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Approach for Reliable Evaluation of Drug Proteins Interactions Using Surface Plasmon Resonance Technology

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The surface plasmon resonance (SPR) biosensor was recently introduced to the analytical biochemical society for measuring small drug−protein interactions. However, the technique has many times been used without specifying the type of enantiomeric form of the chiral drug measured and/or with using a too narrow drug concentration range resulting in biased values of binding coefficients and sometimes even assumptions about single-site bindings although the binding in reality comprises a multisite interaction. In this study we will give guidelines for reliable experimental and methodological approaches to avoid these pitfalls. For this purpose, we also introduce a new tool, based on physical chemistry, to the sensor community; the calculation of the adsorption energy distribution (AED). The AED-calculations reveal the degree of heterogeneity directly from the SPR raw data and thus guide us into a narrower selection of probable models before the rival model fitting procedure. We demonstrate how to measure reliable equilibrium data for the two typically different cases: drug binding to (i) transport (plasma) proteins and to (ii) a target protein. Both the binding of the chiral β-blocker propranolol to α_1-acid glycoprotein (AGP) and that of the anticoagulant warfarin to human serum albumin were heterogeneous, with a few strong enantioselective sites and many weak nonselective sites. We also demonstrate how the multisite binding rapidly falsely turns to single-site as the concentration range is narrowed and how adding dimethyl sulfoxide to the buffer affects multisite drug−protein data. The binding of the enantiomers of the thrombin inhibitor melagatran was investigated on both thrombin and the transport proteins, revealing clear enantioselectivity for thrombin in favor of the active enantiomer, but almost similar binding properties for both enantiomers to the transport protein AGP. The AED-calculations verified that both these system has a unimodal energy distribution and are best described with a homogeneous adsorption model.

The development of single-enantiomer pharmaceuticals has increased drastically over the past decades. Restricted guidelines have been prompted because of later great improvements in the area of enantiomeric separation and analysis by FDA and EMEA.[1,2] Both these guidelines state that enantiomers should if possible be analyzed separately. To determine the effects and side-effects of the separate enantiomers on various receptors and partitioning to transport proteins it is desirable to have a high-resolution method that can study these interactions under physiological conditions.

The use of surface plasmon resonance (SPR) biosensors has been validated for several drug−protein studies,[3−6] but the technique has rarely been used in enantioselective drug−protein studies.[7,8] There are a few SPR-based studies considering chirality but using special techniques.[9−12] In a recent study a HPLC-based method (the perturbation method) was compared with SPR in making a detailed characterization of alprenolol and propranolol enantiomers binding to cellulase protein Cel7a.[7]

Almost a hundred papers using SPR-technology have been published where binding constants have been presented for drug−protein interactions, without considering, or even mentioning, the chiral property of the drug.[5,6,13−18] Only in exceptional cases are enantiomeric[19] differences considered; during 2006 only one among 45 papers concerning small drugs considered enantiomeric differences (ref 7 in this paper). Many of the molecules

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studied have chiral properties, but in the SPR papers it is usually not stated if the analysis was performed on a single enantiomer or on a racemic mixture of both enantiomeric forms.

Furthermore, the drug concentration ranges are often too narrow which results in the conclusion of single-site binding although the interactions in reality are of multisite character.[20−23] In addition, too old-fashioned evaluation tools for nonlinear estimations of parameters are often used. One such method is the Scatchard plot, were only the Langmuir adsorption isotherm model has a linear Scatchard plot. However, if only very low concentrations are used this could lead to false assumptions of Langmuir adsorption isotherms.[22,23] If the dynamic range is increased, the "convincing" linearity is often lost.

In chromatography the combined use of adsorption energy distribution (AED) calculations and Scatchard plots has been most successful to reduce possible adsorption models before the data is fitted to different binding models by a standard nonlinear least-squares method.[24−27] In general a drug will not interact with a protein with only a distinct adsorption energy (K_D) as is assumed in the simple Langmuir adsorption isotherm model. Instead often several different adsorption sites are responsible (e.g., different types of interactions), where each site has a binding energy distribution. A more reasonable approach is therefore to measure the binding in a wide dynamic concentration range often several magnitudes above the therapeutic concentration level of the particular drug. It is then possible to make a complete census of all types of interactions, including those of strong or weak binding energies and low or high binding capacities.[24,25] After the adsorption isotherm acquisition, the AEDs can be calculated from the raw adsorption data of the binding study if the data has sufficient quality (low noise). The AED shows how many different sites are present and estimates each sites equilibrium constant and saturation capacity (amount). We will give a very brief description of the methodology; more detailed information can be found elsewhere.[25,26] The methodology mentioned above has been successfully used with HPLC-based systems for quite some time,[25−27] and should be suitable for SPR analysis as well.

A factor often neglected in binding studies is the effect of solvents. Most components used in the running buffer are naturally added to resemble in vivo conditions, but sometimes organic solvents and additives are used for practical reasons. Although this allows the analysis to be conveniently performed, the results may have little physiological relevance. Historically, mobile phases containing aqueous solvents mixed with organic polar components such as acetonitrile and 1-propanol have been used in combination with immobilized-protein HPLC columns. It is a well established fact that such solvents decrease retention and diminish hydrophobic interactions.[28] It has also been found that such solvents may affect the protein conformation.[29]

Another common solvent is dimethyl sulfoxide (DMSO). This solvent is added in the running buffer to increase drug solubility. Normally, between 2% and 5% is used to obtain an "all around" assay, even when the drug has high solubility in water. The effects DMSO has on the determined adsorption isotherm parameters are surprisingly seldom considered. The issue was addressed by Rich et al. for a single-site interaction, and it was deduced that "...the affinity of the warfarin/HSA interaction increased slightly as the DMSO concentration was increased to 10%...".[5] In their figure we can see the dissociation constant decreased from around 4.1 to around 2.8 when going from 0 to 5% DMSO (Figure 4B in ref 5 of this paper). This result is in strong contrast to Arnell et al.,[7] where 5% added DMSO decreased the binding of the small molecule onto an immobilized cellulase protein using an acetate buffer at pH 6. These contradictory results are reasons why the topic should be further studied.

In this study we use Biacore S51 SPR instrument, optimized for low-molecular compounds,[6] to measure typical chiral drug−protein interactions, in different affinity regions. The substances studied are proven binders to the serum proteins in question. Warfarin is a very well-characterized drug with high affinity to one of the main binding sites on HSA, called the "warfarin site".[30] The drug is an anticoagulant of which the *S* enantiomer has several times the pharmacological activity of the *R* enantiomer. Propranolol is a β-blocker, working as a competitive antagonist to β-receptor sites. Propranolol is administered as a racemate although the majority of its therapeutic activity is tied to the *S* form.[31] Melagatran is another anticoagulant and works as a direct thrombin inhibitor. The molecule has two stereoisomeric centers, but only one form (1*R*, 2*S*) is used clinically.[32]

The overall aim of this paper is to give guidelines on design of experiments, as well as processing and analyzing of the acquired data, to avoid the pitfalls described above. For this purpose, we want to investigate if the new approach recently introduced for HPLC combining AED-calculations with Scatchard plots can be adapted also for the sensor community, and more particular for SPR-raw data. By our new approach, it will be possible to guide the user to a few numbers of very trustworthy models before the rival model fitting procedure and the following statistical evaluation to deduce the best adsorption model. As experimental models we choose chiral drug−protein combinations to exemplify both typical strong and typical weak bindings. We will focus on the importance of addressing chirality correctly, to use a wide concentration range and thorough model discrimination. Finally, the effects of DMSO on chiral selective and nonselective AGP-propranolol interactions at conditions that mimic physiological conditions (PBS) will be studied. Although no general conclusions can be drawn from a single example, we hope to raise the awareness of this issue.

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THEORY

The SPR instrument measures changes in the refractive index at the surface in response units (RU), the results being proportional to the bound concentration of analyte. When a concentration series is injected, the affinity can be calculated by fitting the data to a suitable model. The binding theory is based on the Langmuir adsorption isotherm model,[27] which in SPR-terms can be transposed to

$$R = \frac{C \cdot R_{\max}}{K_D + C} \quad (1)$$

where R is the steady-state response, $R_{\max}$ is the maximum response (response at surface saturation), K_D is the equilibrium dissociation constant, and C is the concentration of the injected analyte. A two-site model is obtained by adding two Langmuirian terms. The resulting model, the bi-Langmuir model, has often been successfully used in chiral systems:[24,25,27]

$$R = \frac{C \cdot R_{\max}^{ns}}{K_D^{ns} + C} + \frac{C \cdot R_{\max}^{es}}{K_D^{es} + C} \quad (2)$$

Indices *ns* and *es* are for the cases "nonselective" and "enantioselective", respectively, since only the high-affinity site is believed to be enantioselective. In this, all nonspecific bindings are approximated with one K_D and $R_{\max}$, common to both enantiomers. To characterize the nonselective binding sites accurately, the data from both enantiomers has to be fitted simultaneously. This is done by finding the parameter vector $\bar{a} = (R_{\max}^{ns}, K_D^{ns}, R_{\max}^{es,S}, K_D^{es,S}, R_{\max}^{es,R}, K_D^{es,R})$ that minimizes the residual squared sum (RSS) of

$$\mathrm{RSS} = \sum_k \left[R_R(\bar{a}, c_{R,k}) - R_R^{\mathrm{meas}}(c_k)\right]^2 + \sum_k \left[R_S(\bar{a}, c_{S,k}) - R_S^{\mathrm{meas}}(c_k)\right]^2 \quad (3)$$

as described in detail by Arnell et al.[7] R_i^{meas} is the measured equilibrium response generated by injecting concentration C_k of the separate enantiomers. $R_i(\bar{a},c_k)$ is given by a binding model, here eqs 1 or 2.

After fitting to several different adsorption isotherm models, an F-test is performed to decide which one is preferable from a statistical point of view.[33] In this study only Langmuir and bi-Langmuir models are used; these are nested models because the bi-Langmuir contains all parameters present in the Langmuir model and two extra parameters. The Fisher ratio for comparing nested models is calculated as

$$F_{(p_2-p_1),(n-p_2),\alpha} = \frac{(n - p_2)}{(p_2 - p_1)} \frac{\mathrm{RSS1} - \mathrm{RSS2}}{\mathrm{RSS2}} \quad (4)$$

The F ratio is based on the RSS obtained, the number of data points, n, used, and the number of adsorption isotherm parameters, p, in the fitting with each model. The critical value F_c (at a given significance level) for the F can be found in statistical tables.[33] If the calculated ratio exceeds the critical value, then model 2 gives a significantly better fit than model 1.

After the data acquisition, the data are fitted to some kind of model to gain physical chemical information of the adsorption process. However, the process of selecting a proper model is seldom discussed or evaluated which is a pity since a wrong model that fits the data well will lead to false mechanistic conclusions. Therefore, we propose a road to ascertain the degree of heterogeneity before the model selection to reduce possible adsorption models. One such approach, based on physical chemistry, involves the calculation of the adsorption energy distribution (AED)[26] which we here intend to adapt to biosensor data. From the Arrhenius equation we know that the equilibrium constant has an exponential relationship to the adsorption energy. The homogeneous unimodal (single energy distribution) Langmuir model often used to estimate equilibrium constants, assumes that all interactions are energetically equal, in other words the energy distribution will be a Dirac function located at energy corresponding to K_D with an area of $R_{\max}$. The heterogeneous bi-Langmuir model will have two different energy sites located at energies corresponding to the two different sites' equilibrium constants with corresponding area $R_{\max}$ for each site. Other adsorption models could have a single tailing AEDs forward lower adsorption energy (Tóth) or forward higher energy (Langmuir−Freundlich). So the heterogeneity could originate from a unimodal unsymmetrical distribution or from several symmetrical or unsymmetrical distributions. If we now expand eq 1 to a continuous distribution of independent sites across a certain range of possibilities, we get this integral equation:

$$R(C) = \int_{K_{D,\min}}^{K_{D,\max}} f(\ln K_D)\, \theta(C, K_D)\, \mathrm{d}(\ln K_D) \quad (5)$$

where θ (C,K_D) is the local adsorption model and f $(\ln K_D)$ is the energy distribution in K_D-space. $K_{D,\min}$ and $K_{D,\max}$ are governed by $C_{\min}$ and $C_{\max}$, respectively, where $C_{\min}$ and $C_{\max}$ are the lowest and highest analyte concentrations measured in the adsorption isotherm.[26] In other words to estimate low energy sites (large K_D) we need high concentration adsorption data, and the high energy sites are measured with low concentration data. In this study we divide the energy space into d ln K_D thick slices and assume that the adsorption process is ideal in every energy slice. The local ideal model used in this study was the Langmuir model:

$$\theta(C, K_D) = \frac{C}{K_D + C} \quad (6)$$

The AED could be calculated using many different methods; in this study we use the expectancy maximization method.[26]

MATERIALS AND METHODS

Instrument. All Biacore experiments, immobilizations, and compound characterizations were performed on a Biacore S51 SPR Instrument (Biacore AB, Uppsala, Sweden) using series S sensor chip CM 5 (Biacore AB, Uppsala, Sweden). Buffers were degassed before the instrument inlet by a connected Degasys Ultimate DU2010 degasser (Uniflows Co. Ltd., Tokyo, Japan). The detector was normalized every day before any analysis was conducted, as recommended by Biacore AB.

Buffer. The buffer used for all AGP and HSA immobilizations was 10 mM phosphate-buffered saline (PBS), pH 7.4, made from PBS tablets (Sigma Aldrich, St. Louis, MO) and filtered through a 0.20 μm disposable NALGENE filter (Nalge Nunc, Rochester,

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NY). The same buffer was used for compound characterizations, except for substances not soluble in water, in which case a modified buffer containing 5% DMSO was used. The PBS concentration was still 10 mM, and the pH was adjusted to 7.4 with HCl to compensate for the DMSO addition. The buffer used for all thrombin assays was PBS-EP, which is a standard 10 mM PBS but with 3.4 mM EDTA and 0.05% polysorbat 20 (P20).

Immobilizations. A new flow cell was immobilized with protein for every compound characterization (a full injection series of both enantiomers). Immobilization of human serum albumin (HSA) and thrombin was performed using a standard amine-coupling procedure (NHS/EDC coupling), see ref 3 and α_1-Acid glycoprotein (AGP) was 2-(2-pyridinyldithio) ethanamine (PDEA) modified on a carboxyl group and immobilized using surface thiol coupling. The thiol coupling procedures is previously described by Löfås et al.[34] Immobilization levels ranging from 8,000 to 12,000 RU for HSA, 7,000−10,000 RU for AGP, and a much lower immobilization level aiming for 1500 RU for thrombin to enable kinetic measurements. All proteins were purchased from Sigma Aldrich, St. Louis, MO.

Sample Preparation. *R*- and *S*-propranolol (Sigma Aldrich, St. Louis, MO), as well as (*R*, *S*)- and (*S*, *R*)-melagatran (kindly provided by AstraZeneca AB), were dissolved and diluted in running buffer. Substances with poor solubility in water, *R*- and *S*-warfarin (Chemoswede AB), were dissolved in 100% DMSO and diluted in 1.05 × PBS to obtain a 1 mM stock solution in 10 mM PBS and with 5% DMSO. The stock was then further diluted in DMSO running buffer. Concentration series from 1 μM to 1 mM with 27 different concentrations, including blank and repeated samples, were used for compound characterization assays.

Compound Characterization. All assays were performed by injecting substances of strictly increasing concentration (i.e., alternating the enantiomers so that both enantiomers of a single concentration were run before the next concentration was injected). All compound characterizations began with three start-up cycles (buffer injections) to stabilize the baseline. Characterizations of water-soluble substances were performed with 60 s of contact time, 30 s of dissociation, washing, and extra washes with buffer followed by a carry-over control (a buffer injection to check for leftover substance in the flow system). Characterizations with DMSO buffer were performed with 60 s of contact time and 30 s of dissociation, followed by washing with buffer, extra washing with 50% DMSO, regeneration with buffer, and carry-over control. Solvent correction cycles were injected before and after the concentration series, by injecting eight different DMSO concentrations in PBS running buffer ranging from 4.5% to 5.8%. All experiments were carried out at 25 °C, at a flow rate of 30 μL/min.

Evaluation. After each compound characterization, all sensorgram curves were inspected visually to detect abnormalities such as air spikes, baseline drift, irregular association, or dissociation. All binding curves with deviant curve profiles were excluded from further analysis. For assays run on DMSO buffer, the sensorgrams were corrected for the DMSO bulk response by data subtraction from the calibration standard curve.[3] 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 from the warfarin-HSA and the propranolol-AGP studies were fitted to the single-site (eq 1) and two-site (eq 2) equations using the Levenberg−Marqardt algorithm, as implemented in Matlab (Mathworks Inc., Natick, MA).

Binding curves from melagatran assays were fitted, using the S51 evaluation software, to the kinetics model for thrombin binding and the steady-state, single-site Langmuir model for AGP binding.

HPLC Study. HPLC analyses were performed using an Agilent 1100 Chemstation equipped with binary pumps, autosampler, diode-array UV detector, and PC workstation (Agilent Technologies, Palo Alto, CA). The column used was a CHIRAL-AGP (100 × 4.0 mm) analytical column (Chromtech AB, Hägersten, Sweden). PBS (pH 7.4) was used as the eluent and for diluting the samples. The buffer was filtered through 0.20 μm disposable NALGENE filters (Nalge Nunc, Rochester, NY) and degassed with 15 min of ultrasound treatment prior to running the assay. The analysis was performed at a flow rate of 1.0 mL/min. UV absorption was measured at 220 nm. Twenty microliters of 100 μM of racemic melagatran (*RS* and *SR* enantiomers) were injected and 100 μL of 5 mM racemic propranolol.

RESULTS AND DISCUSSION

Chiral drug−protein binding studies with SPR must be performed with pure enantiomers, since analysis on racemic mixtures often yield average binding curves and affinity constants.[7] The analytes were here injected in a series of 27 concentrations in a wide range, and the normalized steady-state responses were always measured at a temperature of 25 °C. The analysis was repeated three times for each enantiomer, so that three consecutive injection series and fittings were performed. Fresh sensor chips and injection solutions were prepared each time. The parameters reported are therefore average values of three separate analyses. This ensures that the standard deviations obtained are good measures of the precision of the overall analytical work, not just the precision of the instrument. To make reliable model discriminations, a strategy was proposed that is based on the combined use of Scatchard plots and adsorption energy distributions (AED) on the raw adsorption sensor data. This approach allowed a great reduction in number of possible adsorption isotherms models that could describe the adsorption process before the data were fitted to an adsorption isotherm. Finally, the fitted models were selected based on a statistical criterion (except where stated otherwise).

The binding of substances to serum proteins was divided into different groups in terms of binding strength. Examples were taken from enantioselective drug interactions with the transport proteins HSA, AGP, and the target protein human α-thrombin. The groups were (i) strong, (ii) intermediate, and (iii) weak binders with dissociation constants in the nM, μM, and mM ranges, respectively.

Strong Binders. The binding of melagatran to its target protein, human α-thrombin, is highly enantioselective. Assays on high-immobilization surfaces show very strong binding of the active enantiomer, *RS*-melagatran, and the surface is saturated in the low nM range; in contrast, the binding of the inactive *SR* enantiomer to melagatran is nonexistent, even at concentrations

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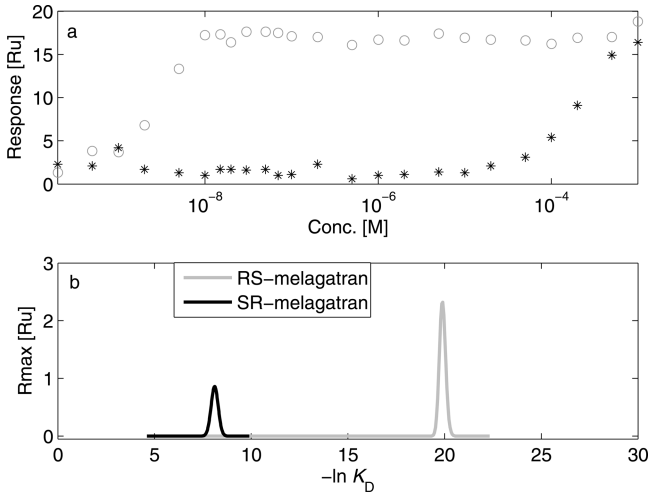

Figure 1. (a) Binding of *RS* (*, black line) and *SR* (○, gray line) melagatran enantiomers to human thrombin versus drug concentration over a broad concentration range at 25 °C. The symbols represent experimental values. (b) Calculated AED (using 300 grid points and 10000 iterations).

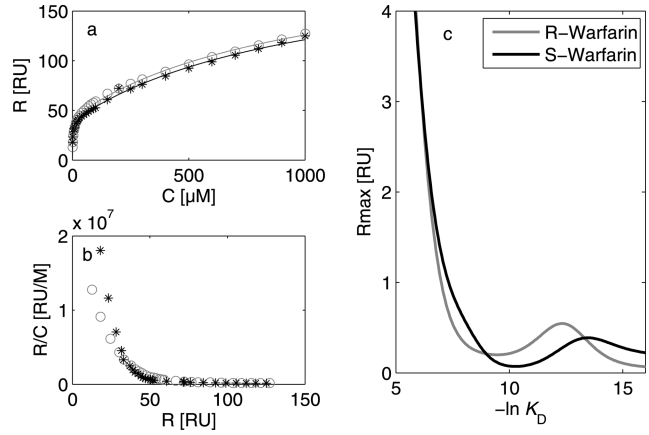

Figure 2. (a) Adsorption isotherm of *R* (*, black line) and *S* (○, gray line) warfarin enantiomers to human serum albumin at 25 °C. Symbols are experimental data, and the solid line is from the fitted model. (b) Corresponding Scatchard plot, and (c) the calculated AED (using 300 grid points and 6 500 iterations).

exceeding 100 μM. This can be seen in Figure 1a, which shows the binding of *RS*-melagatran and *SR*-melagatran versus a million-fold dynamic concentration range. For the highest analyte concentrations some binding of the nonactive enantiomer was observed. This signal was probably caused by contamination of the active enantiomer in the ppm range rather than by nonspecific binding. Otherwise, also the *RS* form should show increased binding in this concentration region.

To evaluate the adsorption process, AED-calculations were made, presented in Figure 1b. Both *RS*-and *SR*-Melagatran had a unimodal AED with dissociation constants of 2.5 nM and 0.4 mM (estimated at the maximum of the AED), respectively. The unimodal AED indicates that a single site model could be used to describe the data, for example, Langmuir or Tóth. Accurate steady-state data could, however, not be acquired at sufficiently low concentrations because of too long association times, so kinetic analysis was used instead.[35] The dissociation constant of the active enantiomer (*RS*) was determined to be 0.7 nM with a standard deviation of 0.06 nM. In the case of strong binders, the effects of the nonactive enantiomer can often be considered as negligible as compared to the active enantiomer.

Intermediate Binders - and Impact of Enantioselective and Nonselective Considerations. The binding of propranolol to AGP and warfarin to HSA, respectively, were evaluated in a wide concentration range (1−1000 μM). As for the strong binder, we first evaluated the raw adsorption data before it was fitted to an adsorption isotherm model.

In the case of warfarin binding to HSA, the Scatchard plot (see Figure 2b) is concave for both enantiomers. A concave Scatchard plots is true for, for example, Tóth, multi-Lagmuir, Freundlich, and Langmuir−Freundlich adsorption isotherm models. Several of the mentioned models are unimodal, which means that the adsorption energy is distributed in a single energy site. This energy site could be homogeneous (Langmuir) or heterogeneous (e.g., Tóth and Freundlich). To further reduce the number of possible models the adsorption energy was calculated, see Figure 2c. The AED-plot reveal that the AED are at least bimodal for both enantiomers of warfarin with a resolved site at high energy and an unresolved site(s) at low energy (cf. Figure 2c). The reason for this is that the highest used concentration in this study is too low to resolve the low energy site. This is not an uncommon problem while characterizing protein phases in HPLC.[24,25] The phenomenon is due to limited solubility of the studied solute in the running buffer. However, it has previously been shown that the unresolved site will not affect the quality on the determined parameters for the resolved high energy sites.[24] The estimated dissociation constant from the AED is 4.4 μM and 1.4 μM for *R*- and *S*-warfarin, respectively. If we now go back and inspect the Scatchard plot we could see that it contains two asymptotes, the first at low q that is associated with the low dissociation constant (high adsorption energy), and the second slope at high q that is associated with the high dissociation constant (low adsorption energy). This kind of Scatchard plot is true for a bi-Langmuir model with a large difference between the adsorption energy sites. The raw adsorption data (Figure 2a) were fitted to a chiral bi-Langmuir model (eq 1) and are presented in Table 1 (third row).

Similar analysis is performed for propranolol adsorption to AGP. In Figure 3 the adsorption isotherm, the Scatchard plot, and the AED are plotted. The Scatchard plot (Figure 3b) is concave with the characteristic two asymptotes mentioned above; indicating that the bi-Langmuir model with a large difference between the two sites could be a good model. The AED calculations (Figure 3c) confirm our observations in the Scatchard plots with a bimodal distribution with dissociation constants at the resolved energy site of 8.7 μM and 11.8 μM for *S*- and *R*-propranolol, respectively. The bi-Langmuir model fitted the raw adsorption data excellently, see Table 1 (second row, without DMSO).

For both substances the *S* enantiomer binds more strongly to the serum protein. An analysis in which a racemic mixture of propranolol was injected instead of the individual enantiomers confirmed that, as expected, the binding curve fitted between the curves of the pure enantiomers (data not shown). From Table 1 it is obvious that the enantioselective high-affinity sites are dominant at low analyte concentrations (being over 100 times stronger than the nonselective sites). However, the number of nonselective low-affinity sites (the saturation capacity is proportional to R_{max}) is 5−10 times

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Table 1. Enantioselective (*R* and *S*) and Nonselective (ns) Parameters for the Binding of Propranolol to AGP with 5%-DMSO and without DMSO and warfarin to HSA at 25°C[a]

analyte-ligand	K_D^{ns} [mM]	K_D^R [μM]	K_D^S [μM]	R_{max}^{ns} [RU]	R_{max}^R [RU]	R_{max}^S [RU]	K_D^R/K_D^S
propranolol-AGP[c]	2.3 (0.8)	39.76 (12.3)	31.30 (7.4)	204 (45)	30.1 (0.8)	29.2 (0.4)	1.26 (0.10)
propranolol-AGP[b]	1.2 (0.1)	10.05 (3.02)	7.73 (2.83)	158 (7)	20.2 (1.1)	20.1 (0.5)	1.30 (0.13)
warfarin-HSA	0.9 (0.1)	2.94 (0.40)	1.39 (0.33)	136 (17)	38.3 (3.8)	33.6 (3.6)	2.15 (0.20)

[a] The values represent the average values and standard deviations for triplicate runs of each substance. [b] Without DMSO in the running buffer. [c] With 5% DMSO in the running buffer.

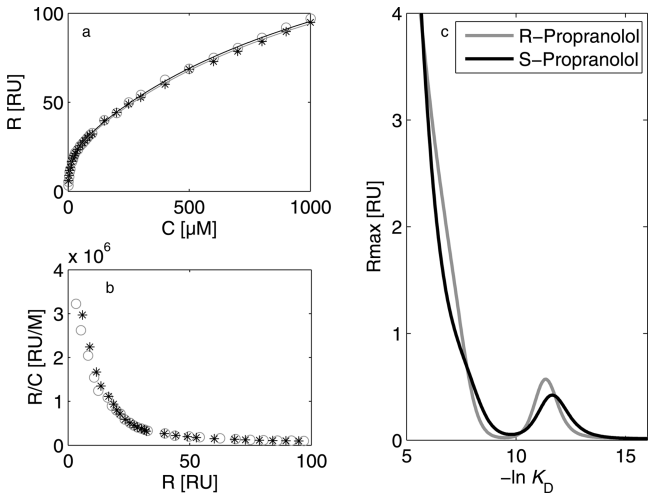

Figure 3. (a) Adsorption isotherm of *R* (*, black line) and *S* (O, gray line) propranolol enantiomers to AGP at 25 °C without DMSO in the running buffer. Symbols are experimental data and the solid line is from the fitted model. (b) Corresponding Scatchard plot, and (c) the calculated AED (using 300 grid points and 30000 iterations).

greater than the number of enantioselective ones, resulting in a non-negligible contribution even at rather low concentrations. The nonspecific binding is more pronounced in the propranolol-AGP system than in the warfarin-HSA system. At infinitesimal concentrations, close to K_D of the enantioselective site, the nonspecific binding, calculated using eq 2 and values from Table 1, still represents approximately 11% of the total binding in the propranolol-AGP system but only 2% in the warfarin-HSA system. At higher concentrations the relative contribution of the nonspecific binding increases in accordance with eq 2. For example, the relative contribution to the total binding from the nonselective sites was around 50% at 165 μM *R* or *S* propranolol and 78% at 1.0 mM propranolol enantiomer. The nonspecific binding originates from the protein and not from the dextran matrix, which was confirmed by running samples on a nonimmobilized surface generating a referenced response of zero. It has been shown, for both propranolol bound to a cellulase protein and for alprenolol (similar to propranolol) bound to AGP,[36,37] that the contribution of the nonselective binding increases at lower pH levels. Under such conditions the nonselective binding has a size similar to that of the specific enantioselective binding, even at infinitesimal concentrations.

A comparison of our results with those of previous studies that used several different techniques, examining both immobilized and free-in-solution proteins, is seen in Table 2. The enantioselective results of the present study fit well with those of previous studies of warfarin-HSA. In the case of propranolol-AGP, we obtained slightly lower affinity than in previous reports. This could be due to the fact that the present study treated immobilized proteins and not free in solution, as the other studies did, but also that we considered nonselective interactions which in previous studies have been poor or nonexistent. Also the immobilization technique will affect the results. It is possible that a milder method by coupling to the carbohydrate portion of the AGP, could give binding constants closer to the free solution.[22,23]

Importance of a Wide Concentration Range. As mentioned above in Table 2, the determined adsorption data are often carried out at different concentration ranges. To point out the importance of using a sufficiently wide concentration range a more detailed analysis was undertaken with the propranolol-AGP system. The model fitting to Langmuir and bi-Langmuir adsorption isotherm model was done repeatedly, and each time the highest concentration was removed from the data set, yielding a fit corresponding to a narrower concentration range. The largest range, the one used to obtain the parameters in Table 1, was 1−1000 μM with 27 injections of each enantiomer, and the lowest range was 1−10 μM with 5 injections of each enantiomer. The residual sum was always smaller for the two-site model regardless of the number of data points used. This is expected since this model has more independent parameters. By applying the F-test one can decide if the improvement is significant, that is, if it is appropriate to assume the more complex model. With very low ranges, the single-site model gave a significantly better fit than the two-site model. For all fittings using a maximum concentration exceeding 20 μM, however, the two-site model gave a significantly better fit.

Thus, in the case of intermediate binders, the nonspecific contributions have a very large effect since they are much more frequent than the high-affinity sites. Although the high-affinity site is populated first, the adsorption to low-affinity sites often affects the adsorption isotherm curvature already at relatively low concentrations. This additional curvature will therefore bias the K_D (equilibrium constant) if a single-site model is erroneously assumed. In this context it is easy to understand the additional source of errors when also neglecting which type of enantiomer is used as often done in the analysis of small drug molecules using SPR-sensors.[5,6,13−18]

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Table 2. Comparison of Previous Studies of the Same Model Systems Using Different Methods

analyte	K_D [μM]	method	T	max conc [μM]	reference
		Previous Warfarin-HSA studies, All Performed at pH 7.4			
R-warfarin	2.94	SPR	25 °C	1000	this paper, Table 1
S-warfarin	1.39	SPR	25 °C	1000	this paper, Table 1
warfarin	2.5	SPR	25 °C	1000	Day, Myszka, 2003[13]
warfarin	3.57	fluorescence spectroscopy	25 °C	50	Dockal M et al., 2000[28]
R-warfarin	3.85	frontal analysis	25 °C	1.5	Loun, Hage, 1994[38]
S-warfarin	2.94	frontal analysis	25 °C	1.9	Loun, Hage, 1994[38]
warfarin	4.05	equilibrium dialysis	37 °C	200	Larsen et al. 1984[39]
		Previous Propranolol-AGP studies, All Performed at pH 7.4			
R-propranolol	10.05	SPR	25 °C	1000	this paper, Table 1
S-propranolol	7.73	SPR	25 °C	1000	this paper, Table 1
R-propranolol	0.37	HPLC	37 °C	2	Xuang and Hage[22]
S-propranolol	0.24	HPLC	37 °C	2	Xuang and Hage[22]
R-propranolol	0.91	HPLC	37 °C	100	Mallik et al.[23]
S-propranolol	0.71	HPLC	37 °C	100	Mallik et al.[23]
Propranolol	1.19	equilibrium dialysis	37 °C	No data	Wong, Hsia, 1983[40]
R-propranolol	2.65	ultrafiltration	37 °C	100	Hanada et al., 2000[41]
S-propranolol	1.58	ultrafiltration	37 °C	100	Hanada et al., 2000[41]
propranolol	8.85	equilibrium dialysis	37 °C	Not mentioned	Urien et al., 1991[42]

To demonstrate the importance of a wide concentration range, we fit Langmuir and bi-Langmuir models to a limited range (1−40 μM, 10 injections per enantiomer) and a full range data set (1−1000 μM, 27 injections per enantiomer) from the propranolol-AGP system, see Figure 4. Using the small range (a,c), it is difficult to judge by eye which model is best. Actually, the Langmuir fit (a) looks very convincing, so without the additional fitting to the bi-Langmuir model (c) and statistical analysis we might have drawn erroneous model conclusions. In the figure it is clear that although the fit to the narrow range data is good, the estimated parameters fail to describe binding in a wider concentration range (see insets of Figure 4a,c), because the binding curves deviate from the high-concentration data points. This was the reason Xuan and Hage recently only found a single site in (HPLC) analysis (cf. Table 2) when investigating the interaction between propranolol enantiomers on immobilized AGP.[22] In a follow-up paper Mallik et al. (group of Hage) determined again the adsorption isotherm for the same system but over a wider concentration range, up to 100 μM.[23] The authors found that a single site adsorption model now was unsuccessful in describing the data from the wider concentration range (see Table 2).

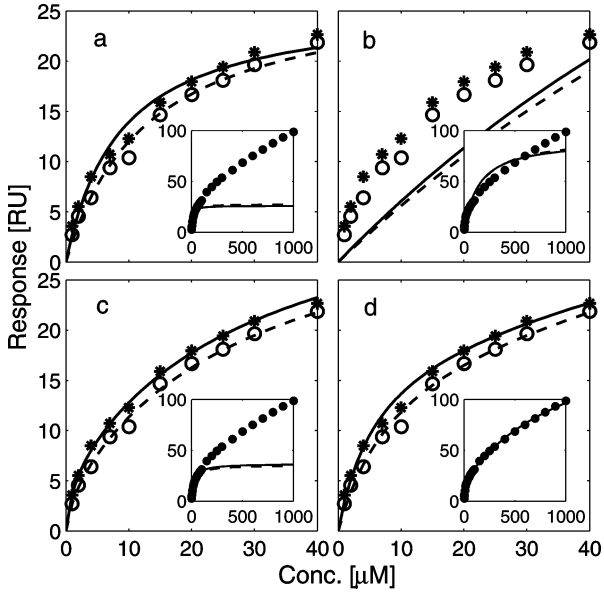

Figure 4. Adsorption isotherms of *R*- (*) and S- (○) propranolol enantiomers to AGP at 25 °C without DMSO in the running buffer. The lines show the best fit to the Langmuir (a,b) and bi-Langmuir (c,d) models using concentration range 2.5−40 μM (a,c) and 2.5−1000 μM (b,d). With a narrow concentration range it is difficult to judge which model is appropriate, and the requirement of a two-site model is evident when the wide concentration range is used.

Obviously the narrow-range analysis yields bad estimates with both models. In Figure 4b,d the results are shown from a fit to the full range data. The single-site model (b) is not flexible enough to give a correct curvature to fit over the whole range. In contrast, the two-site model fits very convincingly. By the use of a wide enough concentration range it is obviously easier to see which model applies. More specifically, the use of a narrower concentration range results in the false assumption of a single-site interaction.

The values of the parameters obtained also depend on the concentration range used. In Figure 5 are the K_D (a) and R_{max} (b) estimates plotted as a function of the maximum concentration used in the fitting. The Langmuir parameter values increase as the data range is increased. The reason for this is that the fractional surface coverage is rather low so that the saturation capacity is extrapolated. The extrapolation error decreases with increasing fractional surface coverage. The bi-Langmuir parameter estimates are unstable in the low range, but once the maximum concentration exceeds approximately 200 μM the K_D and R_{max} estimates for the chiral site in this case no longer depend on the data range used. The bi-Langmuir parameters for the nonselective site do however not converge, indicating how difficult it is to analyze such sites.

Figure 5 shows that very different estimates of K_D and R_{max} will be obtained depending on the concentration range used, whereas the bi-Langmuir model produces accurate results if sufficiently high concentrations are used (although the parameters for nonselective site are not equally accurate). Binding studies are sometimes conducted in the low concentration range using a single-site model, under the assumption that

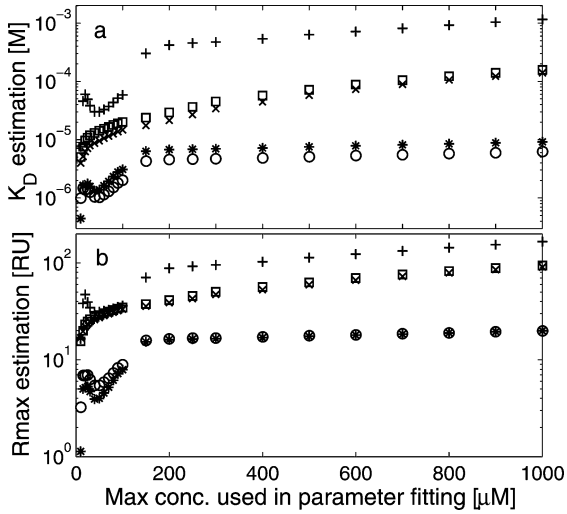

Figure 5. K_D (a) and R_{max} (b) parameters of *R*- and *S*-propranolol in the binding to AGP at 25 °C without DMSO in the running buffer, obtained with different data ranges used in the fitting. The reported parameters correspond to the fitting to all data points between 1 μM and the concentration given by the *x*-axis. Langmuir model parameters: *S*-propranolol (□) and *R*-propranolol (×). Bi-Langmuir parameters: Nonselective site (+), chiral selective site for *S*-propranolol (*) and *R*- propranolol (○).

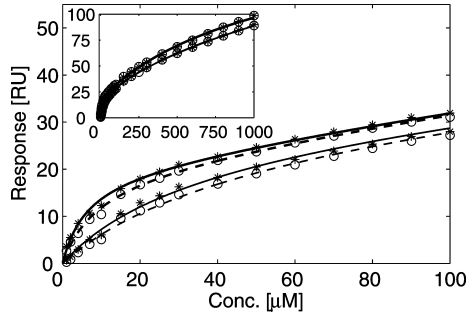

Figure 6. Effects of DMSO on the binding of *S*-propranolol (*, solid lines) and *R*-propranolol (○, dashed lines) to AGP at 25 °C. The inset shows the whole concentration range while the main figure shows a lower concentration range, up to 100 μM. The bold lines are the results with the PBS buffer, and the thin lines represent the binding study performed with PBS and 5% DMSO.

nonselective sites are populated first at higher concentrations and that they do not affect the curvature in the low concentration region. From Figure 5 it can be seen that the parameter values obtained for the high-affinity site with the Langmuir model and a narrow concentration range are indeed in the same order of magnitude as those obtained with a full-range bi-Langmuir analysis. However, judging from the instability (concentration range dependence) of the Langmuirian parameters, it takes quite an amount of luck to obtain accurate results with this approach, although the precision may be good.

DMSO Effects in the Propranolol-AGP Case. The effects of the solvent DMSO in SPR analysis were studied by injecting equal concentration series of propranolol on immobilized AGP, both with and without 5% DMSO in the running buffer (PBS). The results from three consecutive assays are displayed in Table 1, and binding curves are plotted in Figure 6; the inserted figure shows the full concentration range, and the main figure shows a lower concentration range. A shift in equilibrium dissociation

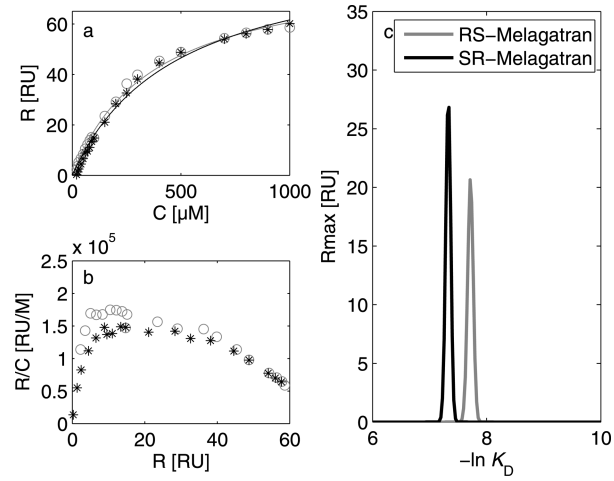

Figure 7. (a) Adsorption isotherm of *RS* (*, black line) and *SR* (○, gray line) melagatran to human thrombin at 25 °C, symbols experimental data, and the solid line is from the fitted model. (b) Corresponding Scatchard plot, and (c) the calculated AED (using 300 grid points and 100000 iterations).

constant is evident at low concentrations (main figure). Evaluation of the equilibrium constants reveals a significant decrease (by about four times) of the enantioselective K_D in the presence of DMSO, whereas the enantioselectivity factor $K_D{}^R/K_D{}^S$ is only slightly affected (cf. Table 1, with DMSO). At analyte concentrations close to that of the enantioselective K_D, the contribution of nonselective binding is approximately 17% of the total binding, calculated using eq 3 and values from Table 1; without DMSO the contribution is only 11%. The results indicate that DMSO may affect the results even more severely than the use of an erroneous binding model does. It is therefore important to realize that physiologically relevant binding constants cannot be calculated accurately in terms of absolute affinities. The 4-fold increase in the dissociation constant is a much more dramatic change, and also is in the opposite direction, than the previous study has found.[5]

Weak Binders. The binding of the thrombin inhibitor, melagatran, was also investigated toward the transport proteins HSA and AGP, respectively. The experiment showed no binding to HSA but some binding to AGP. In Figure 7, the adsorption isotherm (a), Scatchard plot (b), and AED (c) are presented. Interestingly, the AED-calculations contain only one resolved site with dissociation constants of 0.45 mM and 0.65 mM for the *RS* and *SR* enantiomers, this is approximately 10^6 times weaker binding than *RS*-melagatran to thrombin. From the AED we could also estimate the saturation capacity (the sum over the AED) to 90 RU and 106 RU for the *RS* and *SR* enantiomers. Finally, data from duplicate runs were fitted to the Langmuir model, and the dissociation constants for the separate enantiomers were determined to be 0.31 and 0.35 mM for the *RS* and *SR* enantiomers, respectively.

In a previous publication where only one type of drug−protein interaction was investigated, with SPR and HPLC, it was predicted that HPLC should not be useful for the investigation of strong bindings. To get a better knowledge on when HPLC should be abandoned we now compared the above SPR result with HPLC. An injection of a 50:50 mixture of *SR*-melagatran and *RS*-melagatran was performed on a CHIRAL-AGP column, with PBS

mobile phase, yielding a good separation with capacity ratios of $k_{SR} = 12.5$ and $k_{RS} = 26.5$. The selectivity (k_{RS}/k_{SR}) of the chromatographic separation is 2.12, compared with calculated selectivity from AED data of 1.71. These results support the observation from the SPR experiment showing that the physiologically active *RS*-melagatran binds the strongest to AGP. Since retention times are relatively short and since the resolution ability is good, HPLC would be preferable for studying these weak interactions. Using the HPLC perturbation method, it is also possible to determine the enantiomeric affinity parameters from a racemate injection, since they are completely separated in the column. This is not possible in the SPR system, where a racemate generates an average response of the two enantiomers.[7]

Also a propranolol sample was injected into the CHIRAL-AGP column. The binding generated very long retention times around 17 h; the reason is the strong interactions between AGP and propranolol that is approximately 60 times stronger than for the melagatran-AGP. Because of the extreme HPLC retention times, SPR is a better method to use for analysis of intermediate to strong binders. The HPLC analysis time could, however, be shortened dramatically by the use of a column with lower immobilization level, since the retention time is proportional to the ligand concentration. Using the equation below for the Langmuir model we could estimate the retention time of the analyte

$$k = F\frac{q_s}{K_D} \tag{7}$$

where q_s is proportional to the amount of immobilized ligand. Hage used recently a new, less dense, immobilization technique for the investigation of the interactions between propranolol enantiomers and the AGP protein.[22] If eq 7 is applied to the melagatran-trombin interaction (see above) capacity ratios of >10 millions are obtained demonstrating why HPLC is unrealistic for such measurements.

CONCLUSIONS

In this study we have discussed what considerations must be made to obtain accurate results in chiral drug−protein binding analysis with SPR and other techniques. We have shown that the use of an appropriate model is crucial to obtain accurate K_D estimates. To better select the proper model a new methodology was introduced; using this approach the number of possible models prior to the model fitting and statistical evaluation could be considerably reduced. A similar methodology has previously and successfully been used with HPLC data.

With this model selection methodology the raw adsorption isotherm data is first analyzed in a Scatchard plot followed by adsorption energy distribution (AED) calculations. The curvature of the Scatchard plot is dependent on the adsorption process that describes the system. Therefore, Scatchard plots could be used to reduce the possible models. The AED is used to deduce how many different adsorption sites are responsible for the partitioning. These two tools complement each other and make the final model selection easier. Relevant models are finally fitted to the data using nonlinear regression, and the most appropriate model is chosen using a statistical F-test.

It is crucial that the adsorption data is collected in a very wide dynamic concentration range (if possible exceeding 1000). Otherwise it is impossible to decide which model applies. One must point out that if the wrong adsorption model is chosen to describe the system, wrong conclusions about the adsorption process will be drawn (e.g., wrong K_D estimates). A common mistake is to use only very low concentrations in the regression, limited by "physiologically relevant levels". This will often result in the erroneous conclusion that a single-site interaction prevails, instead of a multisite one. To be able to assume single-site interaction from a Scatchard plot and obtain reliable results, we have shown that linearity must prevail over a very wide concentration range.

We measured the binding of some physiologically relevant systems: *R*- and *S*-propranolol to AGP, *R*- and *S*-warfarin to HSA, and *RS*- and *SR*-melagatran to thrombin, AGP, and HSA. Our analysis indicated that a single-site model could be assumed in the *RS*-melagatran-thrombin case (strong binding), and that the *SR* enantiomer did not interact at all with thrombin. On the other hand, both the propranolol-AGP and warfarin-HSA systems were heterogeneous, comprising both high-affinity chiral sites and weak nonselective sites (intermediate binding). Both the Melagatran enantiomers showed no binding at all to HSA whereas a homogeneous, weak affinity, was found for AGP.

The use of the solvent DMSO in drug−protein interaction studies is very common to increase the solubility of the analyte. Surprisingly few researchers seem to consider if, and how, DMSO affects the binding. In the present study we have shown that the propranolol-AGP binding was affected severely (4-fold increase of chiral K_D values) by the presence of 5% DMSO, rendering the results physiological relevance questionable.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

Further details about numerically solving the adsorption energy distribution and the immobilization protocol. This material is available free of charge via the Internet at http://pubs.acs.org.

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