Published in final edited form as:

J Chromatogr A. 2017 October 13; 1519: 64–73. doi:10.1016/j.chroma.2017.08.073.

Studies of Drug Interactions with Alpha1-Acid Glycoprotein by Using On-Line Immunoextraction and High-Performance Affinity Chromatography

Cong Bi¹, Ryan Matsuda¹, Chenhua Zhang¹, Zitha Isingizwe¹, William Clarke², and David S. Hage^{1,*}

¹Department of Chemistry, University of Nebraska, Lincoln, NE, USA

²Johns Hopkins University School of Medicine, Baltimore, MD, USA

Abstract

A method that combined on-line immunoextraction with high-performance affinity chromatography was developed to examine the binding of drugs with α_1 -acid glycoprotein (AGP). Affinity microcolumns containing immobilized polyclonal anti-AGP antibodies were developed that had a capture efficiency of up to 98.4% for AGP and a binding capacity of 0.72 nmol AGP when using a 20 mm $\times$ 2.1 mm i.d. microcolumn. These microcolumns were employed in various formats to examine the binding of drugs to normal AGP and AGP that had been adsorbed from serum samples for patients with systemic lupus erythematosus (SLE). Drugs that were screened in zonal elution experiments for their overall binding to these types of AGP included chlorpromazine, disopyramide, imipramine, propranolol, and warfarin. Most of these drugs showed an increase in their binding to the AGP from SLE serum when compared to normal AGP (i.e., an increase of 13–76%); however, disopyramide gave a 21–25% decrease in retention when the same AGP samples were compared. Frontal analysis was used to further evaluate the binding of disopyramide and imipramine to these forms of AGP. Both drugs gave a good fit to a model that involved a combination of saturable and non-saturable interactions with AGP. Changes in the non-saturable interactions accounted for most of variations seen in the binding of disopyramide and imipramine with the AGP samples. The methods used in this study could be adapted for use in personalized medicine and the study of other proteins or drugs using aqueous mixtures or clinical samples.

Keywords

Alpha₁-acid glycoprotein; High-performance affinity chromatography; Immunoextraction; Drug-protein interactions; Personalized medicine; Systemic lupus erythematosus

* Author for correspondence: Chemistry Department, University of Nebraska, Lincoln, NE 68588-0304 USA. Phone: 402-472-2744; FAX: 402-472-9402; dhage1@unl.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

The binding of drugs with serum proteins is important in determining the transport, excretion and metabolism of many pharmaceuticals in the body [1–3]. Alpha₁-acid glycoprotein (AGP) is a transport protein that is often involved in this process, with this protein binding to numerous basic and neutral drugs in blood [4–6]. Human AGP has an average molecular weight of 41 kDa and a normal serum concentration that ranges from 0.5–1.0 mg/mL [1,4,7]. However, the concentration of AGP can be altered in response to infection, trauma, acute illness, chronic inflammatory disorders, and diseases such as systemic lupus erythematosus (SLE) [4,5]. In addition, AGP has a high carbohydrate content (> 45% of the total weight of this protein), and modifications in its glycan chains have been observed in conditions such as rheumatoid arthritis, SLE, and some types of cancer [5,8,9].

SLE is a severe, chronic autoimmune disease in which the body's immune system attacks healthy tissues [9–11]. This is a highly heterogeneous condition that can affect the skin, joints, kidneys, brain, and other organs [11,12]. The onset of SLE can occur at any age; however, the incidence of this disease is highest in young women of childbearing age [11–13]. Previous studies have noted high plasma levels of AGP in patients with SLE [5]. In addition, a differential glycosylation pattern of AGP (i.e., an increase in bi-antennary glycans, particularly in patients who have accompanying infections) has been reported in SLE [5,9]. This suggests that the binding of some drugs with AGP may change during SLE due to the variation in the concentration of this protein or its glycoform pattern. However, little information exists on how the drug-binding properties of AGP may occur under these conditions.

There are several approaches that have been developed for the analysis of drug-interactions with proteins like AGP. These approaches have included separation methods such as equilibrium dialysis, ultrafiltration, liquid chromatography, and capillary electrophoresis (CE), and non-separation methods such as absorption or fluorescence spectroscopy, calorimetry, and surface plasmon resonance spectroscopy [3,14–19]. High-performance affinity chromatography (HPAC) is a separation method that has been used in various studies to examine drug-protein binding [1–3]. This technique utilizes a biologically-related ligand as the stationary phase and has been widely applied to the examination of drug-protein interactions by using columns that contain immobilized proteins like AGP or human serum albumin (HSA) [1,2,20]. Advantages of this method include its selectivity, speed, precision, ease of automation, and ability to work with small amounts of a target solute or binding agent [1,2,20].

When HPAC is employed for studying drug-protein interactions, an important factor to consider is the selection of the immobilization method to place the binding agent in the column. Several methods have been developed to immobilize AGP in HPAC columns [21–23]. For example, a covalent immobilization method has been described in which mildly-oxidized AGP is attached to hydrazide-activated silica [21]. Entrapment has also been employed, in which the physical containment of a protein such as AGP has been accomplished by using a hydrazide-activated support that is capped with oxidized glycogen [23]. However, these immobilization methods work best with a highly pure binding agent

and cannot be used directly with samples that contain complex mixtures of proteins, as occurs in serum [14].

On-line immunoextraction is an alternative method for column preparation that can be used to both isolate and immobilize a binding ligand that is present in a complex sample like serum [24,25]. Immunoextraction is a technique that utilizes immobilized antibodies against a given target to capture and isolate this target from a sample [24–26]. Recently, a method combining on-line immunoextraction with HPAC has been developed and shown to be a rapid means for examining drug interactions with the major serum protein HSA [24]. In this prior work, polyclonal antibodies against HSA were placed into a column, followed by the adsorption of this protein to the antibodies and the use of this adsorbed protein in drug binding studies, as is illustrated in Figure 1 [24,26].

This report will examine the extension and modification of on-line immunoextraction and HPAC to examine the interactions of drugs with a relatively low concentration protein, such as AGP that has been isolated from normal serum or serum from individuals with SLE. Both zonal elution and frontal analysis experiments will be conducted on the adsorbed samples of AGP and by using various drugs that are known to interact with this protein. The information obtained through this work should provide more detailed information on how the interactions of AGP with drugs may change during SLE. These results should make it possible to determine the advantages of using on-line immunoextraction and HPAC with relatively low concentration proteins like AGP for drug-binding studies. The same method should also be applicable to other types of drug-protein binding studies and in work with additional disease states, as could be used in clinical studies or for personalized medicine [24].

2. Experimental Section

2.1. Materials

The chlorpromazine (98% pure), *R/S*-disopyramide (98%), imipramine (99%), *R/S*-propranolol (99%), *R/S*-warfarin (98%), AGP (from pooled human plasma, 99%, product no. G9885), goat immunoglobulin G (IgG; reagent grade, 95% purity, lyophilized; product no. I5256), human serum (from pooled male AB plasma, sterile-filtered, product no. H4522), poly(ethylene oxide) (PEO; average molar mass, 8,000 kDa), Brij 35 (average molar mass, 1.198 kDa), and Lucifer yellow CH (LyCH, dilithium salt) were from Sigma-Aldrich (St. Louis, MO, USA). The polyclonal anti-human AGP antibodies (goat anti-AGP, affinity-purified, catalog no. GAGP-80A) were obtained from Lee Biosolutions (Maryland Heights, MO, USA). De-identified and pre-existing serum samples from patients with SLE were provided by W. Clarke (Note: this work was determined to be exempt from IRB review by the Johns Hopkins School of Medicine, according to the Code of Federal Regulations - 45 CFR 46.101 b). The Nucleosil Si-1000 (7 µm particle diameter, 1000 Å pore size) was from Macherey Nagel (Düren, Germany). Reagents for the micro bicinchoninic (BCA) protein assay were purchased from Pierce (Rockford, IL, USA). All other chemicals were of the purist grades available. All aqueous solutions were prepared using water from a Milli-Q Advantage A10 system (Millipore, Billerica, MA, USA) and were filtered through 0.20 µm

GNWP nylon membranes from Millipore. The Zeba spin desalting columns (0.5 mL, 7 kDa cutoff) were from Thermo Fisher Scientific (Rockford, IL, USA).

2.2. Apparatus

The affinity microcolumns were packed using an HPLC slurry packer from ChromTech (Apple Valley, MN, USA). The chromatographic system consisted of a DG-2080-53 degasser, two PU-2080 Plus pumps, an AS-2057 Plus autosampler, a CO-2067 Plus column oven, and a UV-2075 absorbance detector from Jasco (Easton, MD, USA). A six-port LabPro valve (Rheodyne, Cotati, CA, USA) was used in the HPLC system to switch between mobile phases. ChromNAV v1.18.04 software and LCNet from Jasco were employed to control the chromatographic system and collect data. The chromatograms and peaks were analyzed by using PeakFit 4.12 (Jandel Scientific, Rafael, CA, USA). Nonlinear regression for the frontal analysis data was conducted by using DataFit 8.1.69 (Oakdale, PA, USA).

The analysis of AGP glycoforms by CE was accomplished by utilizing a P/ACE MDQ instrument (Beckman Instruments, Fullerton, CA, USA). The capillary was maintained at 25°C and the applied potential was -30 kV. This separation used 60.2 cm × 50 µm I.D. fused silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) that had an effective length of 50 cm to the detector. The capillaries were modified by using both static and dynamic coatings of PEO [27]. Absorbance detection on the CE system was carried out at 200 nm.

2.3. Column preparation

Nucleosil 1000-7 silica was converted to a diol-bonded form as described previously [28]. This procedure involved first reacting the silica with 3-glycidioxypropyltrimethoxysilane in pH 5.5, 0.1 M sodium acetate buffer for 5 h at 90°C. The epoxy groups on this support were then hydrolyzed to form diol groups by placing the modified silica in a pH 3.0 sulfuric acid solution and refluxing this slurry for 1 h [28]. A 3 mg portion of affinity-purified anti-AGP antibodies was immobilized onto 300 mg of this modified silica by using the Schiff base method, according to methods reported in Refs. [23,29]. A control support was prepared in the same manner but with no antibodies being added during the immobilization step (Note: No significant amount of binding was noted between the drugs examined in this study and the anti-AGP antibodies, but some drugs did show non-specific binding to the support, for which a correction was made by using this control material). The final supports were stored at 4°C in pH 7.4, 0.067 M potassium phosphate buffer until use.

A micro BCA assay was conducted in triplicate to determine the amount of antibodies that were immobilized to the support for use in immunoextraction. Goat IgG was employed as the standard and the control support was used as the blank [24]. It was determined by this assay that the immunoextraction support had an antibody content of 18.3 (± 0.5) mg per g of silica, where the value in parentheses represents a range of 1 S.D.

The immunoextraction and control supports were downward slurry-packed into separate 20 mm × 2.1 mm i.d. columns at 4000 psi (28 MPa) using pH 7.4, 0.067 M potassium phosphate buffer as the packing solution. These columns were stored at 4°C in the same pH 7.4 phosphate buffer when not in use. All chromatographic experiments were conducted

within 3 months of column preparation and with less than 150 injections/sample applications being made per column.

2.4. Evaluation of antibody microcolumns

The extraction efficiency for the anti-AGP microcolumns was measured by injecting 20 μ L of 5 μ M normal AGP that had been prepared in pH 7.4, 0.067 M phosphate buffer. This sample was applied to an anti-AGP column or a control column at flow rates ranging from 0.05 to 0.50 mL/min and using pH 7.4, 0.067 M phosphate buffer as the mobile phase [24]. A pH 2.5, 0.10 M phosphate buffer was passed through the anti-AGP column at 0.10 mL/min for 20–25 min to elute the bound AGP, followed by re-equilibration and regeneration of this column with the original pH 7.4 buffer, which was applied at 0.10 mL/min for 20–30 min. A correction for the background response for the injected sample was made by also injecting 20 μ L of only the pH 7.4 buffer onto both the anti-AGP column and the control column. Triplicate injections were made for each sample and at each flow rate, and the absorbance was monitored at 280 nm. The peak area for each elution profile was analyzed by using a linear progressive baseline correction and an exponentially-modified Gaussian (EMG) fit.

Various AGP samples (i.e., normal AGP, normal control serum, and serum from SLE patients) were applied via frontal analysis to the anti-AGP columns. A 5 μ M AGP solution was used for the application of the purified AGP sample, as prepared in pH 7.4, 0.067 M potassium phosphate buffer. For the normal serum and SLE serum, 0.50 mL of each sample was first diluted by five-fold with 2.0 mL of pH 7.4, 0.067 M phosphate buffer. A syringe pump was then used to apply each AGP sample to an anti-AGP column at 0.10 mL/min and room temperature while the absorbance of the eluent was monitored at 280 nm. As the AGP sample was applied to the column, a breakthrough curve was obtained. The plateau for this curve was observed within 10 min under these conditions. A pH 7.4, 0.067 M buffer was then applied to each column at 0.10 mL/min for at least 30 min to remove any excess and unbound AGP or other chemicals. The same procedure was performed on a control column using a 5 μ M solution that contained purified, normal AGP to help determine the moles of binding sites that were present for this protein on the anti-AGP column. The data from this latter experiment were analyzed by using a Savitzky-Golay filter (first derivative) and an EMG fit. Any procedures that involved the use of serum samples were performed in BSL-2 facilities and handled according to standard procedures for dealing with materials that may contain blood borne pathogens.

2.5. Chromatographic studies of drug binding to AGP

In the zonal elution studies, 5 μ M of disopyramide, warfarin, imipramine, propranolol or chlorpromazine, and 20 μ M of sodium nitrate (which was used as a non-retained solute) [21,22,24] were prepared in pH 7.4, 0.067 M potassium phosphate buffer. A 20 μ L portion of each sample was applied to an antibody microcolumn containing adsorbed AGP (i.e., prepared as described in Sections 2.3–2.4) and a control column at 37 °C and 0.10 mL/min. These sample and injection conditions were found to provide linear elution conditions for each of the tested solutes. Sodium nitrate was also injected onto the system with a zero-volume union being used in place of the column to correct for the void time of the system.

Triplicate injections were made for each sample. The wavelengths that were used for absorbance detection were as follows: disopyramide, 262 nm; warfarin, 308 nm; imipramine, 250 nm; propranolol, 288 nm; chlorpromazine, 254 nm; and sodium nitrate, 205 nm. The retention time or elution time of each peak was found by using a linear progressive baseline correction and an EMG fit. A correction for the non-specific binding by each drug was made by using the retention measured on the control column (e.g., 19–26% of the total retention factor measured for disopyramide on an antibody microcolumn that contain adsorbed AGP).

Frontal analysis was performed in triplicate at 37 °C by continuously applying ten solutions that contained 0.5–100 µM disopyramide or imipramine in pH 7.4, 0.067 M potassium phosphate buffer to an anti-AGP column containing adsorbed AGP or to a control column at 0.10 mL/min. The retained drug was later eluted by applying only the pH 7.4 buffer onto the columns. The control column was used to correct for the void time and any non-specific binding due to interactions between the drug and support (e.g., 12–13% of the total binding measured for disopyramide on an anti-AGP column containing adsorbed AGP). The mean point of each breakthrough curve was determined by using a Savitzky-Golay filter (first derivative) and an EMG fit.

2.6. Estimation of AGP adsorbed to anti-AGP columns

The AGP that had been adsorbed to a column containing immobilized anti-AGP antibodies was eluted off by using pH 2.5, 0.067 M potassium phosphate buffer at 0.10 mL/min for 25 min. A peak for elution of the AGP appeared within 5 min after the mobile phase was switched to the pH 2.5 buffer. The column was then regenerated by equilibrating it with pH 7.4, 0.067 M phosphate buffer at 0.10 mL/min for at least 30 min. A series of standard AGP solutions were prepared in this pH 7.4 buffer and with AGP concentrations that ranged from 2.0 to 4.0 mg/mL. A 5 µL portion of each standard AGP was injected in triplicate onto the regenerated anti-AGP column and later eluted using the pH 2.5 buffer. The area of each eluted peak for the AGP standards was determined by using a linear progressive baseline correction and an EMG fit. The amount of AGP that had been bound to the anti-AGP columns was estimated by comparing the corresponding peak areas for the eluted AGP with the results that were obtained for the AGP standards on the same columns. The result was used in Sections 3.2–3.4 to normalize the specific retention factors and drug binding data for the amount of adsorbed AGP that was present in the anti-AGP microcolumns.

2.7 Serum pretreatment and analysis by CE

The CE method was a modified version of that described in Ref. [27], with the addition of acid precipitation and desalting steps for sample pretreatment. The acid precipitation of AGP from serum was performed according to a previous method [30] by using a 65 µL portion of serum that was combined and mixed with 130 µL of 0.5 M perchloric acid (Note: Use appropriate chemical hazard precautions when handling or using perchloric acid). The resulting sample was centrifuged at room temperature and 6,600 rpm for 10 min, with 130 µL of the supernatant then being placed into a Zeba spin desalting column and spun for another 2 min at 6,600 rpm. The filtrate was diluted with water to a total volume of 5 mL for CE analysis. The samples were electrophoretically injected by applying a potential of –5 kV

to a PEO-coated capillary for 5 min; a 0.5 g/L solution of LyCH in water was also applied by hydrodynamic injection after the sample at 0.1 psi for 2 s for use as a mobility marker.

3. Results and discussion

3.1. Evaluation of anti-AGP microcolumns

The extraction efficiency of the antibody microcolumns was examined by injecting 20 μ L of a 5 μ M normal AGP sample onto 20 mm $\times$ 2.1 mm i.d. anti-AGP microcolumns and control microcolumns at various flow rates. The extraction efficiency was obtained by comparing the non-retained peak areas that were obtained for AGP on these anti-AGP and control microcolumns. As is shown in Table 1, the extraction efficiency for AGP by the anti-AGP microcolumns increased from 91.4 ($\pm$ 0.6)% to 98.4 ($\pm$ 0.3)% as the flow rate decreased from 0.50 mL/min to 0.05 mL/min. Based on these results, a flow rate of 0.10 mL/min was employed in all of the following chromatographic studies. This condition gave an extraction efficiency of up to 95.3 ($\pm$ 0.2)% while also allowing relatively short analysis times to be achieved when the anti-AGP microcolumns were used with adsorbed AGP in drug binding studies (e.g., less than 3–12 min for the zonal elution experiments and less than 15 min for the frontal analysis experiments that were performed with drugs later in this report). The anti-AGP microcolumns and adsorbed AGP had both good stabilities and reproducibilities in their retention times and breakthrough times under the mobile phase conditions that were used in this study. For instance, a variation of less than $\pm$ 4–7% (1 S.D.) was noted in the retention factor for chlorpromazine over the course of three months when using either a single sample of adsorbed AGP or multiple adsorption/elution cycles for AGP on the anti-AGP microcolumns.

The binding capacity of the anti-AGP microcolumns for AGP was estimated by using frontal analysis. To do this, a 5 μ M solution of normal AGP was applied at 0.10 mL/min to a 20 mm $\times$ 2.1 mm i.d. anti-AGP microcolumn and a control microcolumn, as is illustrated in Figure 2. The binding capacity obtained for a typical anti-AGP microcolumn was 0.72 ($\pm$ 0.03) nmol AGP, or 10.4 ($\pm$ 0.4) nmol AGP/mL column volume. This result agreed with observations made in a previous study, in which binding capacities ranging from 0.34 to 0.42 nmol protein (or 9.8–12.1 nmol/mL column volume) were measured for normal HSA and glycated HSA on 10 mm $\times$ 2.1 mm i.d. microcolumns that contained anti-HSA antibodies [24].

The results from a BCA protein assay indicated that the support in a 20 mm $\times$ 2.1 mm i.d. anti-AGP column contained 4.0 ($\pm$ 0.1) nmol of antibodies. Combining these results with the frontal analysis data meant that 18 ($\pm$ 1)% of the immobilized anti-AGP antibodies was capable of binding to AGP. This result was consistent with a range of 13–16% that has been reported for the binding of HSA to anti-HSA antibodies on a similar support and using the same immobilization method [24]. The apparent loss of antibody activity in these columns could be the result of steric hindrance as the antibodies bind to a protein such as AGP or HSA, or it could reflect an actual loss of activity due to improper orientation or multisite attachment of the antibodies during the immobilization process [23]. It might be possible in future work to increase the level of this apparent activity by using more site-selective methods for antibody immobilization or by optimizing the spacing of the immobilized

antibodies on the support to minimize steric hindrance between neighboring antibodies [23,25].

3.2. Zonal elution studies for adsorbed AGP and various drugs

Zonal elution experiments were conducted with the anti-AGP microcolumns to give an initial comparison of the binding by various drugs (see Figure 3) with adsorbed samples of AGP. Several basic drugs from various classes that are known to bind to AGP were considered in these experiments, including disopyramide (pK_a , 10.4; an antiarrhythmic agent), chlorpromazine (pK_a , 9.2; an antipsychotic agent), imipramine (pK_a , 9.5; an antidepressant), and propranolol (pK_a , 9.6; a beta blocker) [6,31]. Warfarin (an acidic drug and anticoagulant; pK_a , 5) was also used as a model drug in this set of preliminary studies [6,31]. These drugs have reported binding constants for AGP that range from 10^4 – 10^6 M⁻¹ at or near a physiological pH [6,31]. This range of affinities has been shown in prior work to be suitable for the elution of drugs from columns containing immobilized serum proteins under isocratic conditions and in the presence of a pH 7.4 buffer, such as was employed in this study [1,2]. Table 2 summarizes the normalized retention factors that were obtained for each of these drugs on microcolumns that contained various types of adsorbed AGP, as prepared by using the equivalent of 0.5 mL for serum or an aqueous AGP sample. These results were corrected for the non-specific binding of the drug to the support (i.e., by using the retention data obtained on the control column) and were normalized for the protein content of the column by dividing the specific retention factor for each drug by the amount of AGP that was adsorbed to the antibody microcolumns (see Section 2.6).

A comparison was made between the results for the antibody microcolumns containing AGP from an SLE patient, containing normal AGP from a purified sample of this protein, or containing AGP that was adsorbed from a control sample of pooled normal serum. Some typical chromatograms that were obtained in this comparison are shown in Figure 4. The retention obtained with the normal purified sample of AGP was consistent with that observed with AGP from normal serum at the 98% confidence level for imipramine and at the 95% confidence level for all of the other drugs that were tested. The order of the retention factors for these drugs on the columns containing the adsorbed samples of normal AGP also gave good general agreement with the order of affinities that have been previously reported for these drugs with AGP [6]. It was found that the retention increased significantly (at the 95% confidence level) and by 13–76% for most of the drugs in going from the normal AGP to the AGP that was adsorbed from SLE serum, as was seen for imipramine, chlorpromazine, propranolol and warfarin. However, a 21–25% decrease in retention, which was significant at the 95% confidence level, was seen for disopyramide under the same conditions. Based on these results, disopyramide and imipramine were selected for use in further studies because these drugs represented two different scenarios for the changes in their interactions with AGP in going from the normal samples to the SLE sample.

3.3. Frontal analysis studies with disopyramide

In the next set of studies, frontal analysis was employed to further examine the interactions between disopyramide and normal AGP or AGP from an SLE serum sample. Figure 5 shows some breakthrough curves that were obtained when using a 20 mm × 2.1 mm i.d. anti-AGP

microcolumn and containing an adsorbed sample of normal AGP. The moles of applied drug that were required to reach the mean position of each breakthrough curve (m_{Lapp}) was determined and used along with the applied concentration of the analyte or drug [A] to generate binding isotherms. Various binding models (see Table 3 and Figure 6) were compared to these data to determine the type of interactions that were present for disopyramide with the adsorbed AGP [32]. These fits also made it possible to determine the number of binding sites that were present for this drug with the adsorbed AGP and the equilibrium constants for these interactions [24,32].

A one-site binding model, as based on Eq. (1) in Table 3, gave only a relatively modest fit to the data for disopyramide and the adsorbed AGP. The results for normal AGP are illustrated in Figure 6(a). This particular fit had a correlation coefficient of 0.9699 ($n = 10$), a non-uniform distribution of the data points about the best-fit line, and a sum of the squares of the residuals of 1.3×10^{-20} . A two-site binding model and a mixed-mode model were also fit to these binding data, as described by Eqs. (2–3) and as shown in Figure 6(b–c). Table 4 summarizes the best-fit results that were obtained for disopyramide with normal AGP, as well as the correlation coefficients, sums of the squares of the residuals and precisions of the binding models that were obtained. The two-site and mixed-mode models both gave higher correlation coefficients than the one-site model (i.e., 0.9996 for both models), lower sums of the squares of the residuals (1.8×10^{-22} , a difference significant at the 95% confidence level), and a random distribution of the data points about the best-fit line. All of these factors indicated that either of these two models was better at describing this system than a one-site binding model.

Although the two-site and mixed-mode models initially appeared to give identical fits to the disopyramide binding data for normal AGP, differences were found when comparing the precisions of the resulting best-fit parameters. As is shown in Table 4, these two models gave similar values for the association equilibrium constant and moles of binding sites that were estimated to be present for the first saturable site for disopyramide on the normal AGP (i.e., the values listed for K_{d1} and m_{L1} in Table 4). However, the additional binding parameter for the mixed-mode model had much higher precision than the two additional parameters that were required in the two-site model (i.e., relative precisions of $\pm 2.6\%$ vs. $\pm 170\text{--}180\%$, respectively). This difference indicated that the mixed-mode model gave a better description of this data set. The same type of model has been found in prior work to be the best description for interactions by disopyramide and a number of other drugs (e.g., propranolol) with immobilized or soluble AGP [33–35].

Similar frontal analysis studies were carried out with disopyramide on antibody microcolumns that contained AGP which had been adsorbed from normal serum or an SLE serum sample (i.e., the same samples as used in Table 2). Among the three binding models that were tested, the mixed-mode model was consistently found to provide the best fit for disopyramide with each type of adsorbed AGP. The correlation coefficients that were obtained with this model and the AGP for the normal and SLE serum samples were 0.9966–0.9997 ($n = 9\text{--}11$) and the sums of the squares of the residuals for disopyramide with both AGP/serum samples was 2.0×10^{-22} . In these fits, only random variations of the data points about the best-fit lines were also again noted.

These fits indicated that disopyramide had two types of interactions with the AGP: a saturable interaction and a non-saturable, partition-like interaction. The first, saturable interaction probably took place with the polypeptide chain of AGP, as has been determined to be the case for other drugs with this glycoprotein [36]. One possible source of the non-saturable interaction may be the glycan chains of AGP, because the binding of drugs such as disopyramide has been observed in prior work to change as the degree of glycan branching in AGP is altered in some disease states [5,37,38]. The presence of a difference in the glycans of the normal and SLE-derived AGP was confirmed by using CE [27], as is illustrated in Figure 7 for the serum samples that were also used in Table 2 and for the frontal analysis studies with disopyramide. During this analysis there was an observed shift in the distribution of the glycoforms in the AGP that was obtained from normal serum versus SLE serum. The various glycoform bands that were obtained for these samples each differed by a charge of one unit, with the charge in each band increasing as the migration time decreased [27]. The shift in the profile that was seen between the normal and SLE-derived AGP (i.e., to glycoforms with lower charges and longer migration times) could have been obtained by a net decrease in the sialic acid content and degree of branching in the AGP [27].

Table 5 summarizes the binding parameters that were obtained by the mixed-mode model for disopyramide with either normal AGP or AGP from a sample of SLE serum. The normal AGP had an estimated association equilibrium constant that ranged from $7.4 (\pm 1.7) \times 10^5$ to $9.0 (\pm 1.6) \times 10^5 \text{ M}^{-1}$ for disopyramide at its saturable site, which was in good agreement with previous values that have been reported for the same interaction with soluble AGP [35]. The association equilibrium constant measured by frontal analysis for disopyramide with the AGP from SLE serum was $6.7 (\pm 1.3) \times 10^5 \text{ M}^{-1}$. Although there may have been a slight decrease of 9–26% in this value when going from normal AGP to SLE-derived AGP, only the difference in going from normal and purified AGP to the AGP from SLE serum was significant at the 95% confidence level. No appreciable variation was seen in the amount of these saturable sites for the various types of AGP when the results in Table 5 were normalized for the amount of AGP that was adsorbed within the anti-AGP microcolumn.

A large change was observed in the value of the binding parameter nK_a for disopyramide and the non-saturable interactions of this drug with AGP. This value decreased from $1.10\text{--}1.34 \times 10^4 \text{ M}^{-1}$ for normal AGP to $0.26 (\pm 0.03) \times 10^4 \text{ M}^{-1}$ for the AGP from SLE serum. The size of these values was consistent with the general range of 10^4 M^{-1} that has been previously estimated for these interactions with soluble and normal AGP [35]. The change seen in this present study for the nK_a value of disopyramide was significant at the 95% confidence level and corresponded to a 4- to 5-fold decrease in going from normal AGP to the SLE-derived AGP. This decrease in the strength of the non-saturable interaction agreed with the zonal elution results in Section 3.2, in which the retention for disopyramide decreased for adsorbed AGP from the *same* SLE sample when compared to the adsorbed samples of normal AGP. These results suggest that changes in the non-saturable interactions, rather than changes in binding at the saturable site, were responsible for much of the decrease in overall binding that were seen for AGP with disopyramide for the SLE sample.

3.4. Frontal analysis studies with imipramine

The interactions between imipramine and anti-AGP microcolumns containing adsorbed samples of AGP were also investigated by using frontal analysis. Figure 8 shows typical fits to the various models that were obtained for imipramine, as illustrated here for AGP that was isolated from SLE serum (Note: This was a separate serum sample from that used in Section 3.3 for disopyramide but which gave comparable values to the normalized retention values that are listed in Table 2 for the various drugs that were used in this study). Table 4 includes the best-fit parameters that were acquired for the same binding models when used to examine the interactions of imipramine with normal AGP. Similar patterns to the fits seen in Figure 8 and Table 4 were observed for imipramine with the other AGP samples that were investigated in this study.

As was seen for disopyramide, the mixed-mode and two-site binding models consistently gave higher correlation coefficients for the imipramine data than the one-site model (e.g., see Table 4). The ranges of these correlation coefficients for the various AGP samples were 0.9993–0.9999 and 0.9997–0.9999 for the mixed-mode and two-site models, respectively, and 0.9991–0.9995 for the one-site model. The mixed-mode and two-site models also gave smaller residuals and a more random distribution of these residuals about the best-fit lines, as demonstrated by the insets in Figure 8. In addition, the sum of the squares of the residuals for one or both these models was significantly lower, at the 95% confidence level, than the value obtained with the one-site model in each type of AGP sample. All of these results were consistent with the trends seen earlier for the binding of disopyramide with AGP and agree with previous observations made for the binding of imipramine with soluble AGP under similar conditions [35].

A comparison was next made of the fits for the mixed-mode and two-site models. These two models gave similar correlation coefficients and residuals for their fits; however, the mixed-mode model gave much higher precisions for its best-fit parameters. For example, the results for K_{a1} and m_{L1} for imipramine and the two-site model in Table 4 had variations in their values of ± 63 –124%, while the comparable values in the mixed-mode model had variations of ± 35 –36%. A similar trend in the precision was observed for the other AGP samples that were used in this study. As was the case for disopyramide, these trends indicated that a mixed-mode model was a better description for the interactions of imipramine with AGP than a two-site model.

Table 5 summarizes the best-fit parameters that were acquired with the mixed-mode model when fitting the frontal analysis data for imipramine on microcolumns that contained adsorbed samples of normal AGP or AGP from SLE serum. The correlation coefficients were 0.9993–0.9997 ($n = 10$ –11) and 0.9999 ($n = 12$), respectively, when a mixed-mode model was fit to these data, and the corresponding sums of the squares of the residuals were 6.2 – 18×10^{-20} and 6.2×10^{-21} . The association equilibrium constants measured for the saturable site of imipramine on AGP varied from 2.1 – $2.2 \times 10^4 \text{ M}^{-1}$ for the samples of normal AGP to $2.6 \times 10^4 \text{ M}^{-1}$ for the SLE sample of AGP. These values were not significantly different at the 95% confidence level and were only slightly lower than previous estimates in the range of 10^4 – 10^5 M^{-1} that have been obtained for the binding of imipramine with normal AGP when using one-site or mixed-mode models [35]. No

significant change was also seen in the amount of saturable sites for imipramine for the various types of AGP that were tested when these values were normalized for the amount of adsorbed AGP.

However, the value of nK_a that was obtained for the non-saturable interactions of imipramine did show an increase of 1.6- to 4.2-fold in going from values of $2.8\text{--}7.5 \times 10^4 \text{ M}^{-1}$ for normal AGP to a value of $11.7 (\pm 0.6) \times 10^4 \text{ M}^{-1}$ for the AGP from SLE serum. This increase was significant at the 95% confidence level, with the results for normal AGP being consistent with a prior estimate made for imipramine with soluble AGP [35]. This change was consistent with the zonal elution experiments discussed in Section 3.2, which showed that stronger retention was present for imipramine on a microcolumn that contained AGP from SLE serum versus normal AGP. As was the case for disopyramide, the correlation between the zonal elution behavior and frontal analysis results suggested that the observed changes in the interactions of imipramine with normal versus SLE-derived AGP may have resulted from variations in the carbohydrate chains of this glycoprotein [5].

4. Conclusion

This report investigated the combined use of HPAC and on-line immunoextraction with antibody microcolumns to study binding by the transport protein AGP with various drugs. This approach was used to adsorb AGP directly from aqueous samples or serum, with an extraction efficiency of up to 98.4% at an application flow rate of 0.05 mL/min (or 95.3% at 0.10 mL/min) and a binding capacity of 0.72 nmol AGP for a 20 mm $\times$ 2.1 mm i.d. microcolumn. The anti-AGP microcolumns and adsorbed samples of AGP gave good reproducibility in their retention behavior when used over the course of up to three months. In addition, these microcolumns and this approach provided results within 3–15 min of sample injection or application.

These columns were first used in zonal elution studies to screen and compare the binding of several drugs with AGP. This was done by using both purified AGP and AGP that had been isolated from the equivalent of 0.5 mL of normal or SLE serum. Most of these drugs, including imipramine, showed an increase in their binding to the AGP from SLE serum when compared to normal AGP; however, disopyramide gave a decrease in retention when the same AGP samples were compared. Frontal analysis was used to further evaluate the binding of disopyramide and imipramine to these forms of AGP. Both drugs gave a good fit to a model that involved both saturable and non-saturable interactions with AGP. Changes in the non-saturable interactions accounted for most of variations seen in the binding of disopyramide and imipramine with the normal vs. SLE-derived AGP samples. It was suggested that these changes in the non-saturable interactions may be related to structural changes in the carbohydrate groups of AGP, as was supported by analysis of the glycoform patterns by CE. Further studies are planned or are in progress to look at these structural changes in more detail and to see if similar changes in binding occur with other drugs that are known to interact with AGP.

It was shown through these studies how on-line immunoextraction and HPAC can be combined and used to directly place a relatively low concentration serum protein such as

AGP within an affinity column and to use this adsorbed protein for drug screening or detailed binding studies without the need for extra procedures for protein purification or immobilization. The ability to directly isolate and use such a protein from serum makes this method attractive as a less time-consuming alternative to the covalent immobilization methods that are often used to prepare HPAC columns for drug-protein binding studies [1,2]. In addition, the antibody microcolumns that were developed in this study were sufficiently stable and robust to allow even a single anti-AGP microcolumn to adsorb AGP from various samples and to carry out studies with many drugs or drug concentrations for each application of AGP. The use of these methods and tools is not only limited to AGP but could be extended to the study of alternative drug-protein systems by using other antibodies to prepare the microcolumns that are used for on-line immunoextraction. In addition, these methods could be adapted for use in personalized medicine, as demonstrated here in the work with SLE samples, or for examining other types of interactions in biological or clinical samples [2,20].

Acknowledgments

This work is supported by National Institutes of Health under grant R01 GM044931. R. Matsuda was supported, in part, by a fellowship under the University of Nebraska-Lincoln (UNL) Molecular Mechanisms of Disease program, and Z. Isingizwe was supported through the National Science Foundation under the UNL Chemistry Department's Research Experience for Undergraduates (REU) program.

References

1. Hage DS, Anguizola J, Barnaby O, Jackson A, Yoo MJ, Papastavros E, Pfaunmiller E, Sobansky M, Tong Z. Characterization of drug interactions with serum proteins by using high-performance affinity chromatography. *Curr Drug Metab*. 2011; 12:313–328. [PubMed: 21395530]
2. Hage DS, Anguizola JA, Jackson AJ, Matsuda R, Papastavros E, Pfaunmiller E, Tong Z, Vargas-Badilla J, Yoo MJ, Zheng X. Chromatographic analysis of drug interactions in the serum proteome. *Anal Methods*. 2011; 3:1449–1460.
3. Vuignier K, Schappeler J, Veuthey JL, Carrupt PA, Martel S. Drug–protein binding: a critical review of analytical tools. *Anal Bioanal Chem*. 2010; 398:53–66. [PubMed: 20454782]
4. Fournier T, Medjoubi-N N, Porquet D. Alpha-1-acid glycoprotein. *Biochim Biophys Acta*. 2000; 1482:157–171. [PubMed: 11058758]
5. Ceciliani F, Pocacqua V. The acute phase protein alpha₁-acid glycoprotein: a model for altered glycosylation during diseases. *Curr Protein Pept Sci*. 2007; 8:91–108. [PubMed: 17305563]
6. Israili ZH, Dayton PG. Human alpha-1-glycoprotein and its interactions with drugs. *Drug Metab Rev*. 2001; 33:161. [PubMed: 11495502]
7. Zsila F, Iwao Y. The drug binding site of human alpha₁-acid glycoprotein: Insight from induced circular dichroism and electronic absorption spectra. *Biochim Biophys Acta*. 2007; 1770:797–809. [PubMed: 17321687]
8. Doak K., Flaws J., Cruickshank, Y., Smith, KD. Alterations in the glycosylation pattern of alpha-1-glycoprotein may be diagnostic for the detection of breast cancer and/or reducing the effect of chemotherapy in vivo. In: Schweizer, LS., Krebs, SJ., editors. *Oligosaccharides: Food Sources, Biological Roles and Health Implications*. Nova Science Publishers; New York: 2014. p. 143-172.
9. Mackiewicz A, Marcinkowska-Pieta R, Ballou S, Mackiewicz S, Kushner I. Microheterogeneity of alpha 1-acid glycoprotein in the detection of intercurrent infection in systemic lupus erythematosus. *Arthritis Rheum*. 1987; 30:513–518. [PubMed: 3593435]
10. Lacki JK, Samborski W, Mackiewicz SH. Interleukin-10 and interleukin-6 in lupus erythematosus and rheumatoid arthritis, correlations with acute phase proteins. *Clin Rheumatol*. 1997; 16:275–278. [PubMed: 9184265]

11. Mathian A, Hie M, Cohen-Aubart F, Amoura Z. Targeting interferons in systemic lupus erythematosus: current and future prospects. *Drugs*. 2015; 75:835–846. [PubMed: 25940912]
12. Ghodke-Puranik Y, Niewold TB. Immunogenetics of systemic lupus erythematosus: a comprehensive review. *J Autoimmun*. 2015; 64:125–136. [PubMed: 26324017]
13. Rigante D, Esposito S. Infections and systemic lupus erythematosus: binding or sparring partners? *Int J Mol Sci*. 2015; 16:17331–17343. [PubMed: 26230690]
14. Burton, ME., Shaw, LM., Schentag, JJ., Evans, WE. *Applied Pharmacokinetics & Pharmacodynamics: Principles of Therapeutic Drug Monitoring*. Lippincott; Baltimore, MD: 2006.
15. Hage DS. Chromatographic and electrophoretic studies of protein binding to chiral solutes. *J Chromatogr A*. 2001; 906:459–481. [PubMed: 11215902]
16. Matsuda R, Bi C, Anguizola J, Sobansky M, Rodriguez E, Vargas Badilla J, Zheng X, Hage B, Hage DS. Studies of metabolite-protein interactions: a review. *J Chromatogr B*. 2014; 966:48–58.
17. Vuignier K, Veuthey JL, Carrupt PA, Schappler J. Global analytical strategy to measure drug-plasma protein interactions: from high-throughput to in-depth analysis. *Drug Discov Today*. 2013; 18:1030–1034. [PubMed: 23603634]
18. Garcia JJ, Ghaly ES. Protein binding of glipizide using equilibrium dialysis technique: effects of hydrogen ion concentration, drug concentration and ionic strength. *P R Health Sci J*. 2001; 20:31–34. [PubMed: 11394214]
19. Tang DQ, Li YJ, Li Z, Bian TT, Chen K, Zheng XX, Yu YY, Jiang SS. Study on the interaction of plasma protein binding rate between edaravone and taurine in human plasma based on HPLC analysis coupled with ultrafiltration technique. *Biomed Chromatogr*. 2015; 29:1137–1145. [PubMed: 25545282]
20. Hage DS, Anguizola JA, Bi C, Li R, Matsuda R, Papastavros E, Pfaunmiller E, Vargas J, Zheng X. Pharmaceutical and biomedical applications of affinity chromatography: recent trends and developments. *J Pharm Biomed Anal*. 2012; 69:93–105. [PubMed: 22305083]
21. Xuan H, Hage DS. Immobilization of α_1 -acid glycoprotein for chromatographic studies of drug-protein binding. *Anal Biochem*. 2005; 346:300–310. [PubMed: 16225836]
22. Bi C, Jackson A, Vargas-Badilla J, Li R, Rada G, Anguizola J, Pfaunmiller E, Hage DS. Entrapment of α_1 -acid glycoprotein in high-performance affinity columns for drug-protein binding studies. *J Chromatogr B*. 2016; 1021:188–196.
23. Kim, HS., Hage, DS. Immobilization methods for affinity chromatography. In: Hage, DS., editor. *Handbook of Affinity Chromatography*. 2nd. CRC Press; Boca Raton: 2006. p. 35-78.
24. Matsuda R, Jobe D, Beyersdorf J, Hage DS. Analysis of drug-protein binding using on-line immunoextraction and high-performance affinity microcolumns: Studies with normal and glycated human serum albumin. *J Chromatogr A*. 2015; 1416:112–120. [PubMed: 26381571]
25. Hage, DS., Phillips, TM. Immunoaffinity chromatography. In: Hage, DS., editor. *Handbook of Affinity Chromatography*. 2nd. CRC Press; Boca Raton: 2006.
26. Ruhn PF, Taylor JD, Hage DS. Determination of urinary albumin using high-performance immunoaffinity chromatography and flow injection analysis. *Anal Chem*. 1994; 66:4265–4271. [PubMed: 7847629]
27. Zhang C, Hage DS. Glycoform analysis of α_1 -acid glycoprotein by capillary electrophoresis. *J Chromatogr A*. 2016; 1475:102–109. [PubMed: 27863712]
28. Ruhn PF, Garver S, Hage DS. Development of dihydrazide-activated silica supports for high-performance affinity chromatography. *J Chromatogr A*. 1994; 669:9–19. [PubMed: 8055106]
29. Larsson PO. High-performance liquid affinity chromatography. *Methods Enzymol*. 1984; 104:212–223. [PubMed: 6371445]
30. Stumpe M, Miller C, Morton NS, Bell G, Watson DG. High performance liquid chromatographic determination of α_1 -acid glycoprotein in small volumes of plasma from neonates. *J Chromatogr B*. 2006; 831:81–84.
31. Kratochwil NA, Huber W, Muller F, Kansy M, Gerber PR. Predicting plasma protein binding of drugs: a new approach. *Biochem Pharmacol*. 2002; 64:1355–1374. [PubMed: 12392818]
32. Sobansky MR, Hage DS. Identification and analysis of stereoselective drug interactions with low-density lipoprotein by high-performance affinity chromatography. *Anal Bioanal Chem*. 2012; 403:563–571. [PubMed: 22354572]

33. Mallik R, Xuan H, Guiochon G, Hage DS. Immobilization of α_1 -acid glycoprotein for chromatographic studies of drug-protein binding. II. correction for errors in association constant measurements. *Anal Biochem.* 2008; 376:154–156. [PubMed: 18294445]
34. Handa K, Ohta T, Hirai M, Arai M, Ogata H. Enantioselective binding of propranolol, disopyramide, and verapamil to human α_1 -acid glycoprotein. *J Pharm Sci.* 2000; 89:751–757. [PubMed: 10824133]
35. Beeram S, Bi C, Zheng X, Hage DS. Chromatographic studies of drug interactions with α_1 -acid glycoprotein by ultrafast affinity extraction and peak profiling. *J Chromatogr A.* in press.
36. Lambrinidis G, Vallianatou T, Tsantili-Kakoulidou A. In vitro, in silico and integrated strategies for the estimation of plasma protein binding. A review, *Adv Drug Delivery Rev.* 2015; 86:27–45.
37. Kishino S, Nomura A, Itoh S, Nakagawa T, Takekuma Y, Sugawara M, Furukawa H, Todo S, Miyazaki K. Age- and gender-related differences in carbohydrate concentrations of α_1 -acid glycoprotein variants and the effects of glycoforms on their drug-binding capacities. *Eur J Clin Pharmacol.* 2002; 58:621–628. [PubMed: 12483455]
38. Kuroda Y, Kita Y, Shibukawa A, Nakagawa T. Role of biantennary glycans and genetic variants of human α_1 -acid glycoprotein in enantioselective binding of basic drugs as studied by high performance frontal analysis/capillary electrophoresis. *Pharmaceut Res.* 2001; 18:389–93.

Highlights

- Microcolumns were made for the immunoextraction of alpha1-acid glycoprotein (AGP)
- These microcolumns were used on-line in an HPLC method to study drug binding
- Both normal AGP and AGP from serum samples were directly examined by this method
- Many of the tested drugs gave altered binding to AGP in serum from patients with lupus
- This approach allowed detailed studies of the mechanisms of these interactions

Figure 1.
General scheme for using on-line immunoextraction in drug-protein binding studies based on an affinity microcolumn containing immobilized antibodies against a given target protein.

Figure 2.

Typical chromatograms obtained in frontal experiments when continuously applying a 5 μ M solution of purified AGP, in pH 7.4, 0.067 potassium phosphate buffer, to a 20 mm $\times$ 2.1 mm i.d. antibody microcolumn containing immobilized anti-AGP antibodies (dashed line) or a control microcolumn (solid line). The flow rate used for application of the AGP solution was 0.10 mL/min.

Figure 3. Structures of the model drugs that were examined in this study for their binding with AGP that had been adsorbed onto anti-AGP microcolumns. The chiral centers in disopyramide, propranolol and warfarin are indicated by asterisks (*).

Figure 4.

Typical chromatograms obtained during zonal elution studies for the injection of 5 μ M (a) disopyramide or (b) imipramine onto anti-AGP microcolumns containing adsorbed AGP from SLE serum or normal serum. The particular results were obtained at 0.10 mL/min and using a detection wavelength of 262 nm for disopyramide or 250 nm for imipramine.

Figure 5.

Typical chromatograms obtained when using frontal analysis and various concentrations of disopyramide that were applied at 0.10 mL/min to a 20 mm × 2.1 mm i.d. anti-AGP microcolumn containing an adsorbed sample of normal, purified AGP.

Figure 6.

Fits of frontal analysis data to (a) one-site, (b) two-site, or (c) mixed model binding models for disopyramide on anti-AGP microcolumns containing adsorbed samples of normal, purified AGP. The insets show the residual plots for the fit of each model to the experimental data. Each data point in these plots represents the average of triplicate experiments. The values obtained for m_{Lapp} had relative precisions that ranged from ± 2.8 –13.2%, with an average of $\pm 7.0\%$.

Figure 7.

(a) Electropherograms and (b) distribution of glycoform bands obtained for AGP that was isolated from normal serum or SLE serum. The horizontal and vertical error bars in (b) represent at range of ± 1 S.D. in the migration time or % total peak area values, respectively, as obtained for triplicate injections and are similar in size to the symbols used in this plot.

Figure 8. Frontal analysis data fit to (a) one-site, (b) two-site, or (c) mixed-mode binding models for imipramine on anti-AGP microcolumns containing adsorbed samples of AGP from SLE serum. The insets show the residual plots for the fit of each model to the experimental data. Each data point in these plots represents the average of triplicate experiments. The values obtained for m_{Lapp} had relative precisions that ranged from ± 0.6 –13.3%, with an average of $\pm 4.0\%$.

Table 1

Extraction efficiencies measured for AGP at various flow rates on a 20 mm × 2.1 mm i.d. immunoextraction column containing immobilized anti-AGP antibodies^a

Flow rate (mL/min)	Extraction efficiency (%)
0.05	98.4 (± 0.3)
0.10	95.3 (± 0.2)
0.25	93.1 (± 0.5)
0.50	91.4 (± 0.6)

^aThese results were obtained at 37°C and pH 7.4. The values in the parenthesis represent a range of ± 1 S.D. ($n = 3$).

Table 2

Normalized specific retention factors for various drugs on an immunoextraction column containing adsorbed samples of normal AGP or AGP from SLE serum^a

Drug	Normalized specific retention factor (<i>k</i> /nmol AGP) ^b		
	Purified normal AGP	AGP from serum	normal AGP from SLE serum
Disopyramide	1.9 (± 0.2)	2.0 (± 0.1)	1.5 (± 0.1)
Chlorpromazine	34.2 (± 0.8)	34.7 (± 0.6)	39.2 (± 1.5)
Imipramine	3.9 (± 0.3)	4.7 (± 0.1)	5.8 (± 0.3)
Propranolol	3.1 (± 0.1)	3.4 (± 0.1)	4.1 (± 0.3)
Warfarin	2.2 (± 0.3)	2.1 (± 0.2)	3.7 (± 0.2)

^aThese results were obtained at 37°C and pH 7.4. The values in the parenthesis represent a range of ± 1 S.D. (*n* = 3).

^bEach normalized retention factor was calculated by dividing the specific retention factor (*k*) by the moles of AGP that had been adsorbed to a 20 mm × 2.1 mm i.d. immunoextraction column. The specific retention factors were corrected for the non-specific binding of drugs to the support; these corrections for disopyramide, chlorpromazine, imipramine, propranolol, or warfarin were typically 19–26%, 43–56%, 48–64%, 35–48%, or 40–56% of the total retention seen on the immunoextraction columns containing AGP. The amounts of adsorbed AGP for the immunoextraction columns that were prepared using normal purified AGP, normal serum, or SLE serum were 0.35 (± 0.01), 0.41 (± 0.08), and 0.36 (± 0.01) nmol, respectively.

Table 3

Binding models used to examine frontal analysis data obtained for several drugs with various types of adsorbed AGP [32]

Binding model	Predicted Response	
<i>One-site binding model</i> (Single group of saturable sites)	$m_{Lapp} = \frac{m_{L1} K_{a1} [A]}{(1 + K_{a1} [A])}$	Eq. (1)
<i>Two-site binding model</i> (Two groups of saturable sites)	$m_{Lapp} = \frac{m_{L1} K_{a1} [A]}{(1 + K_{a1} [A])} + \frac{m_{L2} K_{a2} [A]}{(1 + K_{a2} [A])}$	Eq. (2)
<i>Mixed-mode model</i> (Saturable + non-saturable sites)	$m_{Lapp} = \frac{m_{L1} K_{a1} [A]}{(1 + K_{a1} [A])} + m_{L2} K_a [A]$	Eq. (3)

Terms: [A], concentration of the applied drug or analyte; m_{Lapp} , moles of applied analyte required to reach the mean position of the breakthrough curve; m_{L1} and m_{L2} , total active moles of binding sites 1 and 2 for the drug or analyte on the immobilized binding agent; K_{a1} and K_{a2} , association equilibrium constants for the drug or analyte at saturable sites 1 and 2; K_a , equilibrium constant for the drug or analyte as part of a non-saturable interaction.

Best-fit parameters obtained by frontal analysis and various binding models for the interactions of disopyramide and imipramine with normal and purified AGP^a

Table 4

Drug & binding model	Highest affinity saturable sites			Lower affinity saturable sites		Non-saturable sites		Correlation coefficient (r)	Sum of squares of residuals ($\times 10^{-22}$)
	m_{L1} ($\times 10^{-11}$ mol)	K_{a1} ($\times 10^4$ M $^{-1}$)	m_{L2} ($\times 10^{-9}$ mol)	K_{a2} ($\times 10^2$ M $^{-1}$)	$m_{L2}K_a$ ($\times 10^{-6}$ L)				
Disopyramide									
One-site model	71 (± 17)	1.7 (± 0.8)	—	—	—	—	—	0.9699 ($n = 10$)	130
Two-site model	9.4 (± 0.9)	97 (± 25)	96 (± 167)	4.2 (± 7.7)	—	—	—	0.9996 ($n = 10$)	1.8
Mixed-mode model	9.8 (± 0.5)	90 (± 16)	—	—	3.8 (± 0.1)	—	—	0.9996 ($n = 10$)	1.8
Imipramine									
One-site model	440 (± 20)	0.95 (± 0.08)	—	—	—	—	—	0.9991 ($n = 11$)	85
Two-site model	8.4 (± 5.3)	49 (± 61)	5.0 (± 0.4)	71 (± 11)	—	—	—	0.9997 ($n = 11$)	27
Mixed-mode model	1.7 (± 0.6)	2.2 (± 0.8)	—	—	10 (± 3)	—	—	0.9993 ($n = 11$)	62

^aThe results were obtained at 37°C and pH 7.4. The values in the parenthesis represent a range of ± 1 S.D. ($n = 3$).

Table 5

Comparison of best-fit parameters obtained by fitting frontal analysis data to a mixed-mode model for disopyramide or imipramine interacting with various types of adsorbed AGP^a

Drug & type of AGP	Saturable sites		Non-saturable sites	
	$m_{L1} (\times 10^{-11} \text{ mol})$	$K_{a1} (\times 10^4 \text{ M}^{-1})$	$m_{L2}K_a (\times 10^{-6} \text{ L})$	$nK_a (\times 10^4 \text{ M}^{-1})^b$
Disopyramide				
Normal, purified AGP	9.8 (± 0.5)	90 (± 16)	3.8 (± 0.1)	1.10 (± 0.03)
AGP from normal serum	7.4 (± 0.5)	74 (± 17)	4.8 (± 0.1)	1.34 (± 0.03)
AGP from SLE serum	10.7 (± 0.7)	67 (± 13)	0.95 (± 0.10)	0.26 (± 0.03)
Imipramine				
Normal, purified AGP	1.7 (± 0.6)	2.2 (± 0.8)	10 (± 3)	2.8 (± 0.8)
AGP from normal serum	3.7 (± 1.1)	2.1 (± 0.6)	35 (± 5)	7.5 (± 1.1)
AGP from SLE serum	2.7 (± 0.4)	2.6 (± 0.4)	42 (± 2)	11.7 (± 0.6)

^aThe results were obtained at 37°C and pH 7.4. The values in the parenthesis represent a range of ± 1 S.D.

^b nK_a is the overall affinity constant for a non-saturable interaction, which was calculated by dividing the best-fit result for $m_{L2}K_a$ by the estimated moles of adsorbed AGP; the amount of adsorbed AGP was as follows: normal and purified AGP, 3.5×10^{-10} mol; AGP from normal serum, 3.6×10^{-10} mol for disopyramide or 4.7×10^{-10} mol for imipramine; and AGP from SLE serum, 3.6×10^{-10} mol.