UPTEC X 05 045
SEP 2005
ISSN 1401-2138

PETER SANDBLAD

Biosensor characterisation of enantioselective drug-protein interactions

Master’s degree project

UPPSALA UNIVERSITET

Molecular Biotechnology Programme

Uppsala University School of Engineering

UPTEC X 05 045	**Date of issue 2005-09**

Author

Peter Sandblad

Title (English)

Biosensor characterisation of enantioselective drug-protein interactions

Title (Swedish)

Abstract

The interaction between purified enantiomers of chiral drugs and human serum proteins was used to validate the surface plasmon resonance-based biosensor technology for the analysis of enantionselective binding. Binding studies were performed on a Biacore S51 instrument and binding constants for the separate enantiomers were determined. The technique showed some variance in repeated experiments but the binding constant ratio between enantiomers was very consistent throughout the study.

Keywords

Biosensor, Biacore, SPR, drug-protein interaction, chiral, enantiomers, plasma proteins

Supervisors

Robert Arnell
Dept of surface biotechnology, Uppsala university, Sweden

Scientific reviewer

Assoc. Prof. Torgny Fornstedt
Dept of surface biotechnology, Uppsala university, Sweden

Project name	Sponsors **Biacore AB**
Language **English**	Security
ISSN 1401-2138	Classification
Supplementary bibliographical information	Pages **27**

Biology Education Centre Biomedical Center Husargatan 3 Uppsala
Box 592 S-75124 Uppsala Tel +46 (0)18 4710000 Fax +46 (0)18 555217

Biosensor characterisation of enantioselective drug-protein interactions

Peter Sandblad

Sammanfattning

Om man är ute en kall vinternatt och ska ta på sig sina handskar kan det vara lätt hänt att man i mörkret gör fel och försöker ta på sig vänsterhandsken på högerhanden. Man märker då att det inte fungerar speciellt bra. Problemet beror på att handskarna förhåller sig till varandra som spegelbild och objekt och bara passar på den hand de är sydda för. Precis samma sak händer ständigt i naturen. De flesta molekyler som finns i levande organismer är ganska komplexa och kan precis som våra händer förekomma i olika spegelbildsformer där den ena passar för vissa saker och den andra för andra saker. På samma sätt fungerar många läkemedel. En högerform av ett läkemedel kan ofta göra samma saker som vänsterformen, men i vissa fall har de helt olika egenskaper. Efter som det är ganska svårt att särskilja högerformen från vänsterformen använder man ofta båda två som läkemedel vid behandlingar. Den så kallade neurosedynskandalen i slutet på 1950-talet är ett exempel på ett sådant fall. Läkemedlet Neurosedyn skulle vara bra för att bota illamående hos gravida kvinnor, men det visade sig att det bara högerformen som kunde binda till receptorn och bota illamåendet. Vänsterformen visade sig å andra sidan kunna binda till helt andra molekyler i kroppen och det var detta som ledde till att 1000-tals missbildade barn föddes.

Sedan neurosedynskandalen har det gjorts mycket forskning om spegelbildsformer och nya tekniker har utvecklats för att kunna separera högerformen från vänsterformen. Under de senaste åren har fler och fler läkemedel utvecklats där det bara är det aktiva formen som ingår men fortfarande finns det många läkemedel med båda formerna på marknaden. För att kunna analysera och förutspå vilka egenskaper de olika formerna av ett läkemedel har, gjordes här ett försök där bindningsstyrkan mellan de olika formerna och mänskliga blodproteiner bestämdes. Detta gjordes med hjälp av en metod som använder optisk mätning för att studera bindning mellan olika substanser. Metoden är välkänd för bindningsstudier men har använts väldigt lite för analys av spegelbildsformer.

Examensarbete 20 p
Civiningenjörsprogrammet i molekylär bioteknik

Uppsala universitet, september 2005

Table of Contents

Introduction

Ever since the thalidomide tragedy in the end of the 1950s when thousands of children were born with birth defects, the concept of chirality has been well known. The tragedy was caused by the "evil mirror twin" of the sedative drug thalidomide, used for treatment of morning sickness for pregnant women[1]. There is awareness today that most chiral compounds have different binding properties and there is a trend towards developing single enantiomeric drugs, but still, the majority of all organic pharmaceuticals sold today are chiral substances and many of these are sold as a mixture of the two enantiomers. The reason to this is the difficulties in separating and analysing the separate enantiomers, but over the last decade improvement has been made in the area of chiral high performance liquid chromatography (chiral HPLC) and asymmetric synthesis of chiral compounds.

The analysis of enantiomeric differences is very important, but still very few studies have been made of characterising the different binding properties of chiral substances. This study uses a surface plasmon resonance (SPR)-based biosensor to study the binding between the different enantiomers of chiral drugs to human serum proteins. The aim of the study is to see whether a biosensor can detect the proposed small differences in binding and to fully characterise the binding between the drug substances in the used model system. Even though few studies of enantioselectivity have been made, numerous studies with drug-plasma protein binding have been reported over the years. Current used techniques for analysis of drug-plasma protein binding include capillary electrophoresis frontal analysis, frontal analysis HPLC, ultra filtration, calorimetry etc.[2].

Chirality

Chirality is a phenomenon present in most organic substances. An object is considered to be chiral if it has a mirror image witch is not superimposable with the original object. A common used example to describe chirality is to look at your hands, if you hold up your hands facing each other you easily see that they are mirror images of each other. If you then try to rotate your hands you will notice that it is impossible to superimpose them (make them look exactly the same, see Figure 1). Next, take two cups of coffee in your hands and hold them by their handles. The two cups will be three-dimensional mirror images of each other with the handles facing away from each other, but if you then rotate one cup 180° (without spilling any coffee) the two cups will be superimposable, or exact copies of each other.

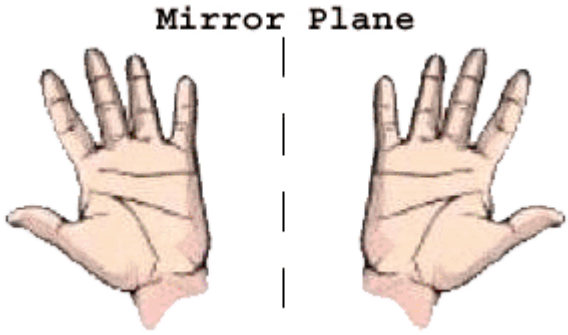


Figure 1. The hands are chiral because they are nonsuperimposable mirror images of each other.

The same discussion can be applied to molecules; a molecule with a nonsuperimposable mirror image is by definition chiral. In order for a molecule to be chiral its centre of chirality (normally a carbon atom) has to be bound to four different groups, if the atom has a double binding or if two of its bound groups are equal the molecules mirror image will always be superimposable and therefore achiral. The two different forms of a molecule are called

enantiomers and are normally labelled with the prefix R or S after the Latin words *rectus* and *sinister* meaning right and left.

In most cases chirality is of very little importance, the two enantiomers will have the same properties in energy, melting point, boiling point, solubility etc, and normally all chiral molecules will occur as mixtures of the two enantiomers, called racemic mixture or racemate. In some aspects the enantiomers will be different, which may have a significant biological importance. The different binding properties of enantiomers have been known since the mid 19th century when Louis Pasteur observed the different optical properties of different crystals of the same substance. During the following years many chiral substances were discovered by their different taste, it is now a well-known fact that many of our olfactory receptors can differentiate between enantiomers. In the end of the 19th century new guidelines for laboratory work appeared that made restrictions for using the mouth for chemical characterisations. When the tradition of tasting the chemicals as a part of the lab work disappeared, it became a lot harder to differentiate between enantiomers, which caused a major drawback for the area of research.

More than half a century later, in the end of the 1950s the research on chirality was again brought to public attention, when it was discovered that the birth defect of almost 10 000 children was caused by the nonactive enantiomer of the sedative drug thalidomide[1]. Since the thalidomide catastrophe, research on chirality has increased. Over the past years techniques have been developed, both for asymmetric syntheses of chiral compounds and separation on chiral stationary phase columns for HPLC systems.

Chirality in pharmaceuticals

Today many drugs available on the market are chiral compounds. More than 50 % of all pharmaceuticals prescribed worldwide are chiral, distributed as either racemic or enantiopure substances. For reasons mentioned above, enantiomeric differences are an important factor when developing new pharmaceuticals. The guidelines for production of chiral drugs from both FDA (U S Food and Drug Agency) and EMEA (European Medicine Agency) have been modified over the past 10 years as the separation and analysis methods have improved. In the recent updates of the guidelines it is stated that since enantiopurification and asymmetric synthesis now days is possible to scale up and that analysis methods are available, investigations of the separate enantiomers should be performed. In cases where enantiomers have different properties the enantiomers should be fully investigated separately except in cases of rapid isomerisation within the body. Furthermore in substances where only one enantiomer is active, the nonactive enantiomer is considered an impurity.[3 4]

The SPR technology

Surface plasmon resonance

The surface plasmon resonance (SPR) technology is a powerful method for monitoring interactions in real time between unlabeled molecules. It is widely known that when light travels from a medium of higher refractive index (e.g. glass) to a medium of lower refractive index (e.g. water) one part of the beam is reflected and the other part of the beam is refracted. Above a certain critical angle of incidence all light is reflected and no light will enter the lower refractive index medium. This is almost true, no light will penetrate the lower refractive index media but a small part of the energy from the light will generate an electric field into the media. This field, known as an evanescent field, is very weak and rapidly decaying with the distance. After about half a wavelength or ~300nm in the case with the laser used in the

Biacore, the field no longer effects its surrounding. If the boarder surface between the two different media is covered with a thin layer of noble metal, e.g. gold, the metal atoms will absorb energy and start to oscillate. These oscillating electrons are also known as plasmons. The degree of oscillation will depend on the angle of the incident light and at a certain critical angle the wave vector will match the wavelength of the oscillating plasmons, which will cause them to resonate. This critical angle is called the surface plasmon resonance angle or more commonly, the SPR angle. The SPR angle can be measured by a photodiode array detector and is seen as the angle that gives an intensity minimum in the reflected beam. Because the metal film is so thin the resonance frequency and therefore the SPR angle will be depend on the refractive index of the medium the evanescent field enters. By having a flow cell very proximal to the gold surface it is possible to detect changes in refractive index of the liquid in the flow cell. This is how the Biacore biosensor works, a ligand is immobilised on the gold surface and an analyte is flushed through the flow cell. If the analyte binds to the ligand, there will be a small change in concentration and therefore refractive index ($n_{water} \approx 1.0$; $n_{protein} \approx 1.3$), which will affect the resonance frequency of the gold surface plasmons and the SPR angle. The changes are very small but with the very sensitive detector that can detect changes down to 10^{-5} degrees it is possible to see very small changes in refractive index. A schematic view of the SPR technology is seen in Figure 2. Besides the refractive index, there are many parameters that affect the SPR angle; therefore it is important that the instrument has a very precise control of temperature and other physical parameters.

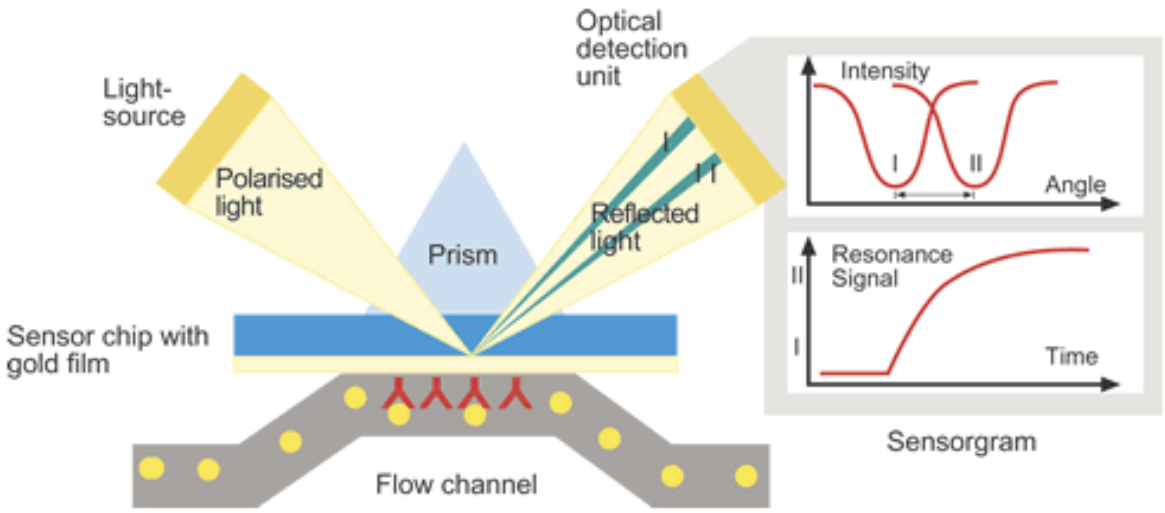


Figure 2. An overview of the SPR-technology, binding of analyte to the immobilised ligand in the flow cell causes a change in SPR angle, which can be monitored in a sensorgram. Figure used with permission from Biacore AB.

The Biacore instrument

The Biacore instrument consists of, most importantly, three essential parts; the IFC, the senor chip and the optical detector unit. The optical unit, as described above, focuses its laser on the gold film of a sensor chip. On the gold film of sensor chip there is a matrix, which is used for coupling ligands. Normally this matrix is carboxymethylated dextran (CM) but other chips are also available. The chip has a limited number of spots for immobilisation, when all spots have been used the chip is discarded and a new chip is inserted. The S-series sensor chip used for Biacore S51 has four spots available for immobilisation. When a chip is inserted to the instrument it is docked to the integrated µ-fluidic cartridge (IFC). The IFC consists of fluid channels and valves that control the buffer and sample flow. When the sensor chip is docked to the IFC a flow cell is formed and this is where the detection occurs. In Biacore S51 there

are two flow cells on the IFC/chip with three detection spots in each flow cell, see Figure 3. The flow cell has two fluid inlets and one fluid outlet. By varying the flow rates in the two fluid inlets, so called hydrodynamic addressing, it is possible to direct the ligand to a desired spot during immobilisation. In this way two different ligands can be immobilised in the same flow cell, thus doubling the screening capacity. The middle spot of the flow cell is always used as reference to subtract the unspecific binding from the response. By having the reference spot in the same flow cell there is no time delay between active and reference spot.

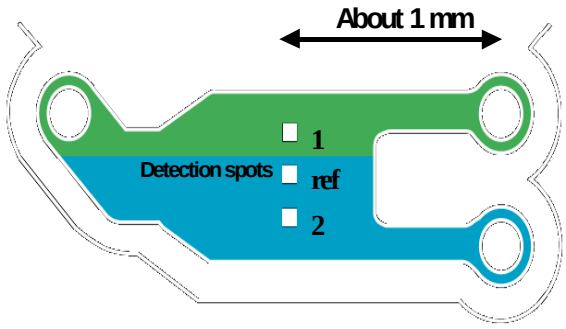


Figure 3. The flow cell in Biacore S51. Hydrodynamic addressing, in this case higher buffer flow in the lower flow cell inlet enables immobilisation on only one spot. Figure used with permission from Biacore AB.

Biological applications

The SPR technology has many advantages over other current used techniques, e.g. the possibility to monitor biomolecular interactions in real time, the possibility to study unlabeled ligands and analytes and the very small amounts of sample needed for the analysis. Furthermore the Biacore biosensor is a fully automated system, which can be loaded with a 384-well plate for automated screenings

For normal interactions between ligand and analyte there is an association phase when analyte is injected over the active surface. During this phase the amount analyte bound to the surface increases until equilibrium is reached. When the injection ends the analyte starts to dissociate from the surface, depending on the binding properties the association and dissociation times needs to be optimised for each assay in order to get enough data for evaluation. If the ligand and analyte binds strongly, they will not dissociate to baseline during dissociation phase. In order to remove all bound analyte the surface must be regenerated before next injection. Injecting a regeneration solution with different pH or ionic strength than the buffer normally does this.

In order to determine the steady state parameters, a compound characterisation is done a concentration series is made from the analyte in question. The series is made of concentrations ranging from below the dissociation constant (K_D) to at least 10-100 times K_D to give accurate and sufficient data for the evaluation of the interaction. When choosing concentrations it is also important that most curves reach equilibrium, steady state, during the association phase. Neglecting this criterion will give more uncertain constants of the steady state affinity. This can be a problem for low concentrations or if the association is very slow. It is also important to study the interaction with high analyte concentrations, especially when looking at weak affinity sites on the ligand. On the other hand, high concentrations might also cause problem by substantial carry-over between injection cycles, which one must bear in mind during analysis. Furthermore the concentration series should have rather many samples; more samples give more data and therefore better approximations of the affinity and kinetics

of the reaction. The concentration series should always contain blank-samples and repeated concentrations to ensure the robustness of the assay.

One problem with using optical biosensors is that different buffers and solvents have different refractive index and therefore gives rise to a bulk response. When a substance is dissolved directly in the running buffer there is no difference in bulk response between the buffer and the sample and the response is directly correlated to the analyte binding. When a substance is solved in DMSO or other solvents this is not completely true. No matter how precise the pipetting is done it is impossible to avoid small changes in solvent concentration between the samples and the running buffer and because of the sensitive detector this bulk refractive index will affect the binding results. To discriminate between the actual molecular interactions and bulk response a solvent correction needs to be done. This is performed by injecting a series of different solvent concentrations. From these injections a standard curve is made by plotting the relative responses (active spot minus reference spot) against the reference spot responses for the different solvent correction injections. By looking at the reference spot response of the injected samples it is then possible to correct the binding responses and generate a corrected binding curve, independent of solvent concentration variations.

Theory

When studying an interaction between two molecules it usually done with affinity or kinetic analysis. The affinity tells us how strong a binding is while the kinetics tells us how fast the reactions is. The affinity of a reaction is much easier to determine than the kinetics, and there are many ways to do this. Examples of methods for determining affinity include ELISA, RIA, and Western blot. From an affinity analysis it is possible to determine how strong an interaction is, normally at steady state, but tells nothing about how the reaction takes place. It is possible to decide the kinetics of an interaction with stopped flow or ELISA techniques by measuring the fluorescence or UV-absorption at multiple times, but this is only possible for some reactions with rate constants within a reasonable range. This is where the Biacore biosensors have a great advantage. Since detection is done in real-time and throughout the whole reaction it is possible to get a good overview of the reaction and see when equilibrium or steady state is reached. From the steady state responses it is possible to determine affinity constants for unlabeled reactants, which is not possible with most other techniques. Knowing the affinity constants gives some information about the interaction, but since the affinity constant is the ratio between the rate constants for association and dissociation, reaction with fast association and dissociation can have the exact same affinity constant as a reaction with slow association and dissociation. For this reason he additional information about the kinetics can be very important when looking at binding of drugs to receptors. Since the detection in the Biacore experiments is continuous with a detection rate of up to 10 Hz it is also possible to extract information about how fast interactants associate and dissociate.

When an analyte interacts with a ligand to form a complex it is usually expressed

$$L + A \leftrightarrow LA. \qquad (1)$$

If the rate constants for association and dissociation are defined as k_a and k_d respectively, the rate equations for the reactions are;

Association: $$\frac{d[LA]}{dt} = k_a \cdot [A] \cdot [L] \qquad (2)$$

Dissociation: $-\frac{d[LA]}{dt} = k_d \cdot [LA]$ (3)

The net rate equation of the complex formation is

$$\frac{d[LA]}{dt} = k_a \cdot [A_{free}] \cdot [L_{free}] - k_d \cdot [LA] \quad (4)$$

In the Biacore instrument flow cell there is a constant flow of analyte during the association phase, which means that the free analyte concentration always will be equal to the injected analyte concentration.

$$[A_{free}] = C \quad (5)$$

The free ligand concentration will depend on how much complex is formed.

$$[L_{free}] = [L_{tot}] - [LA] \quad (6)$$

The formation of a complex is what causes a slight different in refractive index in the flow cell and generating a response in the sensorgram. This response, hereafter mentioned R, is proportional to the bound analyte (or complex) concentration and is expressed in response units, RU. The total ligand concentration, L_{tot}, can also be expressed in RU as the maximum binding capacity of the surface, R_{max}. Combining this information with (4), (5) and (6) gives a new total rate equation where the concentrations are replaced with responses.

$$\frac{dR}{dt} = k_a \cdot C \cdot (R_{\max} - R) - k_d \cdot R \quad (7)$$

At equilibrium, or steady state, there is no net flow and is possible to define an equilibrium dissociation constant, K_D

$$K_D = \frac{k_d}{k_a} = \frac{[L_{free}] \cdot [A_{free}]}{[LA]} = \frac{C \cdot (R_{\max} - R)}{R} \quad (8)$$

Rearranging the expression gives the Langmuir response model[5 6]

$$R = \frac{C \cdot R_{\max}}{K_D + C} \quad (9)$$

This model can be used to calculate R_{max} and K_D values by plotting the binding response against analyte concentration. An initial value for R_{max} and K_D is guessed and a curve is fitted to the data points by iteration, using the Levenberg-Marqardt algorithm. In the case of enantioselective binding, the Langmuir model is not sufficient to describe the interaction with both enantioselective and nonenantioselective binding sites. In these cases a modified Bi-Langmuir model needs to be used. In the special case where only one enantiomer is interacting with the ligand the Bi-Langmuir model is:

$$R = \frac{C \cdot R_{\max}^{ac}}{K_D^{ac} + C} + \frac{C \cdot R_{\max}^{c}}{K_D^{c} + C} \qquad (10)$$

Superscript indices ac and c are for achiral and chiral interactions respectively. To determine the chiral parameters it is enough to look at the data from only that enantiomer, but in order to calculate the achiral parameters, data from both enantiomers should be fitted globally regarding the achiral site. It is also important to keep in mind that the difference between enantiomeric and racemic concentrations during the calculations. For a racemate in solution with a given concentration, the enantiomeric concentration is half that value.

Evaluation of results

The data from a Biacore analysis is shown in a sensorgram where the shift in SPR angle, expressed in response units (RU), are plotted against time. A typical sensorgram consists of a baseline, followed by association, dissociation and regeneration of the surface. However in this study where some of the reactants dissociate very easily, no regeneration is needed to obtain baseline dissociation. A normal sensorgram is shown in Figure 4. If a concentration series is injected over a surface, the sensorgrams obtained can be used to calculate affinity and kinetic parameters.

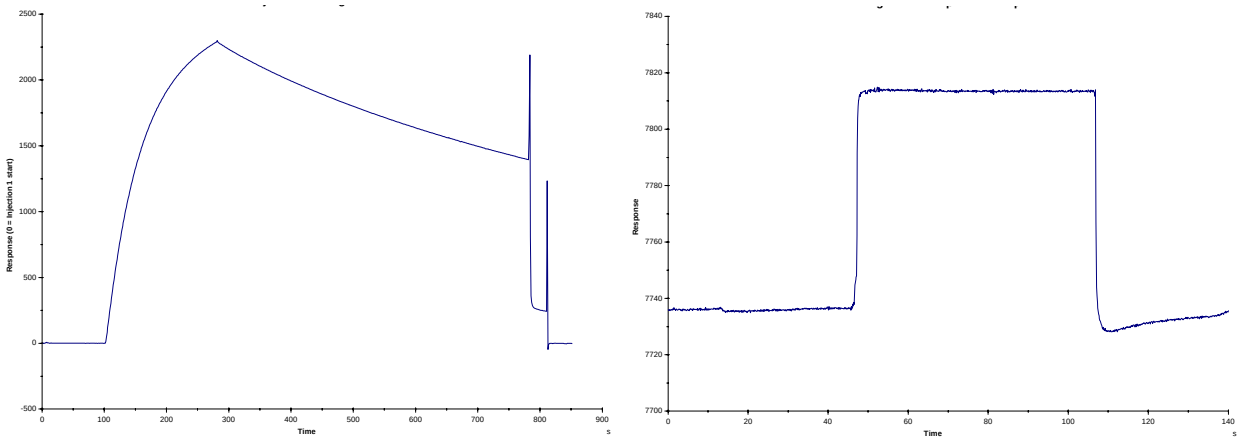


Figure 4. A normal sensorgram. The shift in SPR-angle on the y-axis is plotted in real time against time (x-axis). The left sensorgram shows first the baseline, then association phase until the end of the injection (the peak) when the analyte starts to dissociate. Strong interactions will not dissociate to baseline and require regeneration of the surface. The right sensorgram shows a faster association that reaches steady state instantaneous, the binding is also rather weak and dissociates to baseline without regeneration.

For a normal interaction between ligand and analyte, a simple Langmuir model is used to describe the interaction. The Langmuir model describes how one molecule binds to one other molecule with a constant association and dissociation rate. This model is normally sufficient to describe the system but in some cases a modified model needs to be used, e g when immobilising antibodies which have two binding sites or when immobilising a large amount of ligand which causes rebinding of analyte during dissociation. In this study the binding between low molecular drugs and serum proteins is studied. Previous studies have shown that plasma proteins such as human serum albumin (HSA) and α_1-acid glycoprotein (AGP) can discriminate between enantiomers in their high affinity binding site[7] but that they also have nonenantioselective interaction sites with much weaker affinity which contributes with unspecific binding. In this case the affinity data is fitted to a Bi-Langmuir model (eq 10) that

describes the binding of an analyte to a ligand with two independent binding sites. When using this model all enantioselective binding sites is approximated with one single affinity, and the nonselective ones with another.

DMSO

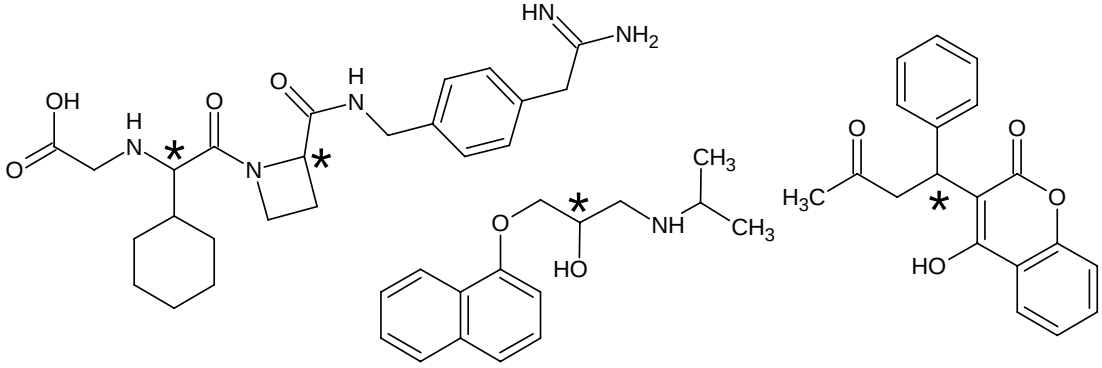
Dimethylsulfoxide, commonly mentioned as DMSO is a very powerful organic solvent. Its ability to solve a great variety of different substances is well known e.g. carbohydrates, peptides and polymers as well as gasses and inorganic salts[8]. DMSO has a unique ability to penetrate physiological barriers, both skin and inner body tissue membranes, and transport solutes over these. This ability gives DMSO a great possibility to act as an inner body distributor for pharmaceuticals, but it also tells us that DMSO must be handled with great caution, since some toxicological effects of high dose treatments has been observed. The pharmacological role of DMSO is under constant debate. It is approved for treatment in some countries for a very limited number of pathological conditions but many researchers and physicians have proposed its positive effect on a variety of diseases, including cancer. Because of its solving properties, the fact that it is water soluble and its relatively low toxicity DMSO is widely used in the pharmaceutical industry for storing drugs and during primary bioscreening. The Swedish medical products agency, MPA, is very sceptic against using DMSO in other than very low concentrations and for a very limited time. In cosmetics and hygiene products it is completely prohibited, in pharmaceuticals low concentrations can be accepted.

Substances

The ligands used are three proteins present in human serum, human serum albumin (HSA), α_1-acid glycoprotein (AGP) and thrombin. Human serum albumin is a 66 kDa protein present in blood serum. Albumin is the most common protein in our blood vessels, and because of its many hydrophobic pockets HSA is an excellent carrier of substances not soluble in water, such as fatty acids and hydrophobic drugs. Since HSA is so common, easy to purify and also essential for life it has been the target of many research studies and is today very well characterised[9]. AGP is an acute-phase protein synthesised mainly in the liver. As the name tells us it is highly glycosylated, which is the major cause to its very low pI (isoelectric point). The protein is a 40 kDa protein (24 kDa unglycosylated) with an pI of about 2.7 and is possibly the most important plasma protein for the distribution of basic drugs[10]. Thrombin is a blood protein activated by factor X as a signal for blood clothing. On activation, thrombin develops a serine protease activity that selectively cleaves the large protein fibrinogen and thereby causing it to form large stringy networks. Active thrombin is a 37 kDa protein produced in vivo from cleavage of prothrombin[11].

The analytes used in this study; warfarin, melagatran and propranolol are all pure enantiomers of commercially available drugs used in treatment, with molecular masses of 300-450 Da. Warfarin is an anticoagulant that has been used in many research studies over the years. Physiologically warfarin is a vitamin K antagonist, which interferes with the vitamin K-dependent production of numerous blood-clothing factors. It is also known as a very strong binder to a major binding site on human serum albumin. Warfarin is still used in medicine although not as much as it used to because of its reactivity to other drugs and difficulties in adjusting the dose. Melagatran is also an anticoagulant, but works in a slightly different way. It is a more direct working anticoagulant, which works late in the clothing pathway. This is done by blocking the active site of activated thrombin, and thus inhibiting thrombin from

passing the signal on to fibrinogen. It is much easier to administer in correct dose and is more inert to other drugs than warfarin. Melagatran is a new drug which should be much more effective than the current available anticoagulants, but it is yet not approved by the FDA. In Europe the substance is sold by AstraZeneca as Exanta. Melagatran has two asymmetrical centres, in this study the reported active form RS-melagatran and its enantiomer will be used, not the other diastereomers. Propranolol is in a class of drugs called beta-blockers. In the body it is a competitive antagonist, which blocks available beta-receptor sites. The drug is used to reduce high blood pressure and to treat irregularities in hart beat. The structure formulas of the used substances are seen in Figure 5 below.



Melagatran Propranolol Warfarin

Figure 5. Chemical structure of the substances used in study. Asymmetrical centres are marked with a star. Molecular weights of substances are 429.5, 295.81 and 308.3 Da respectively.

Materials and methods

Instruments

All Biacore experiments, immobilisations and compound characterisations, were performed on a Biacore S51 (Biacore AB, Uppsala, Sweden). The instrument is specialised in fast kinetics and low molecular compounds. Buffers were degassed prior to the instrument inlet by a connected Degasys Ultimate DU2010 (Uniflows Co Ltd, Japan). Every time a buffer was changed, the "change all solutions" method was used to flush and equilibrate all flow systems. The detector was normalized every day before any analysis by the "normalize detector" method using 70% (w/w) glycerol as recommended by Biacore AB.

Buffers

The buffer used for all immobilisations of AGP and HSA was 10 mM phosphate buffered saline (PBS) pH 7.4 made from PBS tablets (Sigma). The same buffer was used for compound characterisations except for substances not soluble in water, where a modified buffer with 5% DMSO was used. PBS concentration was still 10 mM and pH was adjusted to 7.4 with HCl to compensate for the DMSO addition.

For immobilisations and assays on thrombin, PBS-EP buffer was used (10 mM PBS, 3.4 mM EDTA, 0.05% P20, pH 7.4).

Immobilisations

All immobilisations were performed according to the Biacore handbooks using a suitable chemistry with reagents from Biacore immobilization kit. To avoid the risk of activity loss or contamination by previous substances a new flow cell was immobilised with protein for every compound characterisation (a full injection series of both enantiomers).

Human serum albumin (HSA) was immobilised using amine-coupling procedure. HSA (fatty acid free and essentially globulin free, Sigma) was dissolved in 20mM HEPES pH 7.4 to a stock solution of 1mg/ml; aliquots were stored at -20°C. Prior to injection HSA was diluted to ~15 μg/ml in 10 mM sodium acetate pH 5.0. Immobilization was performed using the immobilisation wizard, a research grade or certified grade CM5 sensor chip (S-series, Biacore AB, Uppsala, Sweden). Surface was activated by injecting a solution of 0.2 M N-ethyl-N'-dimethylaminopropyl-carbodiimide (EDC) and 50 mM N-hydroxysuccinimide (NHS). HSA was injected for 7 min with a 10 μl/min flow rate, the surface was then blocked by injecting 1.0 M ethanolamine-HCl pH 8.5. Finally the surface was washed with two BIAdesorb solutions, first 0.5% (w/v) sodium dodecyl sulphate and then 50 mM glycine pH 9.5. Immobilization levels ranging from 8 000 to 12 000 RU were accepted, no other immobilization levels were used for further experiments.

α-Acidic glycoprotein (AGP) was PDEA-modified and immobilised using surface thiol coupling. Before immobilization, AGP had to be PDEA-modified. 1mg human AGP (Sigma) and 3 mg 2-(2-pyridinyldithio)ethanamine (PDEA) (Biacore AB, Uppsala, Sweden) was dissolved in 1 ml 0.1 M MES-buffer pH 5.0. 50 μl EDC solution was added and mixture was

left to incubate for 10 min at room temperature before changing buffer to 10 mM citrate buffer pH 3.6 by size exclusion chromatography using an equilibrated NAP-10 column (prepacked sephadex G-25 column, Amersham biosciences, Uppsala, Sweden). Modified AGP in citrate buffer was stored in aliquots at -20°C; prior to injection thawed samples were diluted 1:2 with citrate buffer. Immobilization was performed on research grade or certified grade CM5 sensor chip (S-series, Biacore AB, Uppsala, Sweden). Surface was activated by injecting a solution of 0.2 M EDC and 50 mM NHS followed by 40 mM cystamine and 0.1 M DTE solutions. PDEA-modified AGP was injected for 7 min with a 10 µl/min flow rate. The surface was then blocked with 20 mM PDEA solution and then 1.0 M ethanolamine-HCl pH 8.5. The surface was then washed with BIAdesorb solutions 0.5% (w/v) sodium dodecyl sulphate and 50 mM glycine pH 9.5. Immobilization levels of 7 000-10 000 RU was accepted.

The immobilisation of thrombin was performed in two different ways, one for kinetic analysis and one for affinity analysis. To obtain optimal data for kinetics, it is favourable to have rather low immobilization levels. Thrombin from human plasma (Sigma) was dissolved in milliQ water, and desalted on NAP-5 (prepacked Sepharose G-25 column, Amersham biosciences, Uppsala, Sweden) to 0.15 M NaCl. Aliquots were stored at -20°C. Immobilisation was performed on both spots of a flow cell with amine coupling using the aim for immobilization level method. About 600 RU was immobilised on spot 1 and 2300 RU on spot 2. The coupling chemistry was apart from the level aimed for equal to the coupling of HSA with an additional regeneration with 50 mM NaOH after the ethanolamine-HCl blocking.

Sample preparation

Water-soluble substances were dissolved in running buffer PBS (or PBS-EP) to a concentration of 10 mM and were stored in aliquots at -20°C. These were R- and S-propranolol (Fluka 82065 and 82066 resp.) and RS- and SR-melagatran (kindly provided by AstraZeneca, Mölndal, Sweden). Directly prior to assay, samples were thawed and a concentration series was made by diluting the sample in running buffer to cover a suitable range. For assays on HSA and AGP concentration series from 1 µM to 1mM with about 30 different concentrations including blank and repeated samples was used. Kinetic assays on thrombin were run in concentrations ranging from 0.1 to 100 nM with about 20 different concentrations, whereas affinity runs where done with concentrations from 0.1 nM to 1 mM to cover both specific and non-specific bindings.

Substances with poor solubility in water, R- and S-warfarin (Chemoswede AB), were dissolved in 100% DMSO to a concentration of 20 mM and aliquots were stored at -20°C. For every assay thawed samples were diluted 20 times in 1.05 x PBS to get a 1 mM stock solution in 10 mM PBS and with 5% DMSO. The stock was then further diluted in DMSO running buffer to cover concentrations from 1 µM to 1mM in the same way as the water-soluble substances. Also propranolol was run on DMSO-buffer to determine the solvents effect on the binding. For this assay, propranolol enantiomers were prepared in the same way as warfarin

Compound Characterisation

All assays were performed using the compound characterisation wizard, injecting substances in strictly increasing concentration i.e. alternating the enantiomers so that both enantiomers of one concentration were run before the next concentration was injected. All compound characterisations were started with three start-up cycles (buffer injections).

Characterisations of water soluble substances was done at 30 µl/min flow rate by 60 s contact time, 30 s dissociation, wash, extra wash and regeneration with buffer followed by a carry-over control. Characterisations with DMSO-buffer were performed by 60 s contact time and 30 s dissociation time followed by wash with buffer, extra wash with 50% DMSO, regeneration with buffer and carry over control. In each DMSO assay a solvent correction cycle was done before and after the concentration series. Eight different DMSO concentrations in PBS running buffer ranging from 4.5% to 5.8% were injected.

In the study of melagatran on thrombin, the characterization had to be modified because of different association and dissociation rates. Assays were ran on 30 µl/min flow rate and were started with a surface conditioning of 3 times 30 s injection of 2 M $MgCl_2$ followed by three start-up cycles with buffer. Each cycle consisted of 233 s sample contact time, 420 s dissociation, wash and extra wash with buffer followed by 30 s regeneration with 2 M $MgCl_2$ to remove all bound analyte and finally a 180 s stabilization period before next cycle.

Evaluation

After each compound characterisation all sensorgram curves were inspected visually to detect abnormalities in the sensorgrams such as air spikes, baseline drift, irregular association or dissociation etc. All binding curves with deviant curve profile were excluded from further the analysis. For assays ran on DMSO buffer the sensorgrams were corrected for the DMSO bulk response. The corrections were made in the evaluation software according to the following technique; a standard curve was made by plotting the active minus reference spot responses for the eight different solvent correction injections against their reference spot bulk response. This curve should typically cover a range of roughly 1500 RU (from –500 to 1000) in bulk response. This standard curve is then used to correct all detection points in the sensorgram, the principle of solvent correction is seen in Figure 6. The compound characterization method in the evaluation software was then used to analyse the affinity and kinetics of the interaction. In the steady state affinity analysis the active spot minus reference spot response was used as raw data. All reference subtracted curves (or subtracted and corrected if originating from a DMSO assay) were then subtracted with the referenced response from the zero-concentration injections to obtain the “double referenced” data.

The double referenced binding responses were then fitted to a suitable model for the interaction (Langmuir or Bi-Langmuir depending on ligand and analyte) to determine the binding isotherms. The data from the report point table was also exported from the Biacore software and fitted in Matlab (Mathworks Inc., Natick, USA) to the same isotherm model using the same iterative algorithm, Levenberg-Marqardt modified Gauss-Newton algorithm, but ran 1000 times with different initial values. The algorithm was also slightly modified by fitting the values for the nonenantioselective binding site globally, which is not possible in Biaevaluation softwares. This extended analysis was an attempt to globalize the local fitting method in order to possibly obtain more accurate isotherms since it was believed that the enantiomeric differences would be rather small. In the Biacore evaluation software, binding responses were also fitted to a model including an extra offset parameter. This parameter compensates for poor referencing by not forcing the isotherm curve through origin. The different evaluation methods were compared to decide which one to use.

For the interaction between melagatran and thrombin it was also possible to do a kinetic evaluation since the binding was slow enough. This evaluation was performed on compound characterisations ran on low immobilisation levels as described above. Double referenced curves were fitted in Biaevaluation software.

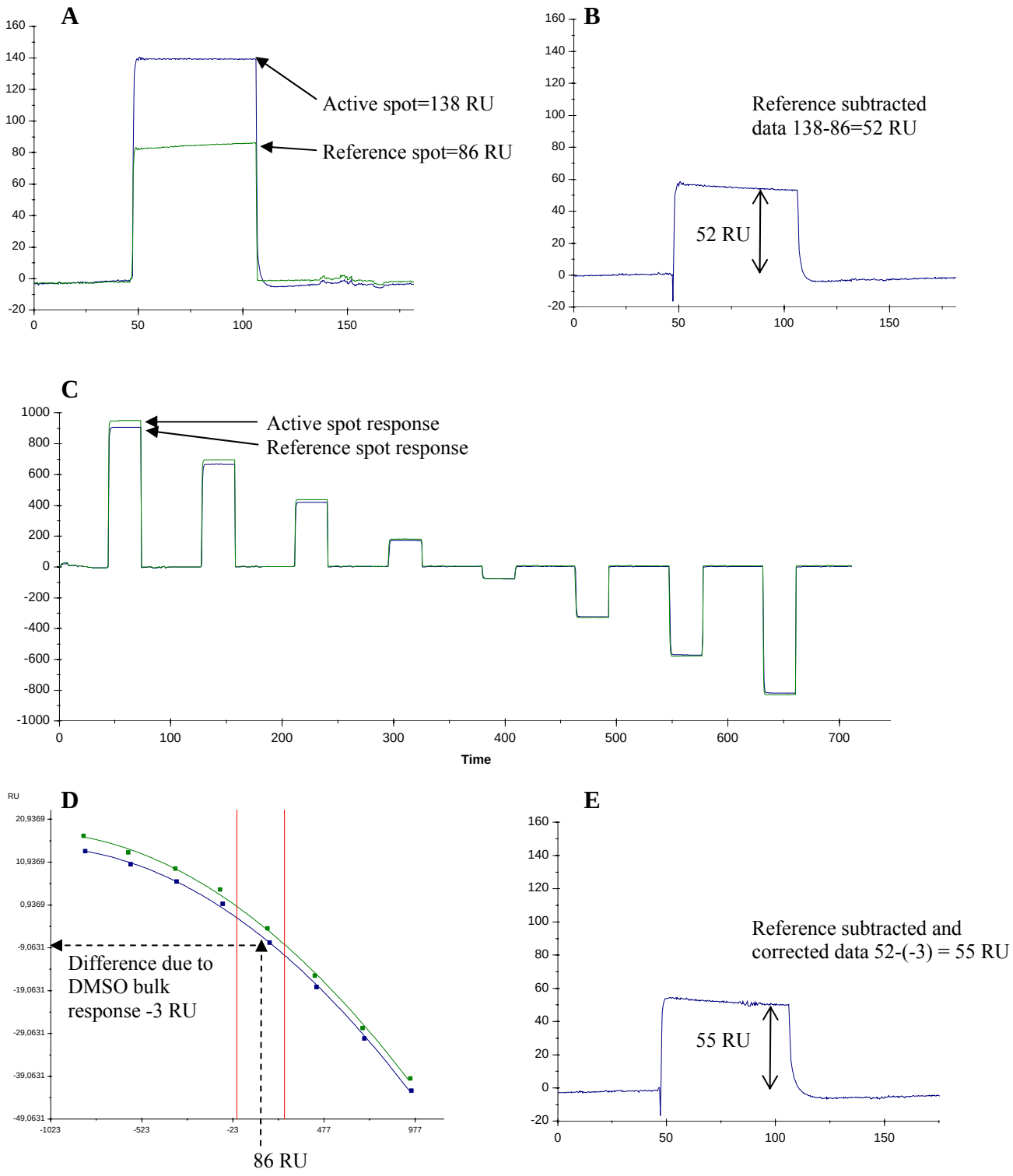


Figure 6. The principle of solvent correction. First reference-subtracted data is generated (A and B) by subtracting the reference spot response from the active spot response for the sample injection. (C) Different concentrations of solvent is injected and (D) a calibration curve is made by plotting the active - reference response against the reference spot response. The correction term is read on the y-axis by reading the calibration curve at the reference response of the sample injection. Red bars show the range of reference spot bulk responses for all samples. (E) Subtracting the solvent correction term from the reference-subtracted data gives the corrected data.

Results

Sensorgrams

Carry-over

Sensorgrams from all compound characterisations were inspected visually and deviating curves were excluded. Examples of an overlay plot of all cycles from a compound characterisation is seen in Figure 7. A carry-over control study was made by plotting carry-over responses against binding responses. Curves from DMSO assays showed a zero carry-over for analyte concentrations up to almost 100µM and increased to a maximum of 10% of the binding response on the highest injected analyte concentrations. For assays run on DMSO-free buffer, the carry over was still low at very low analyte concentration but increased to between 10% and 20% of the binding response for higher concentrations (>10µM). The difference between DMSO and non-DMSO buffers is explained by the additional washing step with 50% DMSO that was not possible in assays run on DMSO-free buffer. To minimize the consequences of carry-over, samples were injected in increasing concentration. To further investigate the carry-over effects, high and low sample concentrations of analyte were injected in replicates and showed very little difference in binding response (<2%) despite the observed carry-over.

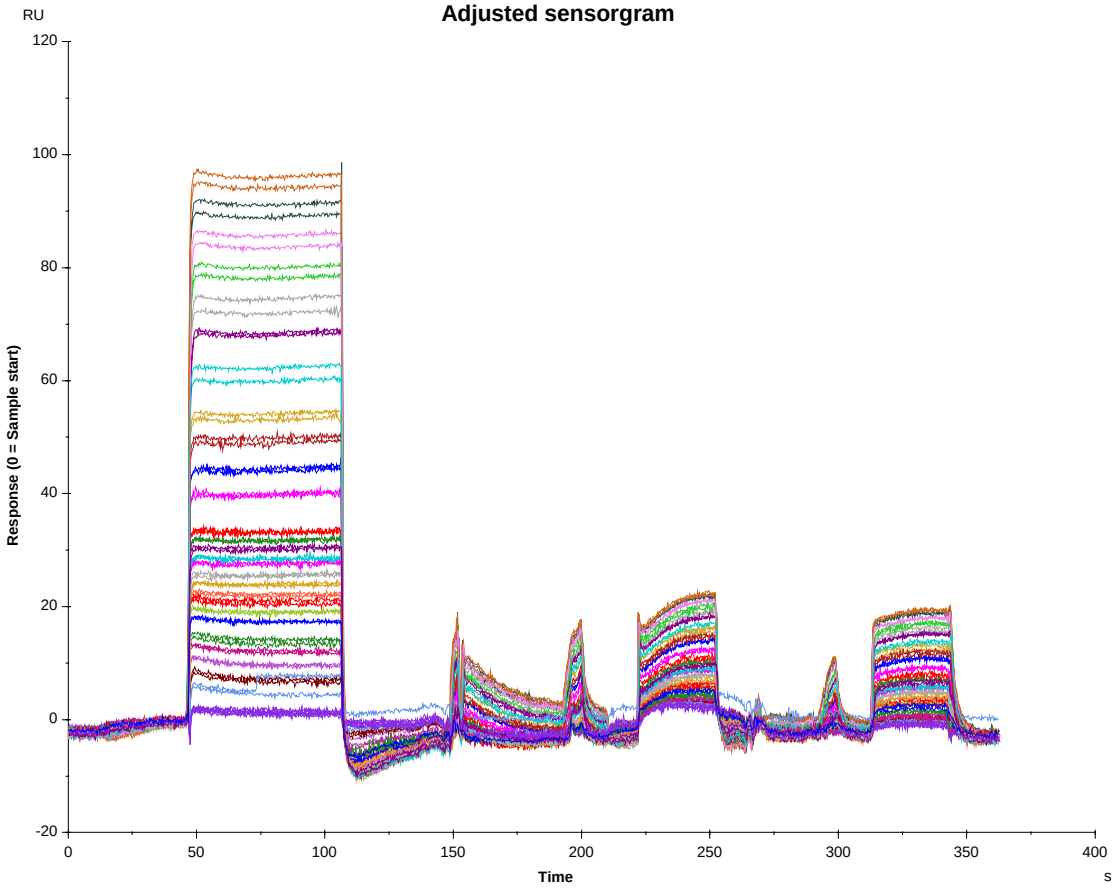


Figure 7. Overlay of the sensorgrams from a complete compound characterization showing the injections of both propranolol enantiomers on an AGP immobilised surface. Injection cycles are coloured by concentration. From left is seen association followed by several washing steps and to the far right a carry-over control.

Solvent correction

Calibration curves from solvent correction cycles with eight solutions between 4.5% and 5.8% DMSO typically ranged from –800 to 1000 RU in the reference flow cell. All samples from all compound characterisations were well within the desired area of rather linear calibration curve, ranging from –300 to 550 RU and with a response span of no more than 500 RU for any full concentration run containing about 60 samples.

Characterisations

Warfarin and propranolol screening

Propranolol and warfarin enantiomers were injected over both HSA and AGP surfaces to roughly determine binding levels. Both enantiomers of propranolol showed good binding for AGP but very low affinity for HSA. Warfarin is previously reported as a very strong binder to HSA, which was confirmed by the primary screening. It was also seen that warfarin had some affinity for AGP, with a high and a low affinity binding site. However the warfarin-AGP binding was weaker than the binding to HSA, and also had lower maximum response. This and the fact that the enantiomeric differences in warfarin-AGP binding seemed very small was the reason to why the full characterisations were focused to warfarin-HSA and propranolol-AGP binding.

Evaluation of evaluation method

Double referenced data (and solvent corrected if necessary), were fitted to the response isotherm equation models Bi-Langmuir and Bi-Langmuir with offset using the Biacore S51 and T100 evaluation softwares. Data was also fitted to a similar Bi-Langmuir model one thousand times in Matlab, starting each fit with different initial value in a reasonable range. Mean values and standard deviation of calculated parameters were compared, see Table 1, and showed that all methods were rather similar; but that Matlab as expected gave slightly lower standard deviations most of the time.

Propranolol					Warfarin			
Parameter	Method	Average	St Dev		Parameter	Method	Average	St Dev
K_D^R	S51eval	12,56	4,69		K_D^R	S51eval	3,66	0,56
K_D^R	T100	12,62	5,02		K_D^R	T100	3,68	0,56
K_D^R	Matlab	10,05	3,02		K_D^R	Matlab	2,94	0,40
K_D^S	S51eval	8,74	2,28		K_D^S	S51eval	1,67	0,34
K_D^S	T100	8,67	2,23		K_D^S	T100	1,66	0,35
K_D^S	Matlab	7,73	2,83		K_D^S	Matlab	1,39	0,33
K_D^R / K_D^S	S51eval	1,41	0,17		K_D^R / K_D^S	S51eval	2,20	0,22
K_D^R / K_D^S	T100	1,42	0,20		K_D^R / K_D^S	T100	2,24	0,26
K_D^R / K_D^S	Matlab	1,32	0,13		K_D^R / K_D^S	Matlab	2,15	0,20

Table 1. Average values and standard deviations for the different evaluation methods.

The K_D and R_{max} values for the unspecific binding, or low affinity sites, are not compared in the table since S51 evaluation software cannot calculate K_D values higher than half the maximum injected concentration. T100 evaluation software can calculate this parameter but comparing this to Matlab fitting gave at least a two-fold higher standard deviations. This could be a result of the different fitting algorithms with global fitting of the low affinity site in

Matlab and local fitting in T100 evaluation software. For the mentioned reasons, Matlab evaluation method was used for Bi-Langmuir affinity calculations. The calculations with offset parameter were the most deviating (data not shown), which can be explained primarily by bad fitting. The measured offset in curves was from below 1 RU to over 10 RU, which should be compared to the high affinity R_{max} of about 20 and 40 RU for propranolol and warfarin respectively. Another factor explaining the bad coherence is the fact that many unknown parameters and relatively small data sets by definition gives more uncertain values. Overall the additional offset parameter seemed to give higher values for K_D indicating weaker binding.

Warfarin and propranolol characterisation

Evaluation of binding curves from warfarin and propranolol show very high affinity for their respective ligand, with rapid association and dissociation. Baseline was obtained without regeneration for both analytes. The AGP baseline was very stable with a baseline drift of less than 1 RU per cycle; HSA was almost as stable showing a baseline drift of about 1-3 RU per cycle. R_{max} varied between different runs because of differences in immobilisation level, but differences were rather small. Values for R_{max} at the high affinity site varied less than 5% for propranolol-AGP and less than 15% for warfarin-HSA. The difference in R_{max} does however not affect the K_D determination. The dissociation constants for the analytes, both their respective enantioselective and their common nonenantioselective (K_D^1) binding site, are presented in Table 2. Values are average values of three complete concentrations series, standard deviations are shown in parenthesis.

Analyte	K_D^1 [mM]	K_D^R [µM]	K_D^S [µM]	K_D^R/K_D^S
Propranolol	1.17 (0.08)	10.05 (3.02)	7.73 (2.83)	1.32 (0.13)
Warfarin	0.86 (0.13)	2.94 (0.40)	1.39 (0.33)	2.15 (0.20)

Table 2. Characterisation parameters for propranolol and warfarin. Values represent the average values and standard deviations for triplicate runs of each substance.

The values for warfarin are rather similar to previously reported KD values determined by chromatography, which were 4.8 and 3.8 µM respectively for R- and S-warfarin[12]. The enantiomeric differences are small, with rather big variations between experiments, but the ratio of the enantioselective dissociation constants is very consistent. Figure 8 shows the fitted curves for propranolol and warfarin both the full concentration range and an enlargement of the lowest concentration area where the enantioselective binding occurs. The low affinity site is of less biological relevance than the high affinity site, since drug concentration never will come close to these high analyte concentrations in the blood vessels. Nevertheless it is important to decide the low affinity site dissociation constant to construct the Bi-Langmuir curve, and it also shows the tendencies for unspecific binding. In all curves, both for propranolol and warfarin, the S-enantiomer binds stronger than the R-enantiomer. In the low concentration graph it is very clear that the slope S-enantiomer curve is steeper than the other.

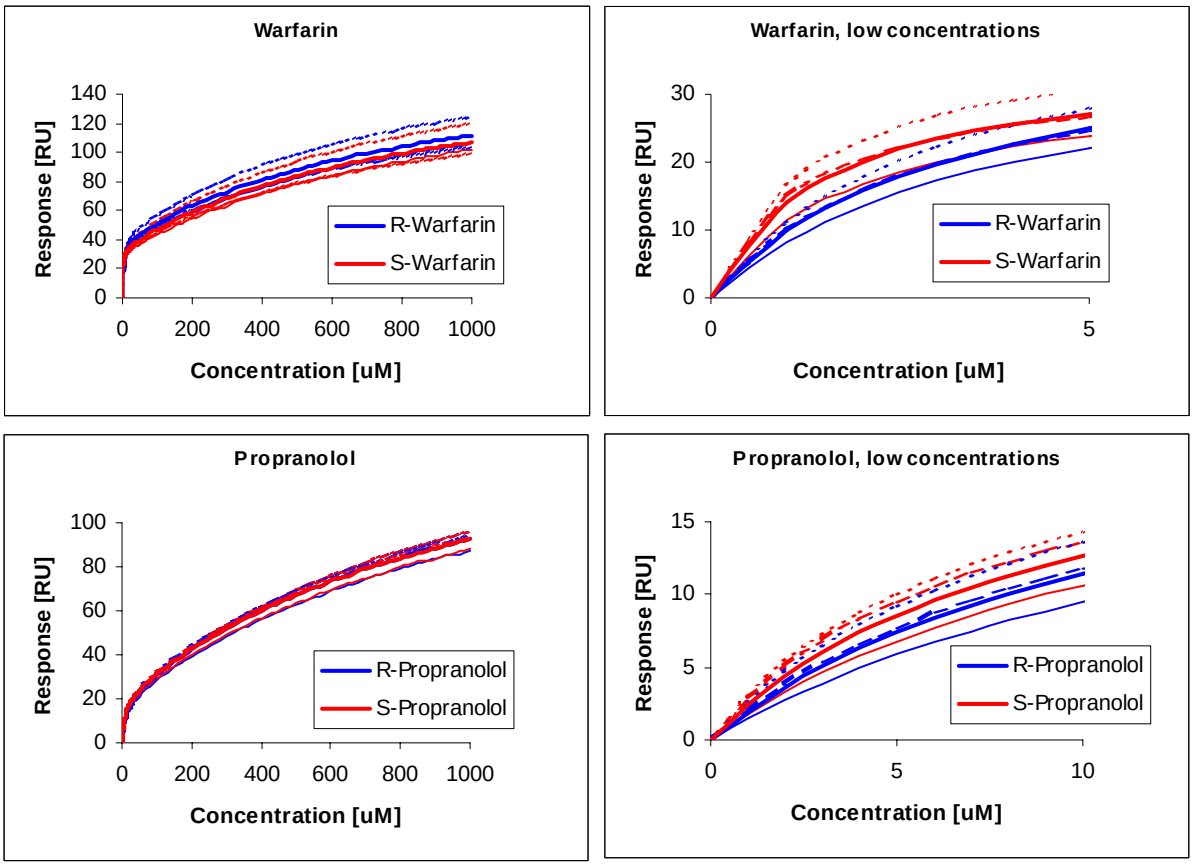


Figure 8. Bi-Langmuir curves of propranolol and warfarin showing full curves to the left and low concentration binding to the right. Normal, dashed and dotted lines are the three repeats of the study and the bold lines are average values.

DMSO effects

To determine what effects the solvent DMSO has on the interaction between analyte and ligand, propranolol was fully characterised with a DMSO assay. Table 3 shows differences in dissociation constants and it is obvious that the interaction is affected by the solvent. K_D values increase four times for both enantioselective sites and a factor two for the nonenantioselective. The propranolol-AGP binding is in other words clearly weakened by the solvent. The ratio between the enantioselective sites is however rather similar.

Assay	K_D^1 [mM]	K_D^R [µM]	K_D^S [µM]	K_D^R/K_D^S
PBS	1.17 (0.08)	10.05 (3.02)	7.73 (2.83)	1.32 (0.13)
PBS +5% DMSO	2.32 (0.79)	39.76 (12.3)	31.30 (7.4)	1.26 (0.10)

Table 3. The solvent effects on propranolol-AGP binding. Values represent average values and standard deviations of triplicate runs.

The calculated curves, using Bi-Langmuir fitting (eq 10), from the DMSO study are seen in Figure 9. Graphs display only the average values, and it is clear that the affinity is highly affected by the presence of DMSO at low concentrations but also to some degree at higher analyte concentrations.

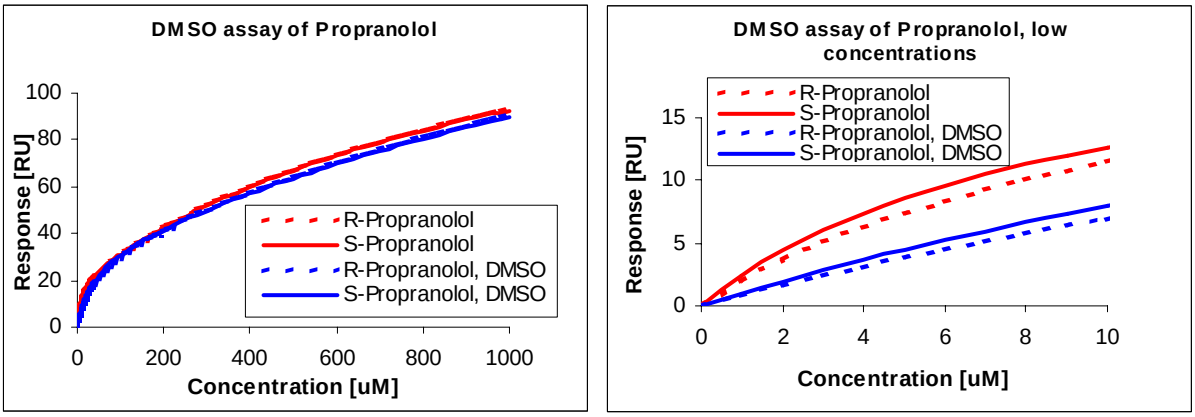


Figure 9. Overlay plot of average values from propranolol runs with (red lines) and without (blue lines) DMSO. The full concentration range is seen to the left and an enlargement of the low concentrations to the right.

Melagatran characterisation

Melagatran injected over immobilised thrombin showed that RS-melagatran (the reported active form and the enantiomer used as pharmaceutical) had very high affinity for thrombin and that the other enantiomer, SR-melagatran, did not bind at all, see Figure 10. The binding properties of melagatran enantiomers was also tested to serum proteins HSA and AGP. The study showed too weak binding levels for HSA to calculate any dissociation constants. Melagatran did however bind to AGP, the experiment was repeated once and the equilibrium dissociation constants were calculated to K_D^R=296 (26) µM and K_D^S=326 (36) µM respectively, standard deviations shown in parentheses.

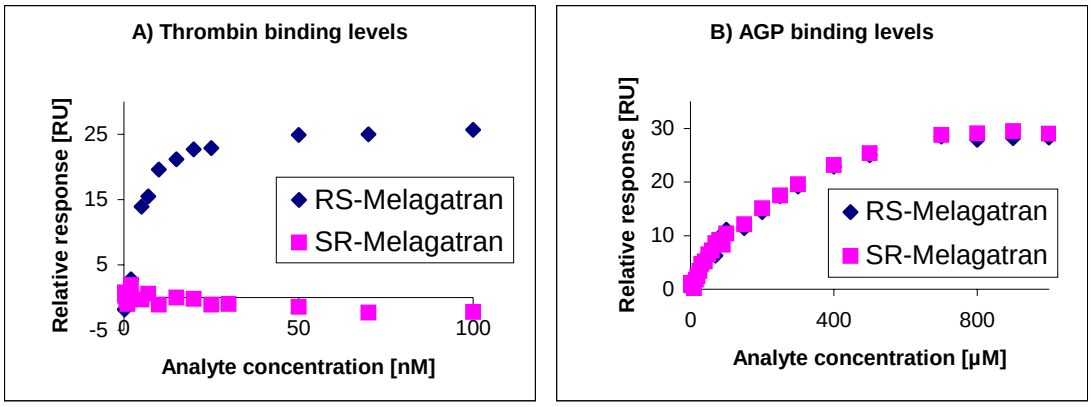


Figure 10. Relative binding responses for melagatran on thrombin (A) and AGP (B). Immobilisation levels for both ligands are ~7000 RU.

The enantioselectivity factor (K_D^S/K_D^R) gives an average value of 1.10 (0.03) for duplicate injections, which means that the both enantiomers have rather similar affinity to AGP. It is hard to say whether there is a significant enantioselectivity or not.

Full concentration series ranging from 0.1nM to 1mM of both enantiomers were injected on thrombin surface with rather low immobilisation level (~2400 RU). The binding responses are seen in Figure 11 and does not show any unspecific binding of RS-melagatran even on very high analyte injections. For SR-melagatran however some degree of unspecific binding is seen but only at very high analyte concentrations (K_D≈400µM). The R_{max} of both enantiomers (specific or non-specific) represent about 75% of the maximum theoretical binding capacity, assumed 1:1 binding.

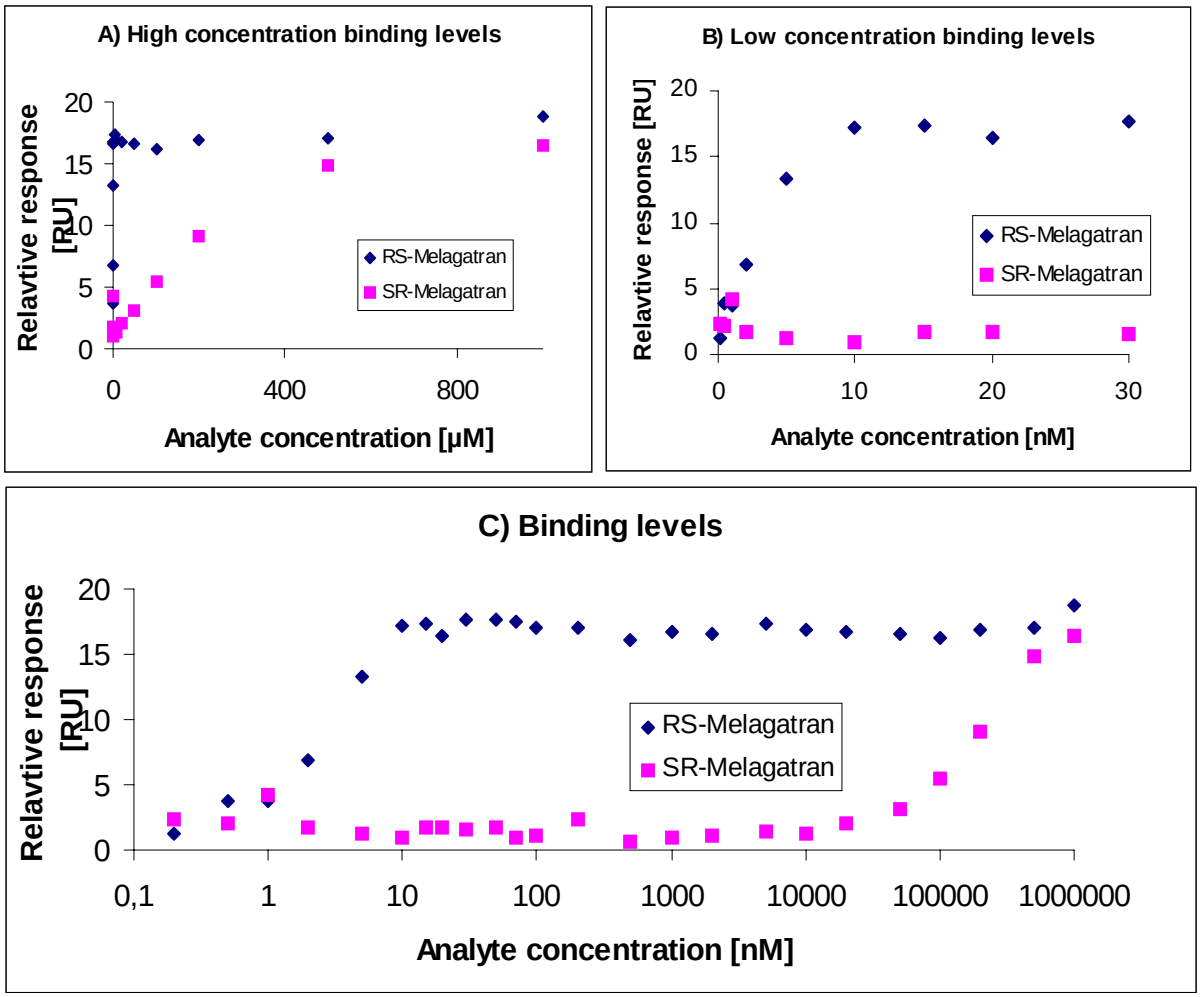


Figure 11. Binding of melagatran enantiomers to thrombin. (A) and (B) are the same figure with different scaled x-axis. (C) is plotted with logarithmic x-axis to give an overview of the 10^5 magnitude difference in binding between the enantiomers.

The determination of binding and kinetic parameters for melagatran-thrombin binding was as previously described[13] very hard to obtain. As seen in Figure 12 the binding curves are more or less straight lines indicating substantial mass transfer. This means that the association of melagatran to thrombin is not seen in the sensorgram. The straight lines represent the free diffusion of melagatran from the flow cell to a place very close to the binding site where the binding is very rapid. Because of the mass transport effect, the curve fitting to obtain kinetic rate constants is impossible. The sensorgram also shows that it is not possible to calculate the steady state dissociation constant since the curves do not reach steady state before saturation of the surface.

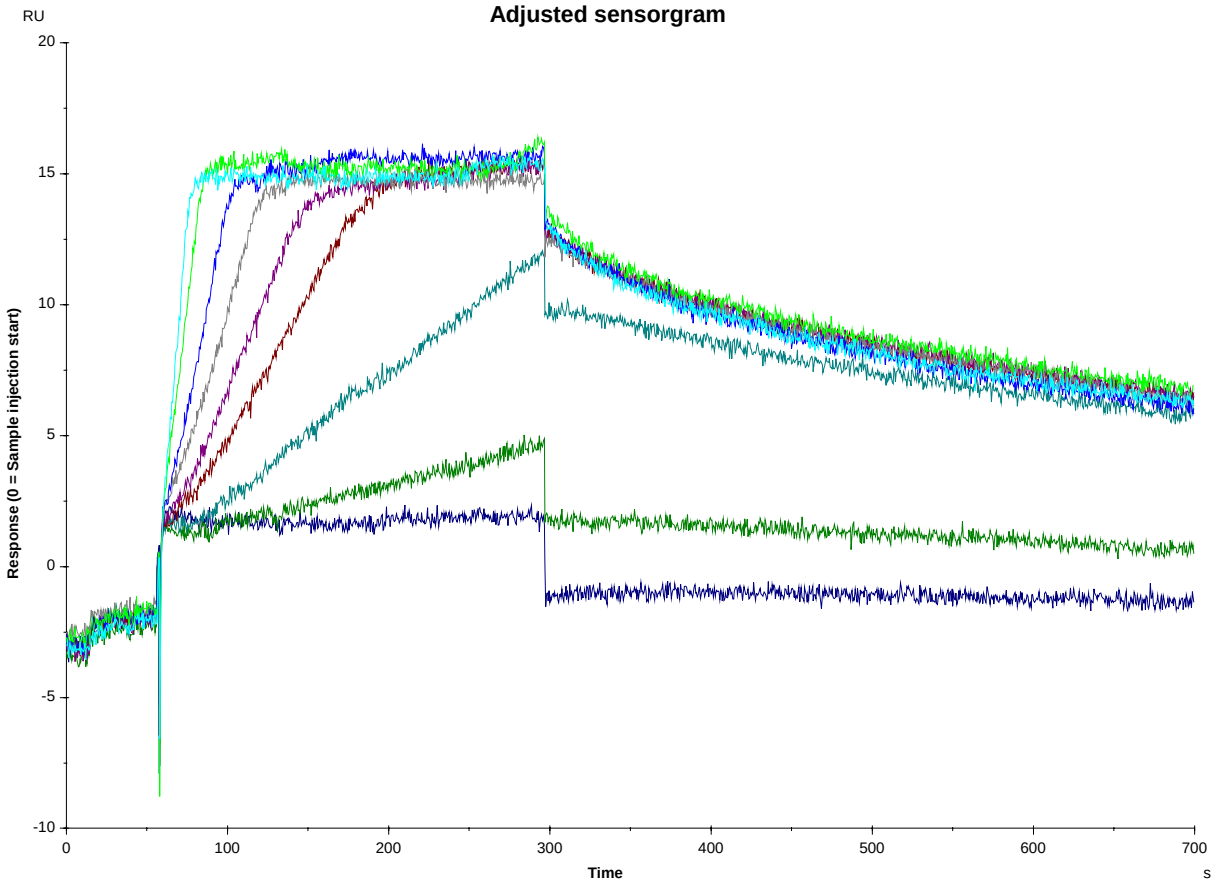


Figure 12. Injections of RS-melagatran on thrombin showing mostly mass transport. The "jumps" seen at the start and end of injection are characteristic bulk responses.

Similar problems were reported by Deinum and colleagues who also had rather poor curves and was unable to obtain reliable rate constants. However the equilibrium dissociation constant calculated from the ratio of the rate constants gave very similar values to those obtained from stopped-flow. Fitting curves within a reasonable concentration range to a mass transport kinetics model generated an average K_D of 0.77 nM.

Discussion

The choice of method for fitting the data can seem irrelevant since all methods gave very similar data, but since the differences in binding were very small the extended analysis should give better data. The evaluation software is designed for rapid analyses, and the globalisation of the method will take much longer time, which might not always be desirable. The other reason to why an external evaluation method was used was very specific for this characterisation. Fitting the high affinity site separately for the two enantiomers but the low affinity site together is besides chiral analysis never necessary and is therefore not possible in the user-friendly evaluation softwares. In this case it is preferable since the nonenantioselective site really becomes nonenantioselective by force.

The determination of dissociation constant was successful and the warfarin values were well in coherence with previous studies. The variation between experiments is rather big, so it is not possible to identify one enantiomer without also injecting the other as a reference. However, the ratio between the enantioselective dissociation constants, 1.3 and 2.2 for propranolol and warfarin respectively was very consistent.

The DMSO study of propranolol showed a massive decrease in binding strength for both enantiomers. This is very interesting, since many assays developed for drug protein interaction are run with DMSO-buffer for all substances whether they are water soluble or not. This is probably good for the comparison of different substances but evidently gives wrong values. This is of course always a problem since no interaction assay is run on serum but assays should be developed to mimic the natural *in vivo* situation as much as possible.

The determination of melagatran-thrombin interactions was not completely successful. The analysis is on the very edge of what the technology can handle, but it should be possible to reach steady state with lower immobilisation levels (maybe around 1000 RU) and longer injection times. It is clear however that the enantiomer to the active melagatran has very low affinity for thrombin. The determination of the high affinity site from kinetic mass transfer fitting gave a KD value of 0.77 nM which is very close to the previously reported values 1.1 nM. The previous study was run on DMSO buffer for the kinetic analysis.

Acknowledgements

This has been a really interesting spring, in which I have met many new people and learned a lot. For this I would like to thank Assoc Prof Torgny Fornstedt and Robert Arnell at the department for surface biotechnology. Thank you both for accepting me for this project and for always taking your time being helpful and friendly. I would also like to thank Björn Persson at Biacore for arranging all practical matters at Biacore and providing me with the help I needed with assay development and the interpretation of data. Finally, to the rest of the staff at the Department of Surface Biotechnology and Biacore AB, Thank You for your friendliness on both professional and other matters.

References

[1] Gordon and Goggin. Thalidomide and its derivatives: emerging from the wilderness. Review article, Postgraduate Medical Journal 79:127-132, 2003.

[2] Østergaard, Heegaard. Capillary electrophoresis frontal analysis: Principles and applications for the study of drug-Plasma protein binding. Review article, Electrophoresis 24, 2903-2913, 2003.

[3] FDA's Policy Statement For The Development Of New Stereoisomeric Drugs. FDA guidelines, http://www.fda.gov/cder/guidance/stereo.htm (14 Mar. 2005).

[4] Investigation of Chiral Active Substances. EMEA Clinical Guideline 3CC29a, http://dg3.eudra.org/F2/eudralex/vol-3/home.htm (23 May 2005).

[5] BIAtechnology Handbook. Biacore AB, 1998.

[6] Kinetic and Affinity Analysis with Biacore. BIAcore training courses, Biacore AB, 2003.

[7] Yang J, Hage D. Chiral separations in capillary electrophoresis using human serum albumin as a buffer additive. Analytical Chemistry, 66(17) 2719-2725, 1994.

8 Balakin, K V. DMSO solubility and bioscreening. Current drug discovery Aug, 2003.

[9] Goodsell, D. PDB molecule of the month. Protein data bank 2003, http://www.rcsb.org/pdb/molecules/pdb37_1.html (27 Apr. 2005).

[10] Götmar, G. Heterogeneous Adsorption in Chiral LC. Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy - 267, Acta universitatis Uppsaliensis, 2002.

[11] Goodsell, D. PDB molecule of the month, Protein data bank 2002, http://www.rcsb.org/pdb/molecules/pdb25_1.html (27 Apr. 2005).

[12] Hage D. High-performance affinity chromatography: a powerful tool for studying serum protein binding. Journal of Chromatography B, 768 3-30, 2002.

[13] Deinum J et. al. A Thermodynamic Characterization of the Binding of Thrombin Inhibitors to Human Thrombin, Combining Biosensor Technology, Stopped-Flow Spectrophotometry, and Microcalorimetry. Analytical Biochemistry 300, 152-162, 2002.