Published in final edited form as:

Anal Bioanal Chem. 2014 October ; 406(25): 6203–6211. doi:10.1007/s00216-014-8081-4.

ANALYSIS OF DRUG INTERACTIONS WITH VERY LOW DENSITY LIPOPROTEIN BY HIGH PERFORMANCE AFFINITY CHROMATOGRAPHY

Matthew R. Sobansky and David S. Hage*

Chemistry Department, University of Nebraska, Lincoln, NE 68588-0304, USA

Abstract

High-performance affinity chromatography (HPAC) was utilized to examine the binding of very low density lipoprotein (VLDL) with drugs, using *R/S*-propranolol as a model. These studies indicated that two mechanisms existed for the binding of *R*- and *S*-propranolol with VLDL. The first mechanism involved non-saturable partitioning of these drugs with VLDL, which probably occurred with the lipoprotein's non-polar core. This partitioning was described by overall affinity constants of $1.2 (\pm 0.3) \times 10^6 \text{ M}^{-1}$ for *R*-propranolol and $2.4 (\pm 0.6) \times 10^6 \text{ M}^{-1}$ for *S*-propranolol at pH 7.4 and 37 °C. The second mechanism occurred through saturable binding by these drugs at fixed sites on VLDL, such as represented by apolipoproteins on the surface of the lipoprotein. The association equilibrium constants for this saturable binding at 37 °C were $7.0 (\pm 2.3) \times 10^4 \text{ M}^{-1}$ for *R*-propranolol and $9.6 (\pm 2.2) \times 10^4 \text{ M}^{-1}$ for *S*-propranolol. Comparable results were obtained at 20 °C and 27 °C for the propranolol enantiomers. This work provided fundamental information on the processes involved in the binding of *R*- and *S*-propranolol to VLDL, while also illustrating how HPAC can be used to evaluate relatively complex interactions between agents such as VLDL and drugs or other solutes.

Keywords

Very low density lipoprotein; Propranolol; Drug-protein binding; High-performance affinity chromatography; Frontal analysis

Introduction

The binding of drugs, low mass hormones and other solutes with serum proteins and lipoproteins can influence the activity, toxicity, delivery and pharmacokinetics of such agents in the human body [1-8]. Information about the type and strength of these interactions can be useful in determining how drugs are distributed after their administration and is of potential interest for the design of personalized dosage regimens [4,8]. In some cases this binding can also be stereoselective, due to the inherent chirality of proteins [9-13].

Propranolol (see Electronic Supplementary Material (ESM) Fig. S1) is a basic, chiral drug that is known to bind several serum proteins and lipoproteins, such as human serum albumin

* Author for correspondence, dhage1@unl.edu.

(HSA), α_1 -acid glycoprotein (AGP), high density lipoprotein (HDL), low density lipoprotein (LDL), and very low density lipoprotein (VLDL) [14-17]. This drug is a non-selective beta adrenergic blocking agent that is used in the treatment of hypertension, angina, arrhythmia, and other disorders [18-20]. Propranolol has a pK_a of 9.45 and a log P value of 3.00, which indicate that propranolol is a basic and relatively non-polar compound [18,21,22]. These properties make a number of interactions feasible between propranolol and lipoproteins. These interactions may include specific interactions with fixed binding regions, interactions with surface phospholipids, or partitioning into the non-polar core of a lipoprotein [7,23-30].

Like other lipoproteins, VLDL is a soluble complex of lipids and proteins (i.e., apolipoproteins). VLDL has the general structure shown in Figure 1 [7,23,24]. An important function of VLDL and other lipoproteins is to transport hydrophobic compounds and lipids, such as cholesterol and triacylglycerols, throughout the body [7,23,24]. Lipoproteins like VLDL are also known to interact with and transport several types of basic or neutral and hydrophobic drugs in the bloodstream [7,23-30]. For instance, it has recently been proposed in work with HDL and LDL that several binding mechanisms, including both saturable interactions with apolipoproteins and non-saturable interactions with the hydrophobic core, may occur between drugs and these lipoproteins [10,14,31].

Numerous methods have been used to examine the interactions of propranolol and other drugs with lipoproteins. These techniques have included equilibrium dialysis and capillary electrophoresis (CE) using high-performance frontal analysis [14-17]; however, only equilibrium dialysis has been used in studying drug interactions with VLDL [14]. High-performance affinity chromatography (HPAC) is an alternative to these techniques and has been used in various studies to examine the interactions of drugs with proteins and other binding agents [3-6,10,31-38].

HPAC is performed by applying a solution or sample of the drug of interest to an HPLC column containing an immobilized binding agent (e.g., VLDL). Evaluation of the subsequent elution profiles can provide data on the equilibrium constants, binding mechanism and number of interaction sites that are present between the drug and immobilized agent. Advantages of this approach include its speed, precision, ease of automation, and good correlation versus solution-phase results and reference methods [3-6,10,31-38].

HPAC has been utilized in previous reports to examine the binding of drugs such as propranolol with HDL and LDL [10,31]. The use of HPAC to perform binding studies of drugs with HDL and LDL has led to the confirmation of a non-saturable binding mechanism, as has been proposed in work with other techniques [14-17], as well as the identification of high affinity binding between propranolol and some apolipoproteins [10,31]. In the case of LDL, this latter process has been found to be stereoselective, with the *R*-enantiomer but not the *S*-enantiomer of propranolol undergoing this type of interaction [10].

In this study, HPAC will be modified and extended to the study of drug interactions with VLDL, using *R*- and *S*-propranolol as model drugs for this work. Propranolol was selected

as a model compound because it is known to interact with VLDL, and some estimates of the binding parameters for this system have been previously reported for soluble VLDL (i.e., based on the use of a non-saturable binding model and racemic propranolol) [14]. The evaluation of propranolol's interactions with VLDL will be examined in this report by preparing and employing columns containing VLDL immobilized to HPLC grade silica. These columns will then be used in frontal analysis experiments to determine the types and strength of interactions that occur between VLDL and *R*- and *S*-propranolol at various temperatures. The results will be compared to prior data that have been obtained with equilibrium dialysis and soluble samples of VLDL [14], as well as with previous results that have been obtained for the same drugs with HDL and LDL [10,14-17,31]. These experiments should provide a more complete description of the mechanisms by which *R*- and *S*-propranolol can interact with VLDL in the circulation. The results of this report should also make it possible to further determine the potential advantages of using HPAC to examine drug binding with lipoproteins such as VLDL, or other types of complex biological interactions.

Experimental

Reagents

The *R*- and *S*-propranolol and VLDL (from human plasma, catalog number L7527, lot no. 036K1143) were from Sigma (St. Louis, MO, USA). Nucleosil Si-1000 silica (7 μ m particle diameter, 1000 Å pore size) was obtained from Macherey Nagel (Düren, Germany). Reagents used for the bicinchoninic acid (BCA) protein assay were purchased from Pierce (Rockford, IL, USA). All other chemicals were of the highest grades available. All solutions used in chromatographic studies were prepared using water from a Nanopure purification system (Barnstead, Dubuque, IA, USA) and were filtered using Osmonics 0.22 μ m nylon filters from Fisher Scientific (Pittsburgh, PA, USA).

Instrumentation

The chromatographic system included a Vici F60-AL injection valve (Houston, TX, USA), an Eppendorf CH-500 column heater (Hauppauge, NY, USA), two 510 Waters HPLC pumps (Milford, MA, USA), and a Waters 2487 UV/Vis absorbance detector. The chromatographic data were collected using Waters Empower software and processed using programs based on Labview 5.1 (National Instruments, Austin, TX, USA). The supports were placed into 100 mm $\times$ 2.1 mm i.d. stainless steel columns by using a slurry packer from Alltech (Deerfield, IL, USA).

Preparation of VLDL silica

VLDL was immobilized onto silica by using the Schiff base method, as carried out by using conditions similar to those described for HDL and LDL [10,37]. The first step in the process was to prepare diol-bonded silica from Nucleosil Si-1000 silica, as described previously [37]. A portion of this diol-bonded silica was also utilized as a control support. In the Schiff base method, the diol-bonded silica was converted into an aldehyde-activated form by placing 0.9 g of this support into 15 mL of a 90:10 (v/v) mixture of acetic acid and water that contained 0.9 g periodic acid. This mixture was sonicated under vacuum for 10 min,

followed by shaking at room temperature in the dark for 1 h. The resulting aldehyde-activated silica was washed five times with water and three times with pH 6.0, 0.10 M potassium phosphate buffer.

Next, approximately 0.45 g of the aldehyde-activated support were placed into 5 mL of pH 6.0, 0.10 M potassium phosphate buffer and sonicated for 5 min under vacuum. A 5 mg portion of sodium cyanoborohydride was added, to reduce Schiff bases that form between the aldehyde support and primary amine groups on a ligand, followed by the addition of 1 mg VLDL. This mixture was gently shaken at 4 °C and protected from light for 8 days. The resulting VLDL support was washed four times with pH 7.4, 0.067 M potassium phosphate buffer. A 17.9 mg portion of sodium borohydride was dissolved into 12 mL of pH 7.4, 0.067 M potassium phosphate buffer, and 2 mL portion of this solution was added slowly to the VLDL support slurry to reduce any remaining aldehyde groups that were still present on the silica. This mixture was shaken for 90 min at room temperature. The final VLDL support was washed six times with pH 7.4, 0.067 M potassium phosphate buffer and stored in the same buffer at 4 °C until use.

The protein content of the VLDL support was evaluated by using a BCA protein assay and bovine serum albumin as the protein standard, as used previously with supports containing HDL and LDL [10,31]. All samples and standards for this assay were prepared in pH 7.4, 0.067 M potassium phosphate buffer. The VLDL silica samples were examined in triplicate, using diol-bonded silica as the blank. All sample and standard solutions were filtered through a 0.22 µm nylon filter prior to obtaining the final absorbance readings for this assay.

Chromatographic studies

Columns containing the VLDL silica or control support were downward slurry packed at 3500 psi into separate 100 mm × 2.1 mm i.d. stainless steel columns using pH 7.4, 0.067 M potassium phosphate buffer as the packing solution. These columns were stored in this pH 7.4 buffer at 4°C when not in use and were equilibrated with this buffer at the specified temperature before each chromatographic experiment. All mobile phases were filtered through Osmonics 0.22 µm nylon filters and degassed under vacuum prior to use. The elution of *R*- and *S*-propranolol from these columns was monitored at 225 nm.

Zonal elution studies were initially conducted on the VLDL column and control column to examine the stability of the immobilized VLDL. These experiments were carried out by repeatedly injecting 20 µL of a 25 mM solution of *R*-propranolol that was dissolved in pH 7.4, 0.067 M potassium phosphate buffer. The mobile phase in these studies was pH 7.4, 0.067 M potassium phosphate buffer, which was applied at 1.0 mL/min. These experiments were conducted at 37°C over the course of approximately 30 h. The retention of time for each peak was determined by utilizing Waters Empower 2 Software (Waters Corporations, Milford Massachusetts).

Frontal analysis studies were carried out on the VLDL columns and control column for both *R*- and *S*-propranolol and in triplicate at 20 °C, 27 °C or 37 °C. A flow rate of 0.5 mL/min was used in these experiments, as has been shown in previous studies with HDL and LDL columns to be suitable for drug binding studies and to have no significant impact on the

measured binding capacities or equilibrium constants [10,31]. A pH 7.4, 0.067 M potassium phosphate buffer was utilized in these studies as the mobile phase for both sample application and elution under isocratic conditions. A total of nine solutions, within the linear range of the detector, were applied to each column. These solutions contained 0.2-25 μ M of *R*- or *S*-propranolol. The results were processed by integration and using programs written in Labview 5.1 to determine the moles of drug that were required to reach the mean breakthrough time at a given concentration of the applied drug [3]. The breakthrough times obtained for the control column were subtracted from those measured at the same drug concentration on a VLDL column to correct for the void time and non-specific binding of propranolol to the support, as described in prior work with HDL and LDL columns [10,31]. The degree of non-specific binding observed on the VLDL columns was typically in the range of 7-15% of the total breakthrough time for *R*- and *S*-propranolol. The frontal analysis results were then fit to various binding models by using non-linear regression and Origin 9.1 software (OriginLab, Northampton Massachusetts).

Results and Discussion

General properties of VLDL support

The VLDL support was first examined by using a protein assay to find the quantity of lipoprotein that was immobilized and contained in a typical HPAC column. Based on this assay, the VLDL support was determined to contain 1.23 ($\pm$ 0.03) mg apolipoprotein per gram silica. This result corresponded to 15.4 ($\pm$ 0.4) mg or 2.05 ($\pm$ 0.05) nmol of VLDL per gram silica, based on an average molar mass of 7.5×10^6 g/mol for VLDL [14] and a typical apolipoprotein content for VLDL of 8% (w/w) [24]. The total amount of VLDL in each HPAC column was estimated to be 0.27 nmol, as based on the apolipoprotein content of the support, the column dimensions, and the packing density of the support. This amount of immobilized VLDL (15.4 mg, or 2.05 nmol, per gram silica) was lower than the 28 mg (12 nmol) LDL per gram silica and 68 mg (380 nmol) HDL per gram silica that have been previously obtained with these other lipoproteins [10,31]. However, as will be seen later, this VLDL content was still in a range that was suitable for drug binding studies.

There were several reasons for the lower lipoprotein content of this VLDL support when compared to prior LDL or HDL supports. For instance, VLDL has a much larger size compared to these other lipoproteins (typical diameter: VLDL, 30-80 nm; LDL, 18-25 nm; HDL, 5-12 nm) [24]. This larger size meant a support with a larger pore size (but also a lower surface area) had to be used to immobilize VLDL. In this study, silica with a nominal pore size of 1000 Å (100 nm) was used to immobilize VLDL, while supports with pore sizes of 500 Å and 300 Å, respectively, were used in the prior work with LDL and HDL [10,31]. In addition, the greater expense and relatively low solubility of VLDL resulted in a smaller amount of this lipoprotein being combined with the support during the immobilization process (i.e., a ratio of 2.2 mg VLDL per gram silica in the starting mixture, compared with 16.7 mg LDL per gram silica or 100 mg HDL per gram silica).

The stability of the VLDL support was examined through both zonal elution and frontal analysis studies. Repeated injections of *R*-propranolol were first made onto a VLDL column under controlled temperature and flow rate conditions. This VLDL column gave

reproducible retention for *R*-propranolol over several weeks and the equivalent of 30 h of operation at 1 mL/min (i.e., at least 2.8 L of mobile phase, or 9.3×10^3 column volumes). During these experiments, *R*-propranolol had an average retention time of $3.2 (\pm 0.3)$ min and an average retention factor of $9.5 (\pm 0.9)$, with only random variations being noted in the individual results. Frontal analysis studies that were conducted on a fresh VLDL column demonstrated similar stability and reproducibility over the course of several months and more than 160 measurements that involved the application of at least 3.4 L of the mobile phase (i.e., 1.13×10^4 column volumes). These data indicated that the VLDL support had a similar stability to what has been seen for HDL and LDL supports [10,31] and that such a support was suitable for use in long-term, multiple drug binding studies. The stability of these columns and their small size resulted in a method for drug binding studies that required significantly less binding agent than equilibrium dialysis or even CE over the course of a large number of experiments [14-17]. For instance, an HPAC column that contained 0.27 nmol VLDL used the equivalent of only 1.7 pmol VLDL per sample application over the course of 160 experiments.

Examination of binding mechanisms for *R*- and *S*-propranolol with VLDL

The binding of *R*- or *S*-propranolol to the VLDL support was evaluated by frontal analysis. Examples of typical breakthrough curves that were obtained on a VLDL column are shown in Figure 2. Depending on the concentration of the applied analyte, the mean position of the breakthrough curves appeared within 2 to 7 min of sample application and could be measured with a typical precision of ± 1 to 2%. Similar analysis times have been noted in work with LDL columns of the same size [31] and were roughly twice as long as the times needed with HDL columns that were half this size [10]. In all of these HPAC studies, the individual run times were shorter than the typical analysis times of 16 min that have been reported when using CE to examine drug interactions with lipoproteins [15] and were much shorter than the six hours that have been used to perform drug-lipoprotein binding studies by equilibrium dialysis [14].

The amount (in moles) of drug needed to reach the mean position of each breakthrough curve (m_{Lapp}) was determined by integration of these curves. These values were then used along with the known concentration of the applied drug ($[D]$) to generate a double-reciprocal plot of $1/m_{Lapp}$ versus $1/[D]$, as demonstrated in Figure 3. When a single type of binding site is present for the drug on the immobilized binding agent in such a column, this type of plot should result in a linear relationship for a system with relatively fast association and dissociation kinetics compared to the time scale of the experiment [8]. When multiple binding mechanisms are present, this type of plot should instead show deviations from a linear response (e.g., at large drug concentrations, or low values of $1/[D]$, for a multi-site interaction) [8]. As is illustrated in Figure 3, each of the double reciprocal plots that were generated for *R*- and *S*-propranolol at 20 °C, 27 °C and 37 °C produced negative deviations from a linear response at high analyte concentrations. This behavior indicated that multiple binding mechanisms were present for *R*- and *S*-propranolol with VLDL. Similar behavior has been observed between *R*- or *S*-propranolol with HDL [31], and for *R*-propranolol with LDL [10].

The frontal analysis data were next examined in more detail through the use of additional binding models and non-linear plots of m_{Lapp} versus $[D]$ (see examples in Figure 4). Four binding models were tested for use in describing the interactions between *R*- or *S*-propranolol and VLDL. For instance, several previous studies based on equilibrium dialysis or CE have used non-saturable partitioning to describe the interactions between propranolol and other drugs with lipoproteins [10,14-17,31]; this was considered in this current study by using a single nonsaturable interaction model. A second type of interaction that may occur is site-specific and saturable binding, as has been noted for *R*- or *S*-propranolol with HDL and for *R*-propranolol with LDL [10,31]. The possibility of multiple site-specific binding locations was also considered by using a model based on two groups of saturable sites [10,31]. In addition, a mixed-mode model was examined in which a single, saturable site and a group of non-saturable interactions were present. The equations used to represent each of these models are provided in the ESM. For plots like those shown in Figure 4, the correlation coefficients, residual values, and the distribution of the data about the best-fit line were used to evaluate the goodness of fit for each model. The corresponding association equilibrium constants, binding capacities, or global affinity constants that were obtained for each model are summarized in the ESM.

Both of the models that used a single type of interaction (e.g., the non-saturable interaction model and the model based on a single group of saturable sites) gave reasonably good correlation coefficients when fit to the frontal analysis data (in Figure 4, $r = 0.9570$ for nonsaturable interactions, $r = 0.9992$ for single-site saturable binding). However, the residual plots for the non-saturable model gave a non-random pattern of data points about the best-fit line, as demonstrated by the inset in Figure 4(a). Furthermore, both of these models gave lower correlation coefficients than were obtained for the two-site or mixed-mode models for the same data, and a lower sum of the squares of the residuals (e.g., $0.017\text{--}1.59 \times 10^{-17}$ for the non-saturable or one-site saturable model vs. $0.86\text{--}6.01 \times 10^{-19}$ for the two-site or mixed-mode models). These results supported the conclusion drawn from the double-reciprocal plots that multiple types of interactions were occurring between *R*- or *S*-propranolol and VLDL.

A comparison of the more complex interaction models in Figures 4(c-d) indicated that similar fits and residual plots were generated when using a two-site saturable model or a mixed-mode model based on one set of saturable sites plus a non-saturable interaction. However, the correlation coefficient obtained for the mixed-mode model was slightly higher than the value for the two-site saturable model ($r = 0.9998$ and 0.9994 , respectively). In addition, the equilibrium constants provided by the mixed-mode model were much more precise than those obtained for the two-site saturable model (see ESM). These results indicated the mixed-mode model was the most likely mechanism of interaction for both *R*- and *S*-propranolol with VLDL. The same conclusion was reached at all of the temperatures that were considered in this study. In addition, these results were consistent with recent models that have been proposed for the binding by *R*- and *S*-propranolol with HDL and for *R*-propranolol with LDL over the same range of temperatures [10,31].

Determination of equilibrium constants and number of interaction sites

Once it had been determined that both *R*- and *S*-propranolol were binding to VLDL through a mixed-mode model, this model was used to provide more details on these interactions. Table 1 summarizes the binding parameters that were obtained for this model at pH 7.4 and temperatures ranging from 20 °C to 37 °C. For instance, the single-site saturable interaction of *R*-propranolol with VLDL had an association equilibrium constant (K_{a1}) of $7.0 (\pm 2.3) \times 10^4 \text{ M}^{-1}$ at 37 °C, while the interaction between *S*-propranolol and VLDL at the same temperature had a statistically equivalent value (at the 95% confidence level) for K_{a1} of $9.6 (\pm 2.2) \times 10^4 \text{ M}^{-1}$. The K_{a1} value for *R*-propranolol with VLDL varied from only 7.0 to $9.2 \times 10^4 \text{ M}^{-1}$ between 20 °C and 37 °C and the K_{a1} for *S*-propranolol ranged from 4.6 to $9.6 \times 10^4 \text{ M}^{-1}$ over this temperature range. For both propranolol enantiomers, no significant variations in K_{a1} were seen at the 95% confidence level over this temperature range and for most of these values, with the only exception being a possible decrease in the value obtained for VLDL with *S*-propranolol at 27 °C. There was also no significant difference at the 95% confidence level between the overall set of values obtained for the two enantiomers, as determined by using a paired Student's *t*-test.

The amount of the saturable binding sites was consistently in the range of 7.8 to 25 nmol for *R*- and *S*-propranolol on the VLDL column and at 20 °C to 37 °C (average: *R*-propranolol = 12 nmol, *S*-propranolol = 16 nmol). This value was 5.4- to 17-fold larger (average, 9.7-fold larger) than the moles of VLDL particles that were estimated to be present in the column. This result was not surprising because it has been proposed in prior work with HDL and LDL that this type of saturable binding occurs with apolipoproteins [10,31], which can have many copies present on a large lipoprotein particle such as VLDL [24].

VLDL particles can contain apolipoproteins B-100, C-I, C-II, C-III, and E [7]. Some or most of these apolipoproteins are also found in HDL or LDL. For instance, LDL can contain apolipoprotein B-100, and HDL can contain apolipoproteins A-I, A-II, C-I, C-II, C-III, D, and E [7]. The K_{a1} values that are listed in Table 1 for *R*- and *S*-propranolol with VLDL are similar to or only slightly lower than the values of $1.1\text{--}1.9 \times 10^5 \text{ M}^{-1}$ that have been measured under the same pH and temperature conditions for the saturable binding by propranolol with HDL (e.g., see results in Table 2) [31]. This suggests that apolipoproteins which are common to HDL and VLDL may be responsible for part or most of these saturable interactions (e.g., apolipoproteins C-I, C-II, C-III, and E) [7].

Although VLDL and LDL can both contain apolipoprotein B-100, the stereoselectivity that has previously been observed for the binding of *R*- and *S*-propranolol to LDL (and proposed to be due to apolipoprotein B-100) [10] was not detected in this current study with VLDL. In addition, the K_{a1} values that have been measured for *R*-propranolol with LDL are 4.6- to 11.3 times higher than the values seen here for VLDL under the same pH and temperature conditions (e.g., see Table 2) [10]. This lack of stereoselectivity, and the lower binding strength of VLDL, indicates that apolipoprotein B-100 is probably not a significant source of the saturable binding that was observed between VLDL and propranolol. This may be due to the presence of a greater amount of other apolipoproteins in VLDL which can also bind to propranolol. Another possibility is that the presence of these other apolipoproteins may

impact the accessibility and/or conformation of apolipoprotein B-100 in the region at which the stereoselective binding of propranolol occurs with LDL. For instance, the presence of other apolipoproteins (e.g., apolipoproteins C and E) has been shown to impact the ability of apolipoprotein B-100 to bind to enzymes and cell surface receptors through protein-protein interactions [40].

The second type of interaction that was seen for *R*- and *S*-propranolol with VLDL involved non-saturable binding. This interaction was described by an overall affinity (nK_a) at pH 7.4 and 37 °C of $1.2 (\pm 0.3) \times 10^6 \text{ M}^{-1}$ for *R*-propranolol and $2.4 (\pm 0.6) \times 10^6 \text{ M}^{-1}$ for *S*-propranolol. These values ranged from 1.2 to $3.0 \times 10^6 \text{ M}^{-1}$ for *R*-propranolol and 1.8 to $2.5 \times 10^6 \text{ M}^{-1}$ for *S*-propranolol at temperatures between 20 °C and 37 °C. There was no significant difference at the 95% confidence level in the overall set of values obtained for the two enantiomers, as determined by using a paired Student's *t*-test. This type of interaction has been suggested in previous work to describe the partitioning of *R*- and *S*-propranolol or other drugs into the non-polar core of a lipoprotein, or perhaps an interaction with phospholipids on the surface [10,14,15,31]. It is interesting to note that the nK_a values determined in this study for the non-saturable binding of *R*- and *S*-propranolol with VLDL were approximately 30- to 200-times higher than the values of 1.6 - $4.1 \times 10^4 \text{ M}^{-1}$ that have been measured at pH 7.4 and between 4 °C and 37 °C for the same type of interaction of these enantiomers with HDL (e.g., see Table 2) [14,15,31]. In addition, these values were 3- to 17-times larger than nK_a values that have been obtained for *R*- and *S*-propranolol with LDL [10,14,15]. This difference is consistent with a mechanism based on the partitioning of these drugs into the non-polar core of these lipoproteins, because the order of these nK_a values agrees with the fact that VLDL has a much larger portion of hydrophobic components (i.e., cholesterol and triacylglycerides) than either HDL or LDL [7,15,23,24].

Conclusions

This report examined the extension and use of HPAC with immobilized VLDL to examine the binding of drugs such as *R*- and *S*-propranolol to this lipoprotein. It was found through the use of this method that *R*- and *S*-propranolol had a combination of two distinct types of interactions with VLDL. The first of these interactions was non-saturable in nature and probably involved the partitioning of propranolol into the non-polar core of VLDL, as described by an overall affinity constant that ranged from 1.4 - $3.6 \times 10^6 \text{ M}^{-1}$ between 20 °C and 37 °C. The second type of interaction was the result of site-specific, saturable binding and was believed to occur between these drugs and apolipoproteins on the surface of VLDL. The association equilibrium constants for these latter interactions were in the range of 4.6 - $9.2 \times 10^4 \text{ M}^{-1}$ between 20 °C and 37 °C.

The binding parameters that were obtained in this report were in the general range of binding constants that have been reported for propranolol when using a similar mixed-mode model for immobilized HDL and LDL [10,31]. When the same data were examined using a nonsaturable model (see ESM), the resulting overall affinities of 10^5 - 10^6 M^{-1} were consistent with the range that has been reported for racemic propranolol with soluble VLDL and using the same non-saturable model [14]. This report also demonstrated that, despite the possible presence of some apolipoprotein B-100, VLDL did not exhibit the stereoselective

interactions for the propranolol enantiomers that have been seen with LDL [10]. This difference was proposed to be due to the different apolipoprotein content of VLDL and LDL, and may also be related to modifications in the accessibility and/or conformation of apolipoprotein B-100 in the presence of other lipoproteins in VLDL [40].

The results obtained in this report demonstrate the suitability of HPAC as a technique for characterizing mixed-mode binding mechanisms between VLDL and drugs. As noted in prior work with HDL and LDL columns [10,31], this approach can provide analysis times of only a few minutes per run (e.g., see examples in Figure 2). This is a significant improvement over the CE and equilibrium dialysis methods that have been previously utilized in the analysis of drug-lipoprotein interactions [14,15]. The VLDL columns developed in this report were also sufficiently stable to be used for a large number of experiments. These combined features gave this HPAC approach a significant reduction versus equilibrium dialysis and even CE in the amount of ligand that was needed for a large number of experiments.

The use of the same VLDL column for multiple studies eliminated or minimized variations due to batch-to-batch changes in the binding agent preparations. Furthermore, use of the VLDL columns with standard HPLC equipment and detectors provided good limits of detection and relatively high precision in the chromatographic results [3,4,10,31]. Together, these advantages made it possible to reliably obtain data over a variety of drug concentrations. These features, in turn, made it possible to compare many possible binding mechanisms and to identify and compare the mixed-mode interactions of VLDL with *R*- or *S*-propranolol. These features should make similar HPAC columns and methods valuable in future studies that are aimed at examining the binding of additional drugs and solutes with VLDL or with other complex binding agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institute of Health under grant R01 GM044931 and was performed in facilities renovated under grant RR015468-01.

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Figure 1.

General structure of very low density lipoprotein (VLDL). As shown in this figure, the specific types of apolipoproteins that can be present in VLDL are B-100, C-I, C-II, C-III, and E. The arrows show how a drug (D) may interact with such a lipoprotein through either non-saturable binding (D_{ns}) or through saturable binding (D_s).

Figure 2.

Typical frontal analysis results obtained for the application of various concentrations of *R*-propranolol solutions to a 100 mm × 2.1 mm i.d. VLDL column at 0.5 mL/min and 37°C in the presence of pH 7.4, 0.067 M phosphate buffer.

Figure 3.

Double reciprocal plots obtained in frontal analysis studies examining the binding of (a) *R*-propranolol and (b) *S*-propranolol to a 100 mm $\times$ 2.1 i.d. VLDL column at 37°C and in the presence of pH 7.4, 0.067 M phosphate buffer. The linear fits that are shown were obtained using data points in the upper region of this plot, which are designated by the closed squares (■) and cover *R*- or *S*-propranolol concentrations that range from 0.2 to 4 μ M. Data points in the lower regions of these plots (i.e., at higher concentrations of *R*- or *S*-propranolol) showed negative deviations from the linear fit to the upper data points and are represented by open squares (□). Expanded views of the lower regions to the left of these graphs are provided in the insets.

Figure 4.

Fit of various binding models to frontal analysis data obtained for *R*-propranolol on a VLDL column at 37 °C and pH 7.4. The models used in this analysis were as follows: (a) non-saturable interactions, (b) a single group of saturable sites, (c) two separate groups of saturable sites, and (d) a group of non-saturable interactions plus a group of saturable sites. The insets show the residual plots for the fit of each model to the experimental data. The correlation coefficients were as follows ($n = 9$): (a) 0.9570, (b) 0.9992, (c) 0.9994, and (d) 0.9998.

Table 1

Binding parameters obtained for *R*- and *S*-propranolol on a VLDL column at various temperatures^a

Enantiomer & binding model	Temperature (°C)	m _{L1} (mol)	K _{ad} (M ⁻¹)	nK _a ^b (M ⁻¹)
<i>R</i> -Propranolol, Two interactions: saturable + non-saturable	20	0.96 (± 0.47) × 10 ⁻⁸	9.2 (± 4.8) × 10 ⁴	3.0 (± 0.3) × 10 ⁶
	27	1.3 (± 0.8) × 10 ⁻⁸	7.3 (± 4.3) × 10 ⁴	2.9 (± 0.5) × 10 ⁶
	37	1.3 (± 0.5) × 10 ⁻⁸	7.0 (± 2.3) × 10 ⁴	1.2 (± 0.3) × 10 ⁶
<i>S</i> -Propranolol, Two interactions: saturable + non-saturable	20	1.6 (± 0.8) × 10 ⁻⁸	6.9 (± 3.4) × 10 ⁴	2.5 (± 0.5) × 10 ⁶
	27	2.5 (± 0.9) × 10 ⁻⁸	4.6 (± 1.3) × 10 ⁴	1.8 (± 0.4) × 10 ⁶
	37	0.78 (± 0.16) × 10 ⁻⁸	9.6 (± 2.2) × 10 ⁴	2.4 (± 0.6) × 10 ⁶

^aThe numbers in parentheses represent a range of ± 1 S.D. All of these results were measured in pH 7.4, 0.067 M potassium phosphate buffer.

^bThe value for nK_a for a non-saturable interaction was obtained by dividing the best-fit result for m_L/K_a by the estimated moles of VLDL in the column. This latter value was obtained by using the protein content of the VLDL support, determined using an average molar mass for VLDL of 6.9 × 10⁶ g/mol and a typical protein content for VLDL of 8% (w/w) [8,10,12-14].

Table 2
Comparison of binding parameters for *R*- and *S*-propranolol with various lipoproteins at pH 7.4 and 37 °C

Lipoprotein	Type of drug	Binding model [Ref.] ^a	m _{1,1} (mol)	K ₀₁ (M ⁻¹)	nK ₀ (M ⁻¹)
High density lipoprotein (HDL)	<i>R</i> -Propranolol	Saturable site + non-saturable binding [31]	2.2 (± 0.7) × 10 ⁻⁹	1.9 (± 0.8) × 10 ⁵	4.1 (± 0.3) × 10 ⁴
	<i>S</i> -Propranolol	Saturable site + non-saturable binding [31]	4.5 (± 0.2) × 10 ⁻⁹	1.1 (± 0.1) × 10 ⁵	3.7 (± 0.2) × 10 ⁴
	<i>R/S</i> -Propranolol	Non-saturable binding [14]	N/A	N/A	1.60 (± 0.14) × 10 ⁴
Low density lipoprotein (LDL)	<i>R</i> -Propranolol	Saturable site + non-saturable binding [10]	7.5 (± 1.5) × 10 ⁻¹⁰	5.2 (± 2.3) × 10 ⁵	1.9 (± 0.1) × 10 ⁵
	<i>S</i> -Propranolol	Non-saturable binding [10]	N/A	N/A	2.7 (± 0.2) × 10 ⁵
	<i>R/S</i> -Propranolol	Non-saturable binding [14]	N/A	N/A	1.76 (± 0.01) × 10 ⁵
Very low density lipoprotein (VLDL)	<i>R</i> -Propranolol	Saturable site + non-saturable binding	1.3 (± 0.5) × 10 ⁻⁸	7.0 (± 2.3) × 10 ⁴	1.2 (± 0.3) × 10 ⁶
	<i>S</i> -Propranolol	Saturable site + non-saturable binding	0.78 (± 0.16) × 10 ⁻⁸	9.6 (± 2.2) × 10 ⁴	2.4 (± 0.6) × 10 ⁶
	<i>R/S</i> -Propranolol	Non-saturable binding [14]	N/A	N/A	2.87 (± 0.28) × 10 ⁵

^aRef [14] utilized soluble lipoproteins, while Refs. [10] and [31] used immobilized lipoproteins.