ANALYSIS OF DRUG INTERACTIONS WITH LIPOPROTEINS BY HIGH PERFORMANCE AFFINITY CHROMATOGRAPHY

by

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High density lipoprotein (HDL), low density lipoprotein (LDL), and very low density lipoprotein (VLDL) are lipoproteins previously shown to bind many basic and neutral hydrophobic drugs in serum. These interactions impact the distribution, delivery, metabolism, and excretion of drugs and are important in determining drug activity, pharmacokinetics, and toxicity in the human body. Information about drug-lipoprotein interactions and the strength of these interactions can be useful in determining the distribution of drugs following administration.

The research presented in this dissertation uses high performance affinity chromatography (HPAC) and packed columns to study binding of the drug propranolol to immobilized lipoproteins such as HDL, LDL, and VLDL. Through these studies, two types of interactions were identified between the lipoproteins and propranolol and verapamil. The first interaction has a relatively high affinity and likely involves binding of the drug by surface apolipoproteins. This high-affinity saturable interaction was stereoselective for LDL. HDL and VLDL did not exhibit stereoselectivity. The second

type of interaction observed in each lipoprotein had a lower affinity involved partitioning of the drug into the non-polar core of lipoproteins.

Additional work analyzing the theory and experimental conditions needed for the detection of multiple binding mechanisms in HPAC columns when using frontal analysis is also presented. This work focuses on the evaluation of binding models that incorporated both a saturable type of binding and a non-saturable interaction. These evaluations make it possible to determine the experimental conditions that would be required for detection of this type of multi-mode interaction.

These studies demonstrate that HPAC is a useful tool in characterizing mixed-mode interactions, as can occur with complex particles like lipoproteins. The affinity columns containing immobilized lipoproteins allowed these studies to be conducted using the same column for hundreds of experiments with short analysis times. The combined result of these advantages was the ability to quickly obtain precise data over a variety of drug concentrations. The results of these experiments indicate that similar columns prepared with other lipoproteins or biological membranes can be used in similar HPAC binding studies.

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CHAPTER ONE

INTRODUCTION

Portions of this chapter have previously appeared in M.R. Sobansky and D.S. Hage, "Analysis of Drug Interactions with Lipoproteins by High-Performance Affinity Chromatography", In: Advances in Medicine and Biology, Vol. 53, L.V. Berhardt (ed.), Nova Science Publishers, 2012, Chapter 9.

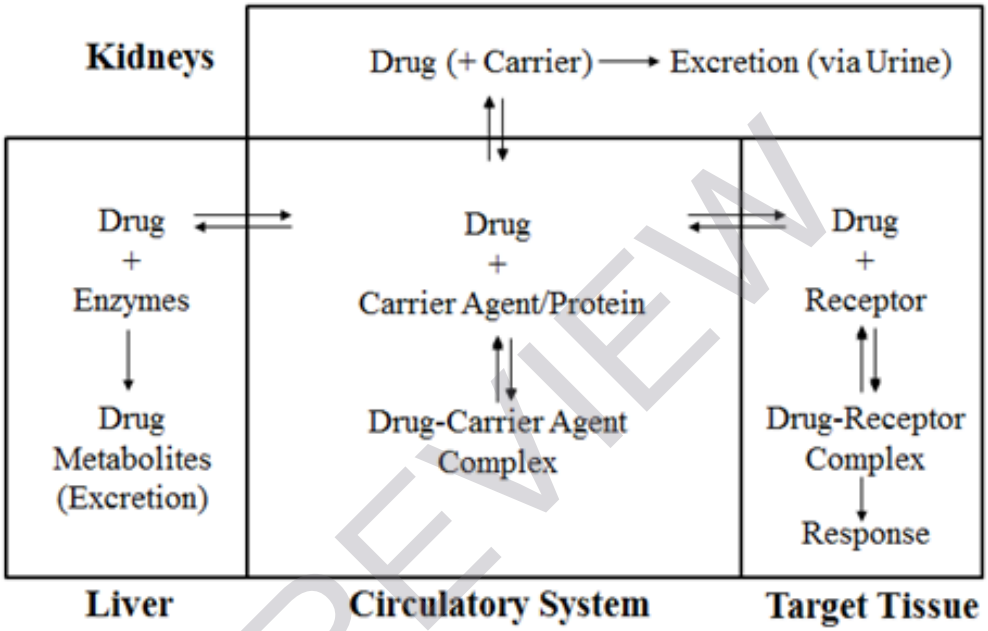
Drug interactions with serum proteins and other binding agents within the blood play an important role in determining the apparent activities of many pharmaceutical agents that have entered the circulatory system. For example, the distribution and pharmacokinetics of numerous drugs within the body is impacted by the binding of these agents [1-3]. Direct and/or indirect competition between a drug and another agent (e.g., another drug or endogenous compound) for the same binding sites on a serum agent may also significantly impact drug-drug or drug-solute interactions [4-8]. Furthermore, the solubility of hydrophobic compounds may be enhanced by the binding of solutes in the blood [9].

The ability of a pharmaceutical agent to illicit a response is significantly impacted by these typically reversible binding mechanisms. This is due to the fact that only an unbound drug molecule contained within the blood is able to reach its receptor and target tissue, to be metabolized by the liver, or to be excreted by the kidneys from the circulatory system. A drug bound to proteins or other agents is generally not available for these processes or to illicit a response [10]. The effects of drugs binding to such

agents in the circulatory system can be illustrated by the general model that is given in Figure 1-1. The interactions between drugs and serum agents are often significant, as demonstrated by the fact that 43% of the 1500 most frequently prescribed drugs have 90% or greater binding to serum proteins and other agents [11]. The frequent and extensive occurrence of drug and serum agent interactions mandates that the evaluation of this binding be an important part of the adsorption, distribution, metabolism, and excretion data that are required by the various health authorities for the approval a new pharmaceutical compound [10].

Lipoproteins such as high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very low-density lipoproteins (VLDL) are a group of binding agents known to interact with several basic and neutral hydrophobic drugs and other solutes in blood [12-23]. Propranolol and verapamil are two examples of drugs that are known to engage in these types of interactions with lipoproteins [2,12-19]. The binding of such drugs with various lipoproteins was the focus of the research in this dissertation. The interactions of such drugs with LDL, VLDL, and HDL have been analyzed previously by means of equilibrium dialysis and capillary electrophoresis (CE) carried out in a frontal analysis mode [20-23].

Figure 1-1. General model for drug interactions with proteins and other binding agents in blood and the relationship of this binding to the ability of a drug to reach its target or to be acted on by the liver and kidneys. This figure is reproduced with permission from Ref. [54].


Kidneys
Drug (+ Carrier) → Excretion (via Urine)
Drug
+
Enzymes
Drug
Metabolites
(Excretion)
Drug
+
Carrier Agent/Protein
Drug-Carrier Agent
Complex
Drug
+
Receptor
Drug-Receptor
Complex
Response
Liver
Circulatory System
Target Tissue

Equilibrium dialysis is often the reference method for the evaluation of drug interactions with proteins or other macromolecules. This method is inexpensive to perform but has several drawbacks. The drawbacks of equilibrium dialysis include its requirement for a large amount of binding agent, the time consuming nature of the test, and its susceptibility to errors arising from leakage of the bound drug fraction through the membrane and/or adsorption of the drug onto the membrane [21]. CE/frontal analysis does not require the use of a membrane and overcomes many of the disadvantages associated with equilibrium dialysis. In addition, CE/frontal analysis provides a relatively quick method that requires relatively small amounts of samples and binding agents [21]. The primary handicap of CE in the evaluation drug interactions is the higher limits of detection that arise when compared to other methods that utilize bench top spectrometers or HPLC systems [24,25].

High-performance affinity chromatography (HPAC) is an alternate technique to equilibrium dialysis and CE/frontal analysis for evaluating drug - protein interactions [3,7-9]. HPAC utilizes high-performance liquid chromatography columns that contain an immobilized binding agent (e.g., HDL, LDL, or VLDL) to which a solution or sample of the drug of interest is applied [26-28]. Based upon past studies, HPAC has shown to be a valuable tool for studying drug interactions with serum proteins as information related to equilibrium constants and the stoichiometry of the interactions occurring within the column can be determined [3,7-9]. As will be demonstrated in this dissertation, the speed and ease of automation make HPAC advantageous when compared to equilibrium dialysis. HPAC provides superior precision when compared to equilibrium dialysis and CE due to the ability of HPAC to use the same preparation of binding agent for a large

number of studies. This feature reduces batch-to-batch and run-to-run variations. Furthermore, HPAC can be interfaced with a variety of HPLC detectors, making it possible to use this method with a wide range of solutes while obtaining low detection limits [10].

HPAC has been employed in many previous studies that have examined drug interactions with serum proteins, such as human serum albumin (HSA) and α_1-acid glycoprotein (AGP) [10]. This technique has also been extended to work with binding agents, such as HDL, LDL, and VLDL in this research [24,25,29]. This chapter describes the basic principles of HPAC and the properties of the lipoproteins that were evaluated, as well as providing an overview of the drugs that were used as model compounds as a means to determine if HPAC was a suitable method for obtaining information regarding drug-lipoprotein interactions. Subsequent chapters within this thesis provide specific details on the experiments that were utilized to determine the nature and strength of drug-lipoprotein interactions, along with the results and significance of these experiments, and potential topics of interest for future work.

Properties of Lipoproteins

Lipoproteins such as HDL, LDL, and VLDL are soluble complexes of lipids and proteins (i.e., apolipoproteins) arranged into a macromolecular structure. The general structure of a lipoprotein is depicted in Figure 1-2. A primary function of these complexes is to transport hydrophobic compounds such as cholesterols, triacylglycerides (triglycerides) and lipids throughout the body [12-14]. Lipoproteins are also known to

interact with and transport several types of basic or neutral and hydrophobic drugs in the bloodstream [12-19,33].

As shown in Figure 1-2, triacylglycerol and cholesterol esters form the non-polar lipid core of a lipoprotein. This core is surrounded by a monolayer of phospholipids and apolipoprotein(s) covering the surface of lipoprotein. The phospholipids and apolipoprotein(s) in this layer are oriented to allow solubilization of the complex. Individual phospholipids are arranged so that the phosphate-containing head of the molecule is on the outer face of the complex while the lipid tail is positioned towards the non-polar core of the apolipoprotein [12,13].

Human lipoproteins have historically been divided into five primary classes based upon density. These five categories are, in order of increasing density, chylomicrons (CM), very low density lipoproteins (VLDL), low density lipoproteins (LDL), intermediate density lipoproteins (IDL) and high density lipoproteins (HDL) [12,14-18]. The properties that differentiate these lipoproteins are summarized in Table 1-1.

In addition to their structural differences, lipoproteins may be divided by the functional role that they serve within the body. Chylomicrons are typically formed following absorption of triacylglycerides and cholesterol within the intestinal tract. Chylomicrons are incorporated into the bloodstream, where free fatty acids are removed by lipoprotein lipase and delivered to various tissues. Chylomicron remnants are then delivered to the liver where they are repackaged as VLDL. VLDL transports endogenous triacylglycerides, phospholipids, cholesterol and cholesteryl esters throughout the body. Lipoprotein lipase removes additional triacylglycerides from VLDL, leaving IDL (i.e., VLDL remnants). IDL is then converted to LDL. LDL carries cholesterol esters formed

in the liver to muscles and other extrahepatic tissues. A process known as reverse cholesterol transport utilizes HDL to remove excess cholesterol from peripheral tissues. The removed cholesterol is delivered to the liver for excretion and recycling [12,13]. The process of lipoprotein transport is depicted in Figure 1-3.

The exact lipoprotein composition and distribution in an individual is dependent on a variety of factors, including sex, age, race, metabolic condition, and disease state [12,13,16]. The typical lipoprotein levels in a healthy fasting adult male are approximately 280 mg/dL HDL, 410 mg/dL LDL, and 150 mg/dL VLDL [13]. Chylomicrons are only present immediately following a meal; therefore, typical fasting levels for this type of lipoprotein are 0 mg/dL [13]. Disruptions in the type and/or concentration of these lipoproteins may result in detrimental health effects such increased risk of cardiovascular disease [12-18].

The transport and distribution of hydrophobic or non-polar compounds of endogenous or exogenous origin is also facilitated by the presence of lipoproteins [12,14,18]. Examples of substances that are transported through interactions with lipoproteins include vitamin E and drugs such as amitriptyline, chlorpromazine, desipramine, imipramine, propranolol, verapamil, quinidine and nilvadipine [5,21,30,31,35-39].

Figure 1-2. Structure of a lipoprotein. This figure is reproduced with permission from Ref. [25].

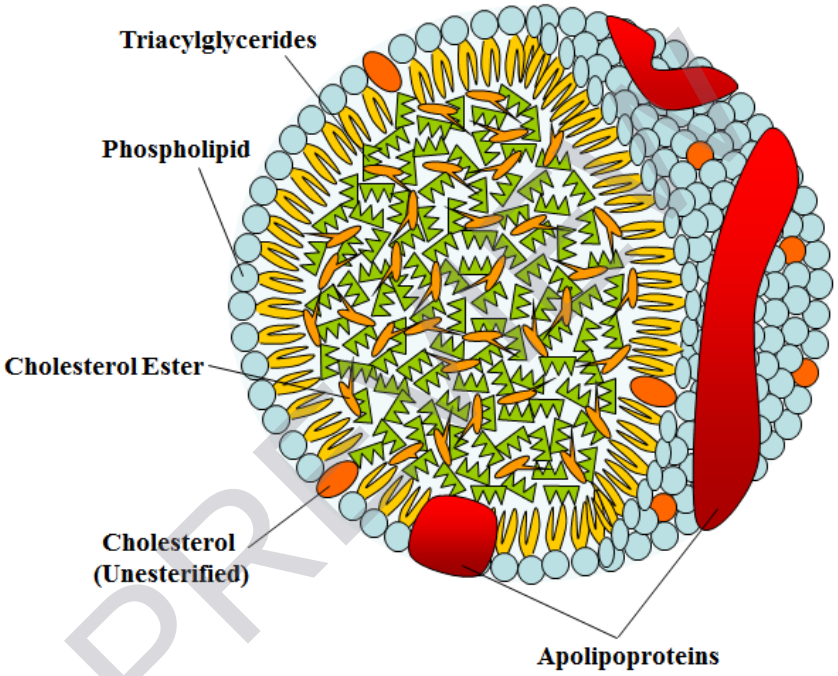

Triacylglycerides
Phospholipid
Cholesterol Ester
Cholesterol
(Unesterified)
Apolipoproteins

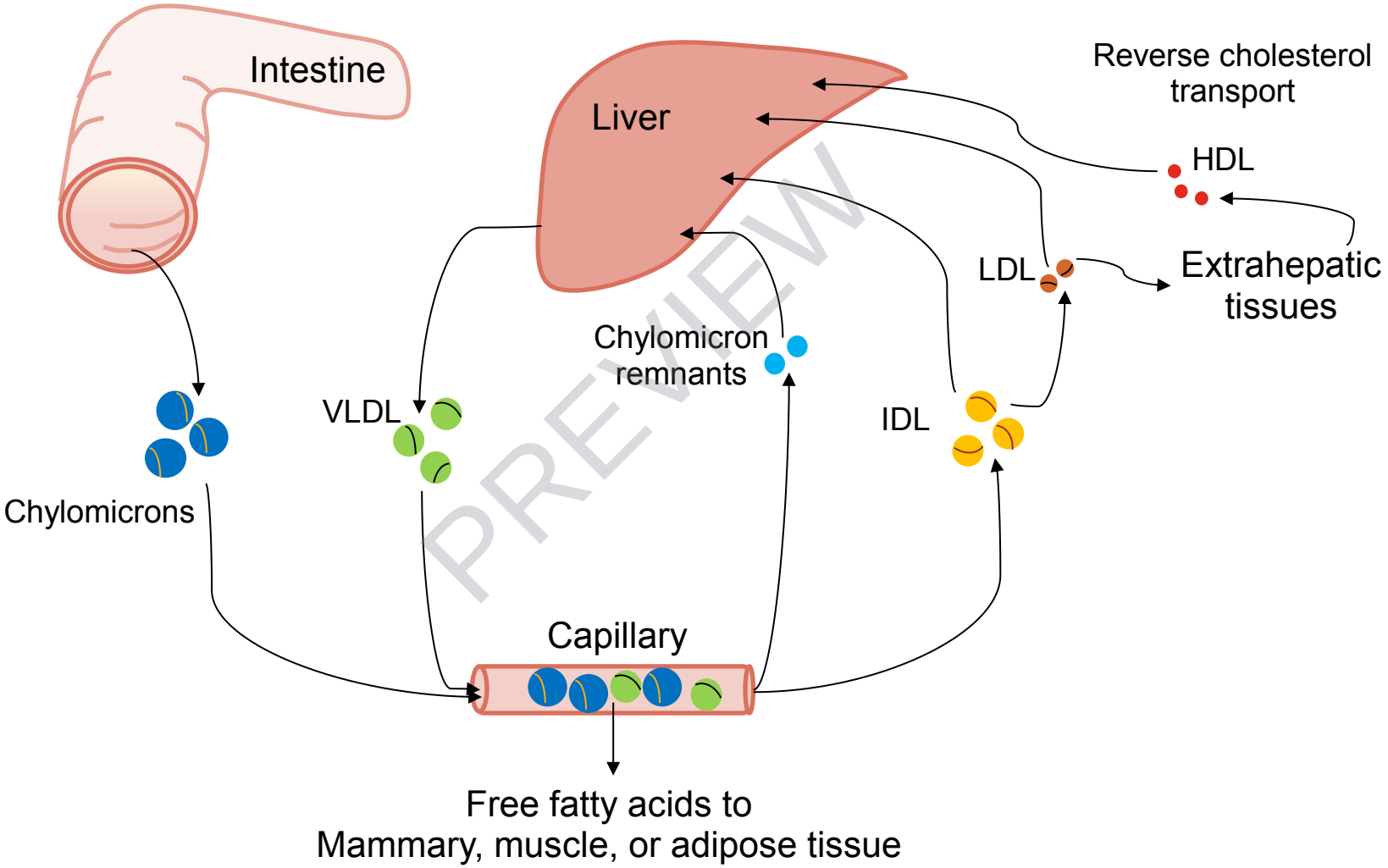
Table 1-1 Typical Properties of Human Lipoproteins

Lipoprotein	Density (g/mL)	Diameter (nm)	Associated Apolipoproteins	Composition (% dry weight)			
				Protein	Cholesterol	Phospholipid	Triacylglyceride
HDL	1.063-1.210	5-15	**A-I**, A-II, A-IV, C-I, C-II, C-III, D, E	55	17	24	4
LDL	1.019-1.063	18-28	**B-100**	23	45	20	10
IDL	1.006-1.019	25-50	**B-100**, C-I, C-II, C-III, E	18	29	22	31
VLDL	0.95-1.006	30-80	**B-100**, C-I, C-II, C-III, E	10	19	18	50
Chylomicron	<0.95	100-500	A-I, A-II, A-IV, **B-48**, C-I, C-II, C-III, E	2	4	9	85

Major associated apolipoproteins are shown in **bold**.

This table is adapted from Ref. [34].

Figure 1-3. The process of lipoprotein transport [34].


Intestine
Liver
Reverse cholesterol transport
HDL
LDL
Extrahepatic tissues
Chylomicron remnants
VLDL
IDL
Chylomicrons
Capillary
Free fatty acids to
Mammary, muscle, or adipose tissue

General properties of model drugs

Two model drugs were used in this work for the evaluation of drug-lipoprotein interactions by HPAC. The first model drug was propranolol (see Figure 1-4). This drug is a basic, chiral drug that is known to interact with several serum proteins and lipoproteins, including HSA, AGP, HDL, LDL, and VLDL [20-23]. Propranolol is a non-selective beta adrenergic blocking agent that is used in the treatment of several disorders, such as hypertension, angina, and arrhythmia [40-42]. Propranolol is basic and relatively non-polar, as indicated by its pK_a of 9.45 and its log P value of 3.00, allowing a number of interactions with lipoproteins feasible for this drug [40,43,44]. These interactions may include interactions with specific binding regions, interactions with surface phospholipids, or partition-based interactions with the non-polar core of a lipoprotein [12-19,33].

The second model drug that was evaluated was verapamil (see Figure 1-5). Verapamil is a calcium channel blocker used to treat hypertension, angina pectoris, and cardiac arrhythmia [22,45]. This drug is basic (pK_a of 8.75) and chiral, with the *S*-enantiomer showing higher pharmacological activity than the *R*-enantiomer [22,46]. Verapamil is also relatively non-polar, with a log P value of 3.79 [47]. These properties make interactions of this drug with lipoproteins possible at specific binding regions, surface phospholipids, or partition interactions with the non-polar core of the lipoprotein.

Figure 1-4. Structure of propranolol. The chiral center is indicated with an asterisk (*).