

Biosensor Analysis of the Interaction between Immobilized Human Serum Albumin and Drug Compounds for Prediction of Human Serum Albumin Binding Levels

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The interactions between a set of drugs, selected on the basis of reported human serum albumin (HSA) binding levels, and immobilized HSA were investigated using surface plasmon resonance technology. Major HSA binding sites were available after immobilization. The intensity of the signal obtained from the interaction of the drug with the HSA surface was correlated with the reported HSA binding level. Drugs were classified into groups corresponding to high, medium, or low HSA binding based on the injection of the drug at 80 μ M concentration. A set of 10 drugs binding to α_1 -acid glycoprotein (AGP) was also investigated and correlated with reported AGP binding data. The throughput of the presented assay is 100 compounds/24 h, and the sample consumption is less than 100 μ L (8 nmol).

Introduction

Many compounds bind reversibly to human serum albumin (HSA), α_1 -acid glycoprotein (AGP), and other serum components. The affinity of a drug toward plasma proteins is an important issue when determining its overall pharmacokinetic profile. A high level of protein binding reduces the free concentration and therefore the physiological activity of the drug. Circulating protein–drug complexes also serve to replenish the free drug concentration, as free drug is removed from the body by various elimination processes, and thereby prolong the duration of drug action. Thus, the level of protein binding is an important factor in the delicate balance between intended physiological activity and potential side effects of the drug.

Ideally the free concentration of each drug candidate should be estimated in the blood from the patient for which the drug is intended. Early estimates of the protein binding level, as well as of other ADME parameters, are now becoming more important in the drug discovery process. Therefore simplified approaches for the determination of protein binding are being considered. A wide variety of methods and experimental conditions are used for this purpose. Methods include equilibrium dialysis, ultrafiltration, ultracentrifugation, spectroscopic methods, affinity and size exclusion chromatography, and electrophoretic methods as reviewed by Oravcová¹ and Hage.² These methods rely either on a separation of bound and free drug or protein or on a change in intrinsic parameters such as the mobility of protein or the spectroscopic properties of a drug–protein complex. To simplify the studies of drug protein interactions it is also common to use single proteins, HSA and/or α_1 -acid glycoprotein, instead of blood or plasma. This approach is particularly useful when many compounds have to be screened for protein binding. Furthermore

it has also been demonstrated that the use of immobilized HSA in high-performance liquid chromatography (HPLC) correctly reflects the binding of drugs to free HSA.^{3,4}

These results encouraged us to investigate the use of biosensor technology for affinity analysis and for ranking of the binding of drug compounds to surface immobilized HSA. The main advantages of biosensor technology (here a surface plasmon resonance, SPR, sensor) are that binding is monitored directly without the use of labels, sample consumption is low, and the analysis is rapid and automated.^{5,6} In this paper we present a new method, based on the injection of compounds at a single concentration, for classifying compounds as high, intermediate, or low HSA binders by comparing their binding levels to that of selected reference compounds.

Materials and Methods

Instrumentation. BIACORE 3000 from Biacore AB, Uppsala, Sweden, was used, Figure 1. Interaction analysis was performed at 25 °C.

Buffers. 67 mM isotonic phosphate buffer with 5% dimethyl sulfoxide (DMSO) pH 7.4 (9.6 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 1.7 g KH_2PO_4 , 4.1 g NaCl to 1 L) was used as running buffer during the assay.

Immobilization of HSA. Sensor chip CM5 was used for analysis. The sensor chip consists of a carboxymethyl-modified dextran polymer linked to a gold-covered glass support.⁷ HSA (Sigma; essentially fatty acid and globulin free, A 3782) at 1 mg/mL was diluted to 15 μ g/mL in 10 mM sodium acetate, pH 5.2 and immobilized to the sensor chip, using amine coupling.⁷ Running buffer without DMSO was used during immobilization. The surface was activated by injecting a solution containing 0.2 M *N*-ethyl-*N*-dimethylaminopropylcarbodiimide (EDC) and 50 mM *N*-hydroxysuccinimide (NHS) for 7 min. HSA was injected during 7 min and the surface was then blocked by injecting 1 M ethanolamine at pH 8.5 for 7 min. 50 mM NaOH was injected in three 30-s pulses to wash off noncovalently bound HSA and to stabilize the baseline. Final immobilization levels were between 9500 and 11000 RU (Resonance Units). The immobilization procedure was followed

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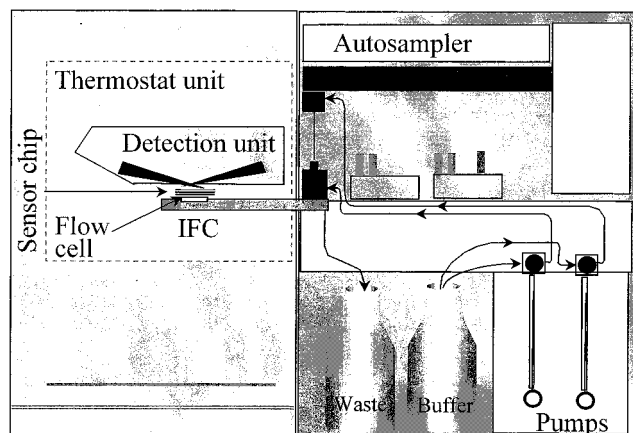


Figure 1. General principle for interaction analysis. A sensor chip surface is placed in contact with the IFC (integrated fluidic cartridge) and the detection unit. Continuous buffer flows through the IFC and over the surface. Samples are injected over the surface, using the autoinjector, and RI changes are detected by the detection unit.

Table 1. Compounds, Molecular Weights, and Literature References to HSA Binding Levels

| compd | MW (Da) | reported binding to HSA (%) | reported binding classified as high, medium, or low | lit. ref. of HSA binding |
|-----------------|---------|-----------------------------|---|--------------------------|
| tipranavir | 603 | 99.9 | H | 8 |
| naproxen | 230 | 99.7 | H | 9 |
| ritonavir | 721 | 99 to plasma | H | 10 |
| sulfadimethoxin | 310 | 98.7 | H | 11 |
| warfarin | 308 | 98 | H | 12 |
| delavirdine | 457 | 96.7 | H | 13 |
| digitoxin | 765 | 96.6 | H | 14 |
| ketanserin | 395 | 93.2 | M | 15 |
| pyrimethamine | 249 | 86.5 | M | 16 |
| diazepam | 285 | 86.1 | M | 17 |
| coumarin | 146 | 83.9 | M | 12 |
| salicylic acid | 138 | 81.7 to plasma | M | 18 |
| rifampicin | 823 | 73.3 | L | 19 |
| phenytoin | 252 | 63.4 | L | 17 |
| prednisone | 358 | 53.7 | L | 20 |
| tolterodine | 325 | 42 | L | 21 |
| quinine | 325 | 34.6 | L | 22 |
| 5-HM | 341 | 23 | L | 21 |
| salbutamol | 239 | 8 to plasma | L | 23 |

by several washes with buffer to equilibrate the HSA surface in the DMSO running buffer. Unmodified dextran was used as a reference surface. To ensure highest sensitivity the SPR detector response was normalized before running the assay, by calibrating the detector at various light intensities under conditions of total internal reflection (automized procedure).

Compounds. Naproxen, sulfadimethoxin, warfarin, digitoxin, diazepam, naproxen, pyrimethamine, coumarin, salicylic acid, rifampicin, phenytoin, prednisone, quinine, salbutamol, dipyridamole, timolol, acebutolol, atenolol, alprenolol, pindolol, metoprolol and propranolol were from Sigma. Ketanserin was from Fluka. Ritonavir was a gift from Björn Claesson, Medivir. Delavirdine, tolterodine, 5-HM and tipranavir were gifts from Pharmacia & Upjohn. Compounds were selected to: (a) cover a wide affinity range toward HSA, (b) include binders to major binding sites, (c) include different classes of compounds, (d) show a range in molecular weight, and (e) if possible be commercially available, see also Table 1. Compounds were diluted from 20 mM DMSO stock solutions to their final concentration in running buffer. Dilution was done in such a way that the pH and the concentrations of both DMSO and buffer substances in samples and running buffer were carefully matched.

Calibration of DMSO Bulk Differences between Reference and HSA Flowcells. The SPR signal reflects changes in refractive index (RI) at the sensor surface. RI changes as a

consequence of binding events close to the sensor surface and are related to the increase of mass on the surface.²⁴ An additional signal is obtained if the injected sample has a RI that differs from that of the running buffer. This signal is referred to as a solvent effect. When the solvent effect is small (in the order of 100 RU) it can normally be eliminated from the total signal by subtraction of the signal from the reference surface.²⁵ However, the introduction of a high-RI solvent such as DMSO can give rise to large shifts in RI during the injection, and mere subtraction of the data from the reference flowcell is no longer sufficient. To correct for these effects a DMSO calibration procedure was adopted (Figure 2c). Buffer solutions with varying concentrations of DMSO (4.5–6%) were injected in sequence over reference and HSA surfaces. This was done at the beginning and end of each experiment, using the same flow rate as for the assay. The responses of the calibration solutions, obtained from the reference surface, covered a range no larger than –500 to +1000 RU relative to the baseline. A DMSO calibration curve was created by plotting the difference in response between HSA and reference flowcells versus the response in the reference flowcell. This curve was used for correcting response levels obtained during sample injection.

Assay Design. Compounds were injected over the reference and HSA flowcell during 1 min at a flow rate of 30 μ L/min. Each cycle consisted of a 1-min waiting period for monitoring of the baseline stability, a 1-min injection of compound, a 35-s undisturbed dissociation phase, and a wash of the flow system, except the sensor surface, with a 1:1 mixture of DMSO and water. Compound responses in the reference flowcell were within the range of the DMSO calibration curve; extrapolation of the calibration curve was not used. An injection of running buffer was made between each sample to check for carry over effects. Eight DMSO calibration solutions were injected sequentially, each during 30 s, at the beginning and end of each experiment.

Data Evaluation. Data obtained in the reference flowcell was subtracted from that obtained in the HSA flowcell (Figure 2). Responses from injections of drug compounds and calibration solutions were extracted between report points set at 10 s before injection start and 10 s before the end of injection. These responses were further corrected for DMSO effects by use of the calibration curves (Figure 2c) and the final response values were used in K_D determinations and in ranking experiments. To get an estimate of K_D and response values at site saturation (R_{max}), measured response values (R_{eq}) obtained with different concentrations (C) of the compound were fitted to the equation:

$$R_{eq}/MW = CR_{max}/(C + K_D) \quad (1)$$

This equation was derived previously for the general case of an analyte binding to an immobilized partner²⁶ and here provides the affinity and the saturation response for a compound that interacts with a single site of immobilized albumin. To facilitate a comparison of responses obtained with compounds of varying molecular weights R_{eq}/MW , i.e., the steady-state response divided by the molecular weight of the compound, was used in the fitting procedure instead of R_{eq} . By using this ratio, the saturation level R_{max} was considered identical for all compounds that bind to a single site of albumin and dose–response curves from several compounds were therefore analyzed simultaneously with R_{max} as a global parameter, i.e., common to all compounds, and K_D as a local parameter, i.e., unique for each compound.

To compare K_D calculated by this method with reported levels of percent (%) bound the equilibrium equation was used: $[HSA][drug]/[HSA - drug\ complex] = K_D$. A concentration of 0.68 mM of HSA in serum was assumed. With x as the total concentration of compound and p as the part bound $(0.68 - xp) \times (x - xp)/xp = K_D$. Following an input of p (p is equal to % bound/100) K_D values were calculated for total drug concentrations ranging from 0.1 nM to 1 mM. Calculated K_D values were largely independent of the total drug concentration. Values for selected compounds are shown in Table 3. In

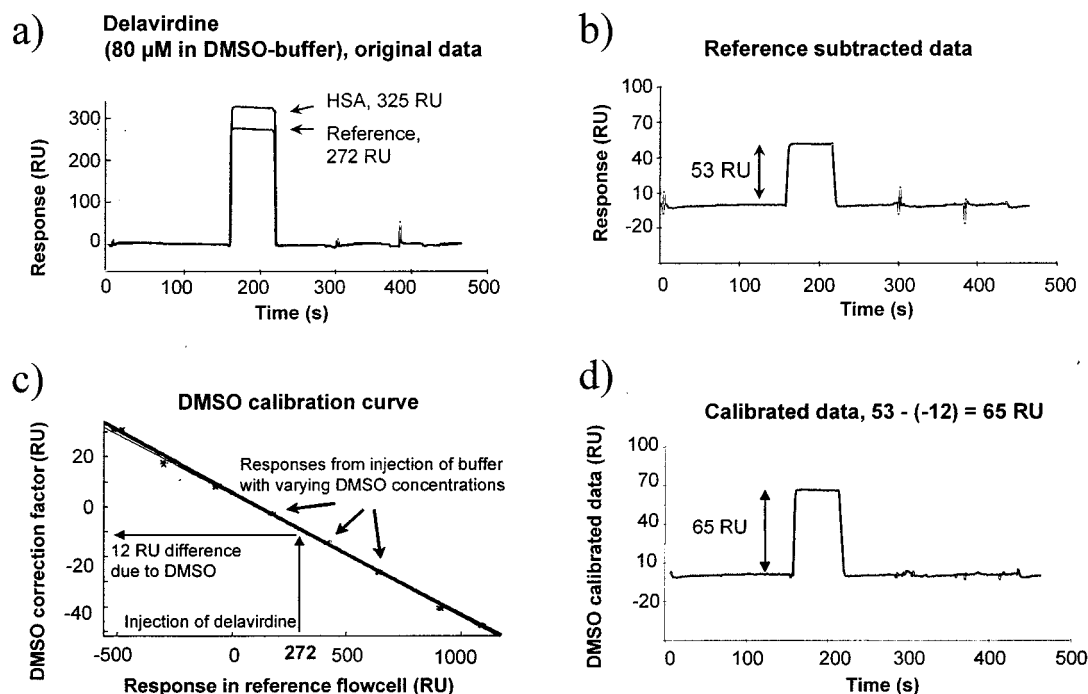


Figure 2. (a) Injection of delavirdine over reference and HSA flowcell. A high response level was obtained since the DMSO concentration of sample and assay buffer was not exactly matched in sample preparation. (b) Reference data subtracted from HSA data. (c) A series of 8 calibration solutions (running buffer with different DMSO concentrations) were run at the beginning and end of each experiment. The responses of the calibration solutions, obtained from the reference surface, covered a range from -500 to $+1000$ RU relative to the baseline. A DMSO calibration curve was created by plotting difference in response between HSA and reference flowcell (DMSO correction factor) versus response in reference flowcell. (d) Compound responses were corrected for DMSO bulk differences via the calibration curves.

ranking experiments responses obtained at $80 \mu\text{M}$ concentration were divided by the molecular weight of the compound to allow comparison of binding levels.

Results

Sensorgrams and Calibration of Solvent Effects.

All compounds tested bound reversibly to immobilized HSA. During injection a steady-state response was reached within a few seconds. After the end of the injection drug compounds dissociated very rapidly and the signal returned to baseline almost immediately (Figure 2a,b). Of the compounds investigated only tipranavir remained bound to the HSA surface for more than 2 min.

To reduce errors in reference subtraction, solvent effects were compensated for by applying a DMSO calibration procedure (Figure 2c). Solvent effects from samples typically ranged from -100 to 500 RU leading to correction factors of -20 to 10 RU. In previous studies remaining solvent-related errors have been shown to be low and between ± 3 RU when solvent effects were as large as 2000 RU.²⁷

Activity and Stability of Immobilized HSA. The major drug binding sites on HSA remained accessible after immobilization of HSA to the sensor surface via its amine groups. This was demonstrated by the binding of warfarin²⁸ to site I (the warfarin site) and naproxen⁹ to site II (the benzodiazepine site) and by binding of digitoxin²⁹ to its site (Figure 3a-c). As expected saturation levels were related to the molecular weight of the compounds. Digitoxin with a MW of 765 Da, warfarin with MW 308 Da, and naproxen with MW of 230 Da approached saturation levels close to 70 , 35 , and 15 RU, respectively. These values indicated that mainly single

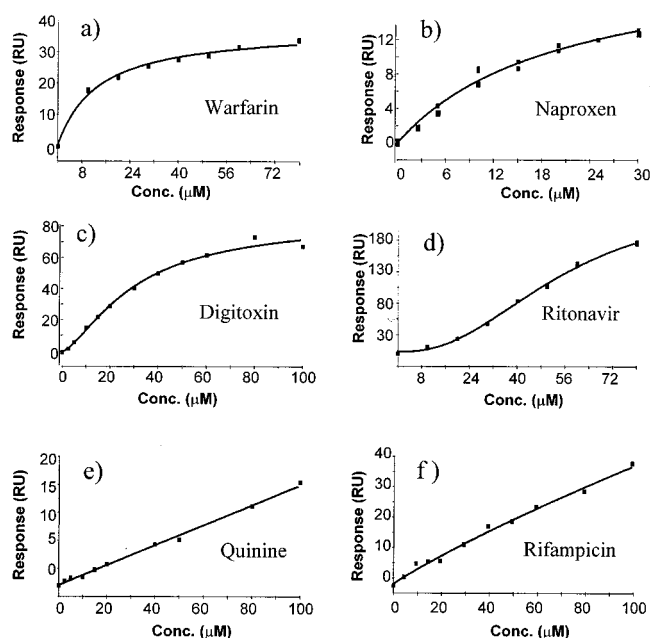


Figure 3. Dose-response curves of six compounds binding to HSA. (a and b) Warfarin and naproxen display monophasic binding curves obtained by fitting data to a steady-state model. (c and d) Binding of digitoxin and ritonavir appears to be biphasic. (e and f) Quinine and rifampicin give linear binding curves. Curves in (c-f) were obtained by four-parameter fits and do not represent K_D fits.

sites were occupied at these concentrations with the exception of ritonavir where the response indicated a stoichiometry of more than one ritonavir molecule/HSA molecule (Figure 3d).

Table 2. Data from Two Ranking Experiments on the Same Surface, the First on the Day of HSA Immobilization and the Second 7 Days Later (warfarin (80 μ M) was injected in duplicate early, in the middle, and late in each assay to check HSA binding activity)

| | response (RU) | |
|---------------------|----------------|----------------|
| | warfarin day 1 | warfarin day 7 |
| early | 37.1 | 38.4 |
| | 38.2 | 28.8 |
| middle | 38.4 | 41.3 |
| | 38.9 | 41.1 |
| late | 39.1 | 41.8 |
| | 39.3 | 41.8 |
| average: | 38.5 | 40.5 |
| standard deviation: | 0.8 | 1.5 |

In ranking experiments warfarin was used as a positive control of HSA activity. Warfarin was injected early, in the middle, and late in each experiment. Reference subtracted and calibrated responses of warfarin from an experiment performed on the day of HSA immobilization and from an experiment conducted 7 days later on the same HSA surface are presented in Table 2. The warfarin response was stable, and on this surface response levels ranged from 37.1 to 41.8 RU with a standard deviation of 1.6 RU.

K_D Determinations. Dose-response curves obtained for warfarin, naproxen, digitoxin, ritonavir, quinine, and rifampicin are shown in Figure 3. For warfarin and naproxen binding appeared monophasic and approached saturation levels at low concentrations. For ritonavir and to some extent digitoxin response levels indicated a biphasic interaction. Quinine and rifampicin gave linear dose-response curves over a wide concentration range, and it was not possible to estimate saturation levels.

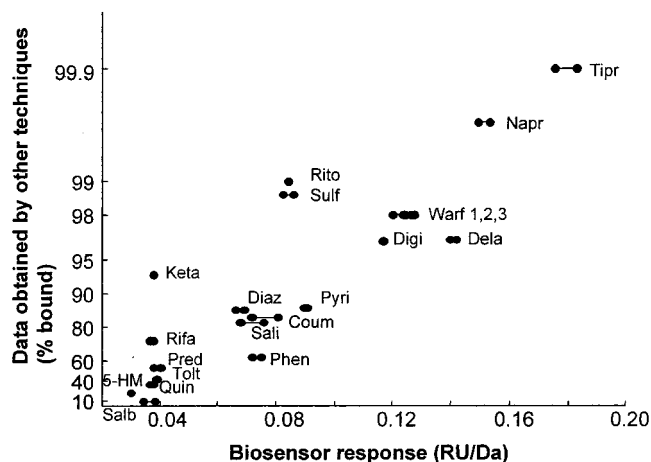
Given the dose-response curves in Figure 3, K_D analysis was problematic. To obtain an estimate of affinity the analysis was simplified by assuming (1) that HSA binding sites were equally available, (2) that only high-affinity sites were populated at concentrations below 40 μ M, and (3) that response levels were normalized by dividing the measured response (RU proportional to g/m^2) with the molecular weight (RU/MW proportional to mol/m^2). The K_D data obtained (from eq 1) with these assumptions showed that compounds with low and medium binding to HSA gave much higher K_D values than compounds with a high level of binding to HSA and suggested a good correlation between reported percent (%) bound and K_D but with limited resolution of compounds with binding levels above 97% bound (Table 3).

Ranking of Binding Levels, Comparison of Biosensor Data with Conventional Techniques. For ranking of compounds we used a simpler approach by measuring the degree of binding at a concentration high enough to give a reasonable signal but low enough to avoid misinterpretation of compounds binding to several binding sites. The binding levels of 19 drugs with molecular weights between 138 and 823 Da and with reported HSA binding levels between 8% and 99.9% were studied through duplicate injections of a single concentration (80 μ M) of each drug. Identical experiments were run on the day of HSA immobilization and 1 week later on the same HSA surface. Figure 4 shows the correlation between binding levels obtained on the

Table 3. Comparison of Literature Data of Percent (%) Bound and Corresponding K_D from the Equilibrium Expression, with K_D Obtained with Biosensor Analysis

| compd | from literature | | biosensor analysis |
|------------|----------------------|-------------------------------|--------------------|
| | % bound ^a | K_D (μ M) ^b | K_D (μ M) |
| naproxen | 99.7 | 2 | 26 |
| ritonavir | 99 | 7 | 21 |
| warfarin | 98 | 14 | 9 |
| digitoxin | 97.6 | 17 | 28 |
| rifampicin | 73.3 | 218 | 195 |
| quinine | 34.6 | 1290 | 556 |

^a Literature references in Table 1. ^b Calculated from percent (%) bound as described in data evaluation.

**Figure 4.** Biosensor data of compounds binding to HSA correlated to data obtained with other techniques, references in Table 1. (Literature data for each compound varies depending upon experimental conditions.)

day of HSA immobilization and the reported degree of HSA binding. To increase the resolution of the high-affinity compounds, values were put on a logarithmic scale and $2 - \log(100 - \% \text{ bound})$ was plotted as a function of biosensor data.

In a screening situation it may be useful to include compounds as markers for medium- and high-level binders. The data set in Figure 4 can then be replotted using only the biosensor data (Figure 5). Using diazepam with a reported binding level of 86.1% and warfarin with reported binding of 98% as marker compounds, drugs could be divided into low-, medium-, and high-level binders.

When tested on the same HSA surface 1 week later the 19 compounds were all classified into the same groups, except for salicylic acid which changed from medium to low on the second analysis. The individual rankings within the group of high-level binders were the same as on the day of immobilization. Within the medium and low group some compounds shifted places, but only salicylic acid switched group from medium to low.

Discussion

Early estimates of pharmacokinetic properties can be used to complement data found on the interaction between a compound and its pharmacological target. The combined data can provide a broader understanding of the functional aspects of the compounds, and this may be useful for developing them into drug candidates. With

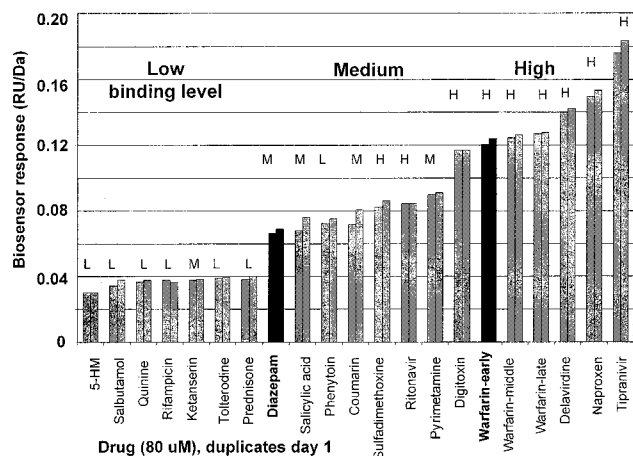


Figure 5. Ranking of binding levels of 19 compounds. The same set of compounds was ranked on the same sensor surface, in duplicate, on the day of HSA immobilization (shown) and in duplicate 1 week later (not shown). Diazepam and warfarin were used as marker compounds to group HSA binding levels into low, medium, or high. Warfarin was injected early, in the middle, and late in each assay as a control of the HSA binding activity. L, M, and H represent the classification into low-, medium-, or high-level binders based on reported literature levels of percent (%) bound.

respect to protein binding it is of particular interest to identify compounds that are highly bound to plasma proteins. We have developed a screening method to rank the binding of compounds to immobilized HSA, based on biosensor technology.

The use of different methodologies and experimental conditions for determination of HSA binding levels varies considerably, and for some compounds used in this study reported binding data was sometimes conflicting, making a fair correlation study difficult. Examples of variations in reported binding levels included warfarin (98.0–99.6%),^{12,30} diazepam (86.1–98.2%),^{17,14} and salicylic acid (81.7–90.5%).^{18,14} For other compounds only one literature value was found, but most likely a similar variation would be found if these compounds were assayed by several methodologies. Despite this it was possible to correlate biosensor data to HSA binding levels determined with other techniques.

The concentration 80 μ M was chosen for the ranking experiments with the purpose of demonstrating ranking of compounds over the entire affinity range. At this concentration the responses obtained for compounds with binding levels lower than 60% were still small, but since many compounds may be of limited solubility and since we wanted to reduce the influence of secondary binding sites, higher concentrations were not used. During preparation of this manuscript we have seen indications that the concentration may be reduced to 10–40 μ M, when focusing mainly on identification of high-level binders. To discriminate between low-affinity binders, an alternative approach with an inhibition study with HSA in solution and the drug target immobilized could be used.

By comparing dose–response curves and considering experimental errors of a few RU, molecular weight-adjusted R_{\max} values obtained with compounds binding to different sites were similar. This led us to assume that the binding sites were equally available. Dramati-

cally different site availability would have resulted in erroneous ranking of binding levels. By using warfarin and diazepam as reference compounds, classification into high-level (>95%) or low-level (<80%) binding was facilitated. Inspection of Figure 5 demonstrates that of the reported high-level binders sulfadimethoxine and ritonavir were classified as medium-level binders and digitoxin was on the border between the medium- and high-level binders. The reported value of 99% bound for ritonavir was obtained in plasma,¹⁰ and it has further been reported³¹ that a 10–20-fold reduction in plasma binding occurs on the addition of AGP, indicating a significant part of the plasma binding is to AGP. It could therefore be argued that in this respect only sulfadimethoxine was an outlier. Our ranking of ketanserin as a low-level binder is in conflict with the reported value of 93.2%.¹⁵ Others have reported a lower binding, 90.6% to plasma,³² which suggests a lower level of binding to HSA, but still the reason for the discrepancy is unknown at this point.

Along with these results, the excellent repeatability of the warfarin data and the stability of the HSA surface make the biosensor method well-suited for use in the prediction of protein binding levels. A particular advantage of the present method was that binding levels obtained with a single injection of the compound could be used for the correlation. One 96-well plate of compounds can be assayed in 24 h. The method is practical, it provides the necessary information, and complicated analysis is avoided. Furthermore ranking experiments can be complemented by competition studies in order to determine site specificity. In such experiments compounds with known site specificity are conjugated with another molecule to increase its mass (and therefore the signal). This marker is mixed with other compounds and a competition for binding site is achieved.³³

Our initial attempts to determine K_D values from the biosensor data showed that some information on the number of binding sites on HSA could be obtained. To calculate data for low-affinity binders when the concentration was well below the K_D concentration R_{eq}/MW was used in the fitting procedure. R_{\max} was then used as a global parameter to constrain the fit. This made K_D values more consistent than individual calculations of R_{\max} values for each compound. But the analysis was also complex due to difficulties in determining K_D for compounds with multiple binding sites. Despite the complexity in affinity determination, the interpretation of dose–response curves for high-affinity binders holds considerable potential for obtaining stoichiometric information.

Since SPR technology measures RI it is natural that future experiments include investigation of how differences in the RIs of compounds affect analysis. Some of the halogens are known to have a high RI, and we have noticed that compounds containing, for instance, bromine may give higher response values than anticipated from the molecular weight. Thus further studies along this line may be used to fine-tune the HSA binding data.

The possibility of studying in similar manner interactions with other plasma proteins such as AGP is obvious and ongoing in our laboratory. The first results from these studies are shown in Figure 6. Biosensor data were correlated against published AGP binding levels

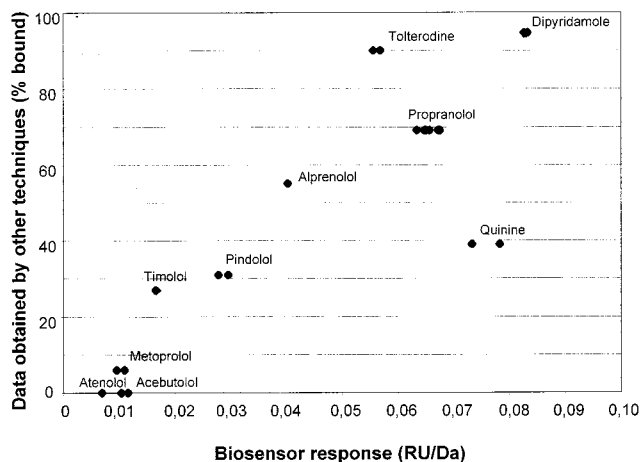


Figure 6. Biosensor data of AGP binding correlated to data obtained with other techniques, references last in Discussion. Prior to immobilization AGP (Sigma G-9885) was modified with PDEA (2-(2-pyridinyldithio)ethaneamine hydrochloride from Biacore). AGP (200 $\mu\text{g/mL}$ in 10 mM Na-citrate, pH 3.6) was then immobilized to a level of 7000 RU using thiol coupling.³⁷ All other experimental conditions were the same as for the HSA correlation study in Figure 4.

of tolterodine,²¹ quinine,²² seven β -adrenoceptor blocking drugs,^{34,35} and dipyrindamole.³⁶ A good correlation of binding was obtained with the exception of quinine which obtained a higher binding level compared to other techniques. All experimental conditions were the same as for the HSA correlation experiments, except the immobilization chemistry of AGP (Figure 6). It is therefore possible to immobilize HSA and AGP in two different flowcells, inject compounds, and obtain binding data to both proteins simultaneously.

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