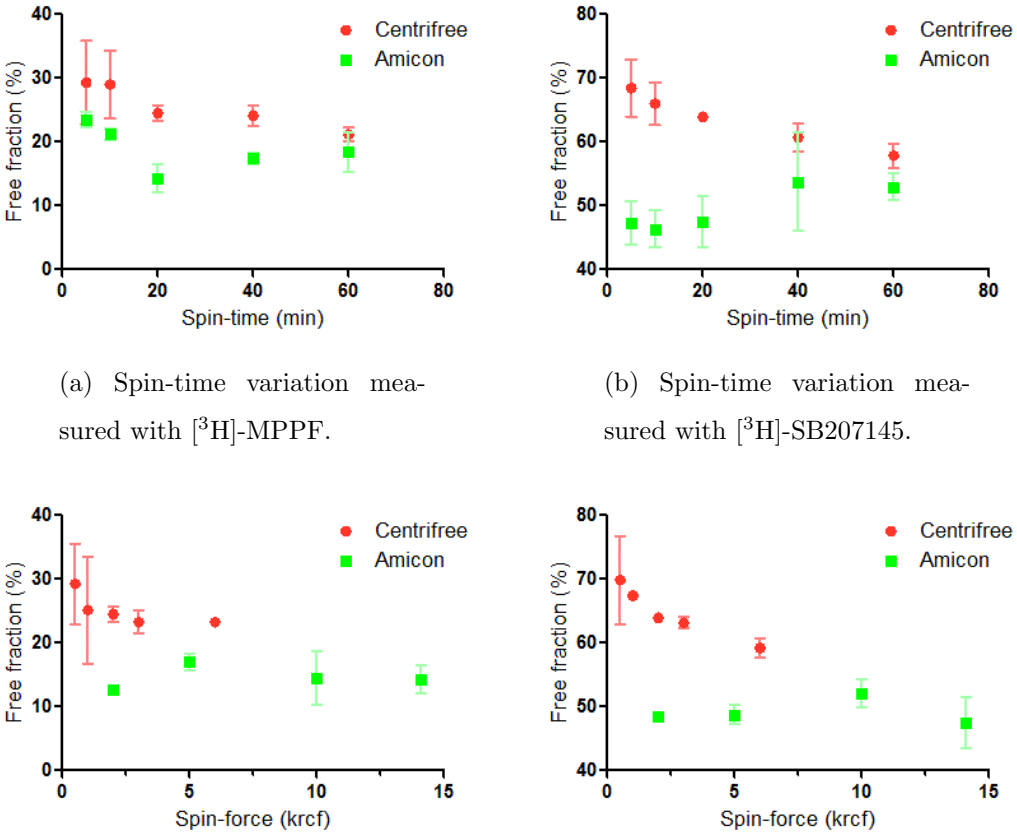


(a) An identity plot of all the UC and ED data.



(b) A zoom of the same data to make data points distinguishable

Figure 21: An identity plot of the data obtained by UC and ED for different test-persons. Plotted with SD, n=3 or noted in the label.



(a) Spin-time variation measured with [^{3}H]-MPPF.

(b) Spin-time variation measured with [^{3}H]-SB207145.

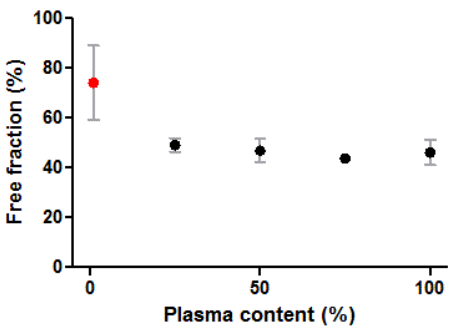
(c) Spin-force variation measured with [^{3}H]-MPPF.

(d) Spin-force variation measured with [^{3}H]-SB207145.

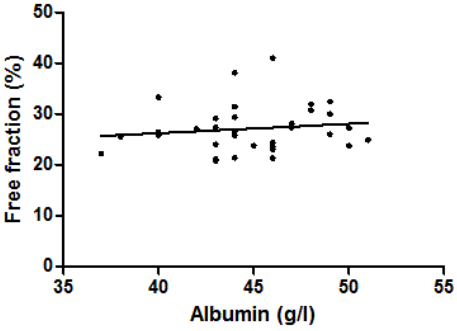
Figure 22: Spin-time and -force variations for [^{3}H]-SB207145 and [^{3}H]-MPPF. Neither compound covers the whole spin-force variation range, as two different centrifuges were used. Plotted with SD, n=2.

23d). Due to lack of Amicon Ultra units, Centrifree® units were used.

UC validation experiment 3: Interindividual variance To estimate whether the varying *Fp*-value of different test-persons' plasma was caused by difference in albumin content, dilutions of a plasma pool were produced and measured for free fraction. The range of dilution was from 1 %–100 % plasma. To furthermore investigate the interindividual variance, data *Fp* and albumin content from previous scans was collected from databases and compared. Plots of the data is shown in figure 24 on page 75.

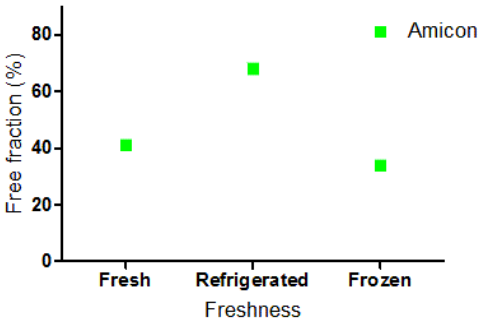


(a) Measurement of *Fp* on dilutions of the same plasma pool. Plotted with SD, n=3.

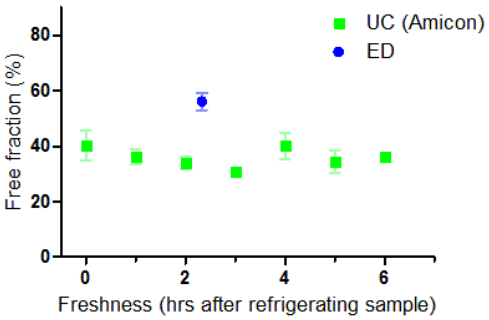


(b) Data collected from databases for 33 test-persons.

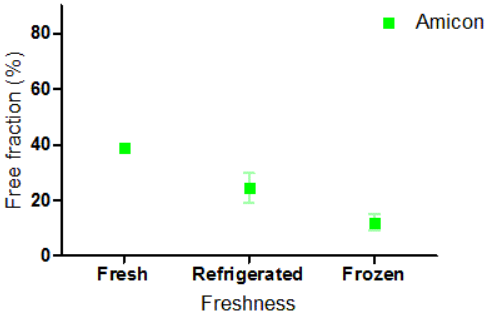
Figure 24: Plots of the interindividual variance experiments. The *Fp* is measured for every test-person, and the albumin content is measured at the hospital's clinical biochemistry department as standard for every test-person as well.



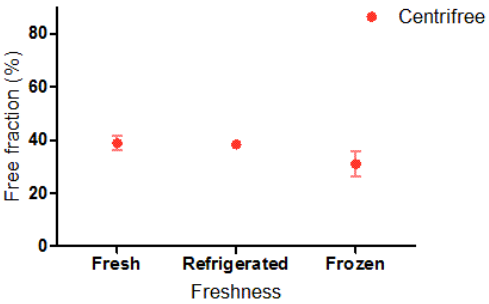
(a) *Fp* measured on fresh, refrigerated and frozen plasma from the same batch. Measured with [^{3}H]-SB207145. Plotted with SD, n=3.



(b) *Fp* measured every hour in the first 6 hours after incubating at 5 °C. Also, one measurement of *Fp* by ED is included as "the true value". Measured with [^{3}H]-SB207145. Plotted with SD, n=3.



(c) FoP3: A re-make of FoP1. Two variables differed: The dichlorvos B solution was added immediately before the radiolabeled compound and stricter temperature control was employed. Measured with [^{3}H]-SB207145. Plotted with SD, n=3.



(d) *Fp* measured with a non-ester to exclude any errors caused by addition of dichlorvos B. Measured with [^{3}H]-MDL100907. Plotted with SD, n=3.

Figure 23: Plots of the freshness of plasma data. The UC-units used can be seen in the legend of the individual plots.

6.3 Discussion

6.3.1 Advantages and disadvantages of the methods

ED requires manual installation of a dialysis membrane, where the user may apply two by accident, or crease the membrane. The chamber volume is specific, so using a poorly calibrated pipette may cause seeping/leakage. This may also happen if bubbles are present in the liquid placed in the chamber prior to closing it. Collecting phases after incubation is relatively easy, but the risk of puncturing the membrane with the pipette tip is present. Furthermore, the dialysis buffer needs to be prepared to mimic physiological conditions, containing the same salts at the same concentration as in human plasma, adjusted to physiological pH. In contrast to this, UC requires only pipetting skills, leaving little room for both systematic and random errors, as the unit is pre-assembled with a membrane that could only willfully be punctured and no buffer is needed.

The volume of the filtrate in UC is dependent on the centrifugal force applied, and for how long. The subsequent analysis of the filtrate must not require more filtrate than is available. With a chromatographic system this should not be an issue, as they generally have low detection limits and can work with low injection volumes.

In both methods, there is a hold-up volume in the membrane. This has little influence in ED, as the equilibrium (Equation 9 on page 19) will not be affected by this. In UC, the membrane may retain the free compound, giving rise to a lower measured free fraction than the true value. This effect should have higher influence on compounds with a low free fraction, than compounds with intermediate or high free fraction. The hold-up volumes

of the Centrifree® units is 10 µl, and of Amicon Ultra it is <1 µl.[15] The membrane may also retain some compounds more than others, making it necessary to validate the method for each compound.

6.3.2 Comparison of methods

The identity plot of ED vs. UC can be seen in figure 21 on page 73. For convenience, a zoom of the full identity plot is shown, to facilitate distinguishing of data points with $Fp \leq 10$ % from each other. The data points are plotted with their SD, n=3 (if n≠3, it is noted in the label). The aim originally was to compare ED to UC by Centrifree® only, but some data points measured with Amicon Ultra have been included as well.

In the identity plot comparing *Fp*-values obtained by ED and UC, it seems that a higher *Fp* yields poorer concordance than lower *Fp* does. The zoom-plot (Figure 21b) shows compounds with *Fp* in the range 0-10 %. The data generally fits the line of identity better than the *Fp*-values higher than 10 %.

A Pearson correlation test yields significant correlation for Centrifree® ($r = 0.8796$ and $p < 0.0001$), but not for Amicon Ultra ($r = 0.08696$ and $p = 0.007562$.)

The data generally looks good, and fits the line of identity. This statement seems to be more true for Centrifree® than for Amicon Ultra. Keeping in mind, that only a few of the compounds have been measured with Amicon Ultra, it seems like this unit generally underestimates the *Fp* compared to both ED and UC by Centrifree®. This is somewhat expected, as the MWCO of Centrifree® allows more molecules to cross the membrane, than does the MWCO of Amicon Ultra. However, it is surprising, that Centrifree® seems

[15] Hold-up volume values are cited from the respective product specification sheets.

to be more comparable to ED than Amicon Ultra, as the MWCO of Amicon Ultra and the dialysis membranes match.

The Centrifree® units have a higher MWCO than the ED-membranes, which could cause higher *Fp*, and are more susceptible to clogging of the membrane, which could cause lower *Fp*. The Amicon Ultra units have the same MWCO as the ED-membranes and are not susceptible to clogging of the memrane. For these reasons, it was expected, that Amicon Ultra would mimic the ED better than Centrifree®, but actually it is opposite.

6.3.3 ED

The equilibrium dialysis is the gold standard of plasma protein binding measurement, to which all other methods are compared. It is assumed, that the measured value of *Fp* actually reflects the value in the plasma – whether this is correct cannot be known for sure. As in the lipophilicity section, the term "gold standard" applies to laboratory bench work, and the better method is the one, that reflects the actual value in the plasma. ED is one of the PPB-methods, that perturb the sample the least, and it is assumed to be the correct measure.

Issues with the method include osmosis, similarity of the phases, quality of the membrane. Should the phases be different in constitution, osmosis will occur, to keep the concentration of each constituent the same on either side of the membrane. This is prevented by using the fixed-volume chambers, and the membrane is permeable to salts. Also, the ability of each phase to accommodate the analyte may be different, if the phases are too different. This is the background for the choice of dialysis buffer – it has to be as similar to plasma as possible, but without proteins. If feasible, it is probably better to use protein free plasma, which could be produced on-site with the

test-person's blood using ultracentrifugation, leaving no difference between the phases, except for protein-content.

The quality of the disposable membranes has to be steadfast to ensure high reproducibility. The membranes used in this project are assumed to be of high and unvarying quality.

Addition of PET-tracer to the plasma has been of varying magnitude. Generally, it has been attempted to add approximately 5 MBq to the 2-3 ml plasma – but this amount is merely chosen on a basis of achieving satisfactory counting statistics. The varying specific activity and concentration of the PET-tracer solutions cause the "5 MBq" to be a quite variable volume. Seen in the light of the Interindividual Variance-experiment (Section 6.1.2), it is not considered to be of influence.

The method is fairly robust, but outliers do occur. All the results were obtained in triplicate, and obvious outliers were omitted.

6.3.4 Ultracentrifugation

Choice of UC-units The UC-units were chosen on the basis of having been used earlier in the research project. An earlier Amicon Ultra-model was also available, but it was decided not to use it, as its membrane is perpendicular to the spin-force.

UC validation experiment 1: Spin-time and -force variation This experiment was conducted to validate the spin-time and -force, and to evaluate the robustness of the method. Also, it is an assumption in UC, that the equlibirum described in equation 9 on page 19 is not perturbed. The results presented in figure 22 on page 74 are discussed in the following paragraphs:

Spin-time variation for [^{3}H]-MPPF See figure 22a: The Centrifree®

units seem to show a slight descending trend, but the high SD of the first two results make the data inconclusive. The Amicon-results fluctuate more, but no trend seems obvious.

Spin-time variation for [^{3}H]-SB207145 See figure 22b: In this plot, the descending trend of the Centrifree®units is more evident, and it seems to converge towards a value, but unfortunately not plateau within the range. The Amicon points seem constant for the first 20 minutes, but then increase towards a higher value.

This convergency may be explained mechanically. During the course of centrifugation, macromolecules and particles in the plasma will accumulate on the Centrifree® membrane, causing decreasing *Fp* with time. For Amicon, it is exactly opposite. As the membrane is placed on the sides of the sample reservoir, it will be "stripped" of macromolecules and particles, which will accumulate in the bottom of the sample reservoir. The effect is not observed in the same experiment with [^{3}H]-MPPF, and it may be caused by the value of *Fp*, which for [^{3}H]-SB207145 is roughly twice the *Fp* of [^{3}H]-MPPF.

Spin-force variation for [^{3}H]-MPPF and [^{3}H]-SB207145 See figure 22c and 22d: There seems to be a general trend for the Centrifree® units; a decreasing *Fp* with increasing spin-force. It is indicated for both [^{3}H]-MPPF and [^{3}H]-SB207145, but for [^{3}H]-MPPF it seems to plateau within the range and for [^{3}H]-SB207145 it does not.

The overall effect of the *Fp* converging towards a value may be explained by clogging of the membrane in the course of centrifugation. This hypothesis is supported by the not-converging values of *Fp* measured with Amicon Ultra units, for which accumulation of macromolecules

and particles on the membrane is mechanically hindered by the design of the unit. The *Fp*-values measured with Amicon Ultra seem constant and consistent.

Statistics One-way ANOVA was performed for all the spin-time and -force variations, and for none of them does p exceed 0.05 indicating that the values are constant. Tukey's multiple comparison test was also performed, and only for one pair of data did p exceed 0.05, namely the spin-time variation using Amicon Ultra units and [^{3}H]-MPPF comparing values for 5 minutes and 20 minutes spin-time.

Purity of solutions Should the compound-solutions used in this experiment be contaminated with radiolytes or other radiochemical impurities, there is no way of predicting if it will in- or decrease the results. The bias should however be systematic, and therefore not relevant when investigating these variations.

Use of frozen plasma When investigating the properties of the UC-units, the trends are of greatest interest. As long as the plasma pool used is the same and treated the same throughout the experiment, there is no need for the plasma to be fresh.

Summary of spin-time and -force variations Generally, the Centrifree® units show greater susceptibility to spin-time and -force variations than Amicon Ultra units. This is attributed to the design of the unit, with Centrifree®'s membrane being perpendicular to the axis of the unit, and Amicon Ultra's being parallel. Even though centrifuge 1, with a fixed angle, was used for Centrifree® in the experiment, which is supposed to decrease macromolecule-buildup on the membrane, it still seems to be an issue.

It was somewhat expected, that the *Fp* would *increase* with spin-force: If small protein-tracer-complexes were forced enough, perhaps the holes in the membrane would not be able to retain them in spite of their MW or perhaps the drug-protein-complex would be affected by the applied force in such a way, that it would dissociate. As the spin-force variations show no upwards trend, the membrane shows the ability to retain the complexes in spite of being applied a higher force than recommended.[16] It does not either seem like the complexes are dissociated by the force.

An important issue is, that the two units do not measure the same. This was expected, as the MWCO of the units do not match. The Centrifree® units seem to measure *Fp*-values comparable to the values obtained by ED – this is unfortunate, as this unit shows the most susceptibility to spin-time and -force variations.

UC validation experiment 2: Pretreatment – freshness of plasma
The refrigerated sample of FoP1 (Figure 23a on page 76) gave twice as high *Fp* as the fresh and frozen samples. The experiment was repeated in FoP3, and the refrigerated sample gave almost half the *Fp* of the fresh sample. FoP3 neither verifies or falsifies the results of FoP1 even though the experiments were conducted in almost the exact same manner.

In FoP2, the first six hours of refrigerating the sample was investigated to clarify the increase in *Fp* between the fresh and refrigerated sample of FoP1, but the increase was not observed, and FoP2 cannot explain, verify or falsify FoP1 or FoP3. Although the experimental circumstances were controlled, the results are inconsistent.

From this, it is concluded, that another effect must be present. Perhaps

[16]For Centrifree®, the recommended applied force is maximum 2,000 rcf, and the maximum applied force in the experiment was 6,000 rcf.

the addition of dichlorvos B is more sensitive than expected. The esterase inhibitor may need time to work before addition of the radio-labeled compound. This effect can eliminated by using a non-ester radio-labeled compound, as it is done in the fourth FoP-experiment.

FoP4 was conducted with [^{3}H]-MDL100907, leaving no need for dichlorvos. The results seem valid and conclusive; the value of Fp does not change with different pre-treatment of the plasma. There might be a slight drop in Fp of the frozen sample, but as plasma from test-persons is rarely or never frozen, this is not of importance.

The [^{3}H]-MDL100907-results are quite good and the [^{3}H]-SB207145-results are not. This is indicative of a bias caused by either the PET-tracer solution or the addition of dichlorvos. The results of FoP4 are in contrast to FoP1 and FoP3. The causes thereof are not easily deducted, but a few possible causes include:

Degradation of dichlorvos The dic B solution was produced on the first day of FoP1 and FoP3-experiments and used on the second day as well. If dichlorvos is not stable in H_2O, the esterase-activity would not be inhibited for the refrigerated and frozen samples. Should the compound degrade, more hydrophilic species will be present in the solution causing a rise in Fp. Experience from earlier scan-dates with [^{11}C]-SB207145, where addition of dic B was forgotten, tells, that Fp in these cases increase. This occurs for the refrigerated sample in FoP1, but not for the frozen, and it does not occur at all in FoP3, so degradation of dichlorvos cannot explain the data alone.

Addition of dichlorvos The addition of the dic B solution is not strictly controlled when it comes to "incubation-time" before addition of the PET-tracer. This may play a role.

Compound-specific pre-treatment effects The possible degradation by refrigerating and freezing the plasma may affect one compound more than the other. The simplistic view of the PET-tracer binding reversibly to proteins may be faulty. As seen in the "Interindividual variance"-experiment (Section 6.1.2 on page 71), the Fp does not change even though only a fourth of the protein content is left, and compound-specific pretreament effects do not seem like a valid explanation.

Reference sample When calculating the Fp measured by UC, the filtrates are compared to a "reference sample", an aliquot of the plasma of the plasma pool. Only a single reference sample is produced, leaving room for undetected random errors occuring when transferring the plasma, weighing the counting vial, adding the scintillation liquid and so forth. This error will however affect each of the FoP-samples in the same direction, and cannot account for the increase *and* decrease of Fp between fresh, refrigerated and frozen samples.

Choice of UC-unit FoP4 was performed with Centrifree® units instead of Amicon Ultra as FoP1/2/3 were, due to lack of Amicon Ultra units. If the change of UC-unit is to explain the different Fp-values, the individual Amicon Ultra units should measure very differently. The standard deviations of the Centrifree® and Amicon Ultra units are however comparable, and the change of UC-unit cannot explain the improved results of FoP4.

Summary of FoP The FoP-experiments with SB207145 are inconclusive, as the data is too incosistent. In FoP2, there are realtively many datapoints, but no increase in Fp is observed. Disregarding FoP1/3, there does

not seem to be any pretreatment effects on *Fp*.

UC validation experiment 3: Interindividual variance Figure 24a on page 75 shows the free fraction measured on dilutions of plasma. The hypothesis is, that the number of binding sites for the PET-tracer is enormours compared to number of PET-tracers. Therefore there should be no change of *Fp* when varying the content of albumin (e.g. diluting the plasma).

Firstly, it is mentioned, that the point at 1 % plasma content has high standard deviation, and is an outlier (Grubb's outlier test, $p<0.05$). This is caused by poor counting statistics,[17] and the point is disregarded. The remaining four points seem consistent. The SDs are acceptable, and the *Fp* is the same for every point. This indicates, that even with only 25 % of the normal protein content in plasma, the number of binding sites is still much higher than the number of PET-tracers.

The picture is a bit different when comparing data collected from different plasma samples, e.g. data from different test-persons. The plot in figure 24b shows data from 33 test-persons. The *Fp*-data were measured by ED, and was collected from the database of the research project. The albumin contents were collected from the hospital's clinical biochemistry department's database, LABKA. Unfortunately, no sample standard deviations or uncertainties were available, and the data can only be evaluated as it is. No trend seems evident, and a Pearson correlation test gives $r = 0.1359$ and $p = 0.4509$ indicating no correlation. The variation in *Fp* for different test-persons must be caused by some other phenomenon than difference in albumin content.

The variation may be caused by natural causes, that are not easily elucidated – or by poor measurements. Considering the degradation of dichlorvos

[17] The blind sample was 9 CPM and the ultracentrifugation filtrates from the 1 % sample were 9, 9 and 10 CPM respectively.

mentioned above, it seems more likely, that the interindividual variance is caused by poor analysis than by natural causes.

6.3.5 Degradation of dichlorvos

It was discovered late in the process, that dichlorvos is unstable in aqueous solutions with pH>2.6 and at pH=7.0, the half-life of dichlorvos is 99 minutes.[22]. As it was assumed that dichlorvos was stable, there has been no strict time-control of production of dichlorvos B-solution nor time-control of addition of dichlorvos B to plasma with SB207145, and the additions have not been logged. This compromises the results of any experiment including SB207145 in plasma, of which the pH=7.4.

The experiments affected are only from the plasma protein binding section in experiments using SB207145, and include:

- All values collected during scan-dates. The data has been used in the identity plot comparing methods, figure 21 on page 73.
- Spin-force and -time variations with [^{3}H]-SB207145, figures 22b and 22d on page 74.
- Pre-treatment experiments with [^{3}H]-SB207145 – FoP1, FoP2 and FoP3, figures 23a, 23b and 23c on page 76.
- Interindividual variance experiment, figure 24a on page 75.

Some experiments are affected more than others, and an extended discussion is needed for each:

Data collected on scan-dates Because the established protocol for SB207145-scan-dates avoids use of old dichlorvos B-solution, the data should not be affected. The solution is usually used within 3 hours,

and with the half-life of 99 minutes, there should still be more than one fourth of the dichlorvos left in the samples.

Scan-dates on which two SB207145-scans are performed could be affected. It is highly likely, that the dichlorvos B-solution produced in the morning for the first scan was used again in the afternoon for the second scan. If the solution was produced at 9 AM and used at 15 AM, it would have undergone 3.6 half-lifes, leaving 8.3 % of its original content. This may or may not still be enough to hinder the esterase activity. Among the data used for the *Fp* vs. albumin-content plot, there are two sets of data from test-persons being scanned two times in one day. Their *Fp*-results are presented in table 9 on page 88.

Table 9: *Fp*-values from scan-dates with one test-person getting scanned twice in one day. Scan 1 is usually around 10 a.m. and scan 2 is usually around 1 p.m.

Person	*Fp* for scan 1	*Fp* for scan 2
1	$20.8\% \pm 0.9\%$	$29.2\% \pm 2.2\%$
2	$30.0\% \pm 3.8\%$	$32.4\% \pm 15.1\%$

It is not statistically reasonable to make conclusions on this small a data set. However indication of scan 2' s results being higher than scan 1's. Especially when taking into consideration, that the triplicate results of person 2's second scan were 15.0 %, 41.2 % and 41.1 %, rendering the first result an outlier (Grubb's outlier test, $p<0.01$) and *Fp* of scan 2 significantly higher than *Fp* of scan 1.

It should be implemented in the research project's protocols, that dichlorvos B-solution should be produced fresh prior to any usage.

Spin-time and -force variations The spin-time and -force variations were conducted in one run spanning a couple of hours, so the results should not be affected.

Freshness of plasma experiments For FoP1, the dichlorvos B-solution was added to the plasma pool on the first day. The refrigerated sample gave a significantly higher value than the fresh sample, indicating that the dichlorvos had degraded overnight.

The frozen sample was not significantly different from the fresh sample indicating, that freezing preserves the dichlorvos.

For FoP2, all the samples were run within 6 hours. Should dichlorvos be degraded significantly within the time period, an increasing *Fp* should be observed – this was not the case.

For FoP3, dichlorvos B-solution was added immediately before the [^{3}H]-SB207145-solution. There was kept no log of production of dichlorvos B-solution, but having in mind, that lack of dichlorvos usually gives increased *Fp*-values, it seems, from the data, that the dichlorvos B-solution was produced fresh on each day.

Interindividual variance experiments (IIV) The IIV-experiment was conducted in the same run as spin-time and -force variations on the same plasma, ie. within a few hours. The *Fp*-results should not be affected by dichlorvos degradation.

Implications of dichlorvos degradation The previous results of *Fp* of second scan on scan-dates with two SB207145-scans cannot be relied on. The protocol should be updated to make sure dichlorvos B-solution is always freshly prepared.

The spin-time and -force-variations conducted with SB207145 should be treated with consideration.

The freshness of plasma experiments, FoP1 is invalid. FoP2 and FoP3 are valid, and the results can be used.

The IIV-experiment is valid. "Second scan *Fp*'s" collected data from the research project's database should be omitted or, alternatively, a new *Fp* vs. albumin-content plot should be made with another PET-tracer to eliminate random error from dichlorvos addition.

6.4 Conclusion

Ultracentrifugation assays were developed, and the results obtained was compared to the results of ED. Great concordance were found between Centrifree®-units and ED in the range $Fp = 0 - 10\%$. For *Fp* higher than 10 % the results are still in accordance, but not as well as in the low range. The Amicon Ultra units, that were only used in a few scans, did not show concordance at all. The correlation between results obtained by Centrifree® and by ED is statistically significant on a 95 % CI.

The Centrifree®-units unfortunately showed susceptibility to change in spin-time and -force, and keeping experimental conditions constant is of importance. The repeatability of the method is acceptable and comparable to the repeatability of ED.

Measuring *Fp* with Centrifree® is a lot faster and easier than measruing with ED, and the methods can be substituted with proper consideration and validation.

7 Conclusion and perspectives

This project was conducted in almost the opposite order of other projects. In order to collect data from scans with [^{11}C]-, [^{18}F]- and [^{123}I]-labeled tracers, it was necessary to begin collecting data even before the methods were validated. The methods were then evaluated, developed and validated on the side.

The lipophilicity-data obtained from different methods was not in accordance, and as the shake-flask method is assumed to be the correct measure, it is concluded that the HPLC column/eluent-system used is not suitable for the measurement of logD.

The PPB-data obtained with Centrifree®-units is in accordance with data obtained with ED, and the methods can be substitued.

The fact, that dichlorvos degrades spontaneously in aqueous solutions with pH>2.6 was discovered quite late in the project process, and for this reason new and improved experiments were not conducted. An improved repetition of the experiments, that include dichlorvos, is desirable.

Collecting *Fp*-data from the database from scans with other PET-tracers than SB207145 could clarify whether the interindividual variance in *Fp* is caused by different test-persons or by the analysis being susceptible to the dichlorvos-addition.

It is now considered by the responsible employee at the research unit to substitute the dialysis buffer for ED with protein free plasma produced by UC.

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References

[1] F. H. P. van Velden, R. W. Kloet, B. N. M. van Berckel, F. L Biuijs, G. Luurtsema, A. A. Lammertsma, and R. Boellard. HRRT versus HR+ human brain PET studies: An interscanner test-retest study. *Journal of Nuclear Medicine*, 50:693–702, 2009.

[2] Corwin Hansch and Albert Leo. *Exploring QSAR – Fundamentals and Applications in Chemistry and Biology*, volume 1. ACS Professional Reference Book, 1995.

[3] Christophe Plisson, Jonathan McConathy, Laurent Martarello, Eugene J. Malveaux, V. M. Camp, Larry Williams, J. R. Votaw, and M. M. Goodman. Synthesis, radiosynthesis and biological evaluation of carbon-11 and iodine-123 labeled 2ß-carbomethoxy-3ß-[4'-((*Z*)-2-haloethenyl)phenyl]tropanes: Candidate radioligands for in vivo imaging of the serotonin transporter. *Journal of Medicinal Chemistry*, 47:1122–1135, 2004.

[4] Alan A. Wilson, Li Jin, Armando Garcia, Jean N. DaSilva, and Sylvain Houle. An admonition when measuring the lipophilicity of radiotracers using counting techniques. *Applied Radiation and Isotopes*, 54:203–208, 2001.

[5] Alan A Wilson, Nathalie Ginovart, Mark Schmidt, Jeffery H. Meyer, Penny G. Threlkeld, and Sylvain Houle. Novel radiotracers for imaging the serotonin transporter by positron emission tomography: Synthesis, radiosynthesis and in vitro and ex vivo evaluation of ^{11}C-labelled 2-(phenylthio)araalkylamines. *Journal of Medicinal Chemistry*, 43:3103–3110, 2000.

[6] Alan A. Wilson and Sylvain Houle. Radiosynthesis of carbon-11 labelled n-methyl-2-(arylthio)benzylamines: Potential radiotracers for the serotonin reuptake receptor. *Journal of Labelled Compounds and Radiopharmaceuticals*, 42:1277–1288, 1999.

[7] Stephen F. Donovan and Mark C. Pescatore. Method for measuring the logarithm of the octanolwater partition coefficient by using short octadecylpoly(vinyl alcohol) high-performance liquid chromatography columns. *Journal of Chromatography, A*, 952:47–61, 2002.

[8] Nellie Gulyaeva, Alexander Zaslavsky, Pamela Lechner, Michael Chlenov, Arnon Chait, and Boris Zaslavsky. Relative hydrophobicity and lipophilicity of b-blockers and related compounds as measured by aqueous two-phase partitioning, octanolbuffer partitioning, and HPLC. *European Journal of Pharmaceutical Sciences*, 17:81–93, 2002.

[9] Nellie Gulyaeva, Alexander Zaslavsky, Pamela Lechner, Michael Chlenov, Oliver McConnell, Arnon Chait, Victor Kipnis, and Boris Zaslavsky. Relative hydrophobicity and lipophilicity of drugs measured by aqueous two-phase partitioning, octanol-buffer partitioning and HPLC. a simple model for predicting blood-brain distribution. *European Journal of Medicinal Chemistry*, 38:391–396, 2003.

[10] Joachim D Krass, Bernd Jastorff, and Hans-Gottfried Genieser. Determination of lipophilicity by gradient elution high-performance liquid chromatography. *Analytical Chemistry*, 69:2575–2581, 1997.

[11] William J. Lambert, Latrelle A. Wright, and Joyce K. Stevens. Development of a preformulation lipophilicity screen utilizing a C-18-derivatized polystyrene-divinylbenzene high-performance liquid chro-

matography (HPLC) column. *Pharmaceutical Research*, 7:577–586, 1990.

[12] Franco Lombardo, Marina Y. Shalaeva, Karl A. Tupper, Feng Gao, and Michael H. Abraham. Elogp oct: A tool for lipophilicity determination in drug discovery. *Journal of Medicinal Chemistry*, 43:2922–2928, 2000.

[13] M. C. Pietrogrande, P. A. Borea, and G. L. Biagi. Lipophilicity measurement of benzodiazepine-receptor ligands by reversed-phase liquid chromatography. comparison between high-performance liquid and thin-layer chromatography. *Journal of Chromatography*, 447:404–409, 1988.

[14] Klára Valkó, Chris Bevan, and Derek Reynolds. Chromatographic hydrophobicity index by fast-gradient RP-HPLC: A high-throughput alternative to log P/log D. *Analytical Chemistry*, 69:2022–2029, 1997.

[15] Chisako Yamagami, Kozue Araki, Kyoko Ohnishi, Kaoru Hanasato, Haruko Inaba, Masahiro Aono, and Akihiro Ohta. Measurement and prediction of hydrophobicity parameters for highly lipophilic compounds: application of the HPLC column-switching technique to measurement of logP of diarylpyrazines. *Journal of Pharmaceutical Sciences*, 88:1299–1304, 1999.

[16] Jørgen Gomme. *Isotopteknik 1 – Radioaktive isotoper og ioniserende stråling i Biologi og Medicin.* GAD, 1997.

[17] OECD guideline for testing of chemicals: Partition coefficient (n-octanol/water), high performance liquid chromatography (HPLC) method, March 1989.

[18] OECD guideline for the testing of chemicals: Partition coefficient (n-octanol/water): Shake flask method, July 1995.

[19] Matthias M. Herth, Vasko Kramer, Markus Piel, Mikael Palner, Patrick J. Riss, Gitte M. Knudsen, and Frank Rösch. Synthesis and in vitro affinities of various MDL 100907 derivatives as potential ^{18}F-radioligands for 5-HT2A receptor imaging with PET. *Bioorganic & Medicinal Chemistry*, 17:2989–3002, 2009.

[20] S. S Zoghbi, R. M. Baldwin, J. Seibyl, D. S. Charney, and R. B. Innis. A radiotracer technique for determining apparent pK_a of receptor-binding ligands. In *XIIth International Symposium on Radiopharmaceutical Chemistry – Abstracts and Programme*, pages 136–138, 1997.

[21] Kuo-Shyan Lin, Manik L. Debnath, Chester A. Mathis, and William E. Klunk. Synthesis and ß-amyloid binding properties of rhenium 2-phenylbenzothiazoles. *Bioorganic & Medicinal Chemistry Letters*, 19:2258–2262, 2009.

[22] F. Benoit-Marqui, C. de Montety, V. Gilard, R. Martino, M. T. Maurette, and M. Malet-Martino. Dichlorvos degradation studied by 31-P-NMR. *Environmental Chemistry Letters*, 2:93–97, 2004.

A Appendix

In the appendix you will find information that was deemed unnecessary in the thesis itself, but may still have relevance to the curious reader.

A.1 Development of HPLC-method for lipophilicity measurements

The first approach was right out of the OECD guideline[17] with 75 % methanol in the mobile phase. The reference compounds (with $\log D_{pH7.4}$ in parenthesis) were acetanilide (1.0), phenol (1.5), benzene (2.1), benzophenone (3.2) and naphtalene (3.6). All compounds eluted within 8 minutes, and acetanilide and phenol coeluted because of too little retention.

Decreasing the methanol-concentration in the mobile phase by increments of 5 % showed separatation of acetanilide and phenol at 55 % methanol. The results however, were better at 50 % methanol, and this percentage was chosen for measurements. At 45 % methanol, the retention time of the most lipophilic compound was app. 60 min, which was considered too long.

After this, more reference compounds were included, see table 2 on page 35, and the calibration was found to be linear in the range $\log D_{pH7.4} = [1.5 : 3.6]$ with an $r^2 = 0.98$ and baseline separation of all compounds. If the range was increased to $\log D_{pH7.4} = [1.0 : 4.0]$ with acetanilide and diphenyl, the correlation coefficient decreased to $r^2 = 0.7788$, with the retention time of diphenyl exceeding 60 minutes. The former range was deemed sufficient for measurements of the potential PET-tracers.

The OECD suggests to use a maximum of 75 % methanol in the eluent. The best methanol-concentration was determined to be 50 % allowing all peaks to be separated, and the most lipophilic compound to be eluted within

one hour.

A C18-column of similar dimensions was also tested, but as it performed poorly, it was not chosen for analysis.

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