A Comparison of Step-Gradient and Sequential Density Ultracentrifugation and the Use of Lipoprotein Deficient Plasma Controls in Determining the Plasma Lipoprotein Distribution of Lipid-Associated Nystatin and Cyclosporine

Kishor M. Wasan,[1,3] Shawn M. Cassidy,[1] Manisha Ramaswamy,[1] Allison Kennedy,[1] Frank W. Strobel,[1] Samson P. Ng,[1] and Tak-Yee Lee[2]

Received July 1, 1998; accepted October 7, 1998

KEY WORDS: lipoprotein distribution; ultracentrifugation; liposomes; nystatin; and cyclosporine.

INTRODUCTION

Plasma lipoproteins are macromolecular complexes of lipid and protein mainly involved in the transport of lipids through the vascular and extravascular body fluids (1) and other processes including immune reactions, coagulation and tissue repair (2–4). Recently, plasma lipoproteins have been implicated in the transport of a number of water-insoluble agents resulting in the modification of their pharmacokinetics, tissue distribution and pharmacological activity (5,6).

The plasma lipoprotein separation techniques currently available were designed to separate, isolate, and purify individual lipoprotein subclasses from plasma and not to determine the lipoprotein distribution of drugs or drugs incorporated within lipid-based vesicles. These techniques including ultracentrifugation, sequential precipitation, size exclusion chromatography, affinity chromatography, fast protein liquid chromatography, and gel electrophoresis are designed to separate plasma lipoproteins based on their differences in density, molecular size, surface charge or protein content (7).

Ultracentrifugation (UC) appears to be the most acceptable and widely used technique in the separation of different lipoprotein subclasses due to its ease of use, equipment availability and reproducibility (7). There are two main types of UC used, step-gradient and sequential density UC. Each UC method has differences in UC time, rotor speed, volume of sample required, and temperature at which the lipoprotein separation occurs, all which may influence the result (7). Numerous investigations establishing the optimal conditions for lipoprotein separation from plasma with minimal contamination or overlap between lipoprotein fractions have been done by our laboratory (Tables 1 and 2) and others (7,8). However, both UC techniques have been used indiscriminately by others in determining the lipoprotein distribution of drugs, such as cyclosporine (9–11), with little thought about possible differences in results. In addition, to date, no specific studies have investigated this possible concern with lipid-based drug products. Furthermore, without the appropriate controls to confirm that plasma lipoprotein distribution of a specific compound is not a function of the technique used, erroneous conclusions about the drug's lipoprotein distribution may be made.

We conducted studies to determine the human plasma distribution of free and liposomal nystatin (Nys) and cyclosporine (CSA) by step-gradient and sequential density UC. Our working hypothesis was that similar drug lipoprotein distribution profiles would be observed when plasma was partitioned into its different lipoprotein and lipoprotein-deficient components by either step-gradient or sequential density UC. These studies were designed to assess the similarities and differences of drug lipoprotein distribution when plasma was partitioned by different UC separation techniques and to develop the appropriate controls that would account for non-specific drug flotation, elution or binding.

MATERIALS AND METHODS

Chemicals and Plasma

Free (Nys) and liposomal Nys (L-Nys), formulated as previously described (12), were provided by Aronex Pharmaceuticals Inc. Radiolabeled CSA ([*mebmt*-β-^{3}H] Cyclosporin A; Specific Activity, 7.39 mCi/mg) was purchased from Amersham Life Science (Buckinghamshire England). Liposomal ^{3}H-CSA (L-^{3}H-CSA) was formulated as previously described by Ouyang and coworkers (13). Drug-free vehicles used to reconstitute Nys, L-Nys, ^{3}H-CSA and L-^{3}H-CSA did not alter lipoprotein lipid and protein composition and concentration (9,12). Methanol, tetrahydrofuran and other organic solvents were purchased from Fisher Scientific Canada (Toronto, Ontario, Canada). Sodium bromide was purchased from Sigma Chemical Company (St. Louis, MO). Normolipidemic fasted human plasma was obtained from the Vancouver Red Cross (Vancouver, British Columbia). Ten μl of 0.4M ethylenediaminetetraacetic acid pH 7.1 (EDTA, Sigma Chemical Company) was added to 1.0 ml of whole blood.

Lipoprotein Separation

Sequential Ultracentrifugation (UC)

Human plasma (2.0 ml) samples were placed into centrifuge tubes, and their solvent densities were adjusted to 1.006-g/ml by the addition of sodium bromide 1.478-g/ml solution and then mixed. These centrifuge tubes were placed in a TLA 100.3 fixed-angle rotor. Following centrifugation (TLA-100 tabletop ultracentrifuge; Beckman Canada) at 100,000 rpm (645,000g; *k factor = 10; *k factor is the relative pellet

[1] Division of Pharmaceutics and Biopharmaceutics, Faculty of Pharmaceutical Sciences, The University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada.

[2] Aronex Pharmaceuticals Inc., The Woodlands, Texas 77381.

[3] To whom correspondence should be addressed. (e-mail: kwasan@unixg.ubc.ca)

0724-8741/99/0100-0165$16.00/0

Table 1. Total and Lipoprotein Plasma Cholesterol (Esterified + Unesterified) and Triglyceride Concentrations of Human Plasma Following Separation by Step-Gradient and Sequential Ultracentrifugation (UC)

Patient profile	Triglyceride rich lipoproteins mg/dl	Low density lipoproteins mg/dl	High density lipoproteins mg/dl	Total mg/dl
Cholesterol (esterified + unesterified)				
Step-gradient UC	20.5 ± 1.8	48.0 ± 4.9	39.0 ± 0.7	124.3 ± 4.8
Sequential UC	17.5 ± 1.0	50.1 ± 1.3	33.4 ± 1.4*	124.4 ± 1.3
Triglyceride				
Step-gradient UC	47.0 ± 5.3	21.1 ± 2.1	32.3 ± 1.3	121.2 ± 7.0
Sequential UC	41.6 ± 2.2	23.5 ± 1.7	23.3 ± 2.3*	124.2 ± 6.5

Note: Data is expressed as mean ± standard deviation; (n = 6).
* $p < 0.05$ vs. step-gradient UC.

efficiency of each rotor) for 18 hours at 4°C the very low-density lipoprotein (VLDL)-rich and VLDL-deficient plasma fractions were recovered. The VLDL-deficient plasma fraction was readjusted to a homogenous density of 1.063-g/ml and respun at 50,000 rpm for 18 hours at 4°C to separate the low-density lipoprotein (LDL)-rich and VLDL/LDL-deficient plasma fractions. The VLDL/LDL-deficient plasma fraction was subsequently readjusted to a homogenous density of 1.21-g/ml and respun at 50,000 rpm for 20 h at 4°C to separate the high-density lipoprotein (HDL)-rich and lipoprotein-deficient plasma (LPDP) fractions (14). Sodium bromide solutions were prepared as previously described (12,14).

Step-Gradient Ultracentrifugation (UC)

Human plasma (3.0 ml) samples were placed into centrifuge tubes, and their solvent densities were adjusted to 1.25-g/ml by the addition of solid sodium bromide (0.34g/mL of plasma). Once the sodium bromide had dissolved into the plasma, 2.8 ml of the highest density sodium bromide solution (density of 1.21-g/ml, which represents the HDL fraction) was layered on top of the plasma solution. Then, 2.8 ml of the second sodium bromide solution (density of 1.063-g/ml, which represents the LDL fraction) was layered on top of the sample, followed by 2.8 ml of the third sodium bromide solution (density of 1.006-g/ml, which represents the VLDL and chylomicron fraction). Upon completion of layering with the sodium bromide density solutions, four distinct regions of progressively greater densities (from top to bottom of the tube) were observed (12). All sodium bromide solutions were kept at 4°C prior to the layering of the density gradient. The centrifuge tubes were placed in an SW 41 Ti swinging bucket rotor (Beckman Canada) and centrifuged at 40,000 rpm (288,000g; k factor = 128), at a temperature of 15°C for 18 hr (L8-80 M; Beckman Canada). Following UC, each density layer was removed using a Pasteur pipette and the volume of each lipoprotein fraction measured.

Nystatin (Nys), ^{3}H-CSA, Lipid and Protein Quantification

Nys was extracted from each lipoprotein and lipoprotein-deficient fraction and the concentration in each fraction determined by high-pressure liquid chromatography methods previously described (12). ^{3}H-CSA recovery within each lipoprotein and lipoprotein-deficient fraction was determined by scintillation counting and calculating the amount of ^{3}H-CSA within each fraction using external calibration/quenching standard curves (9). Total and lipoprotein plasma triglyceride, cholesterol, and protein concentrations were determined by enzymatic assays purchased from Sigma Chemical Co. as previously described (9,12).

Experimental Design

Nys, L-Nys (at 20 μg of Nys/ml), ^{3}H-CSA and L-^{3}H-CSA (at 1 μg of ^{3}H-CSA/ml) were incubated in human plasma and lipoprotein-deficient human plasma for 60 minutes at 37°C. Following incubation, the plasma samples were partitioned into different densities using step-gradient or sequential density UC and each density fraction was assayed for drug by HPLC or radioactivity. In human plasma the 1.0–1.006-g/ml density fraction represents VLDL and chylomicrons, the 1.006–1.063-g/ml density fractions represents LDL, the 1.063–1.21-g/ml density fraction represents HDL, and the greater than 1.21 g/ml density fraction represents the LPDP fraction composed of mostly albumin and alpha-1 glycoprotein. Each lipoprotein fraction was

Table 2. Plasma Lipoprotein Composition of Human Plasma Following Separation by Step-Gradient and Sequential Ultracentrifugation (UC)

	Triglyceride rich lipoproteins			Low density lipoproteins			High density lipoproteins		
	TC/TP (wt/wt)	TG/TP (wt/wt)	TC/TG (wt/wt)	TC/TP (wt/wt)	TG/TP (wt/wt)	TC/TG (wt/wt)	TC/TP (wt/wt)	TG/TP (wt/wt)	TC/TG (wt/wt)
Step-gradient UC	2.0 ± 0.1	4.7 ± 0.5	0.4 ± 0.1	1.4 ± 0.2	0.6 ± 0.1	2.3 ± 0.2	0.3 ± 0.1	0.3 ± 0.1	1.2 ± 0.1
Sequential UC	1.3 ± 0.1*	3.2 ± 0.2*	0.4 ± 0.1	1.3 ± 0.1	0.6 ± 0.1	2.2 ± 0.2	0.5 ± 0.1	0.3 ± 0.1	1.4 ± 0.1

Note: Data is expressed as mean ± standard deviation, (n = 6).
* $p < 0.05$ vs. step-gradient UC; TC, total cholesterol (esterified + unesterified); TG, total triglycerides; TP, total protein; wt/wt, weight/weight.

analyzed for total cholesterol (esterified & unesterified), triglyceride, protein, total cholesterol: total protein ratio, total triglyceride: total protein ratio, and total cholesterol: total triglyceride ratio.

Statistical Analysis

Differences in plasma lipoprotein lipid and protein concentration and composition following separation by step-gradient and sequential density UC and differences in drug distribution following separation using both UC methods were determined by a one-way analysis of variance (InStat; GraphPad Software). Critical differences were assessed by Tukey posthoc tests. Differences were considered significant if p was < 0.05. All data are expressed as mean $\pm$ standard deviation.

RESULTS AND DISCUSSION

A number of preliminary studies have been done in order to establish the optimal conditions (i.e. ultracentrifuge rotor type, length of spin, temperature, rotor speed, sample volume, type of ultracentrifuge method) for lipoprotein separation from human plasma with minimal contamination between different lipoprotein subclasses and aqueous plasma proteins (7,8,14,15). Two methodologies have emerged from these studies, step gradient and sequential UC.

When human plasma was separated into its different lipoprotein subclasses by step-gradient and sequential UC the following differences were observed. HDL cholesterol and triglyceride concentrations were significantly lower following sequential separation than following step-gradient separation (Table 1). Furthermore, the triglyceride rich lipoproteins (which contains VLDL and chylomicrons) total cholesterol: total protein and total triglyceride: total protein ratios were significantly lower following sequential separation than following step-gradient separation (Table 2). All other lipoprotein lipid parameters measured were not significantly different (Tables 1 and 2).

Once it was confirmed that these methods separated lipoproteins in a similar fashion, a direct comparison of their ability to determine the lipoprotein distribution of different hydrophobic compounds was assessed. When human plasma containing Nys, L-Nys (Table 3), ^{3}H-CSA and L-^{3}H-CSA (Table 4) were separated into their lipoprotein and lipoprotein-deficient fractions by step-gradient and sequential density UC significant differences in drug distribution were observed. The rationale for incubating these compounds in lipoprotein-deficient plasma was to determine if drug recovery within each of the different density fractions was a result of the drug's association with a specific lipoprotein and not due to its formulation density.

Upon separation by sequential UC, at least 14% of the initial concentration of Nys and L-Nys incubated in lipoprotein-deficient plasma were recovered in each density fraction (Table 3). In addition, the distribution profile of Nys and L-Nys in lipoprotein-deficient plasma (Table 3) was similar to their distribution profile in human plasma (Table 3). Similar results were observed for ^{3}H-CSA and L-^{3}H-CSA within density fractions less than 1.063 g/ml (Table 4). These findings suggest that we are not able to determine the lipoprotein distribution of these compounds using sequential UC since we cannot distinguish if drug recovered within each density fraction was due to its association with a specific lipoprotein or due to its formulation density.

However, following step-gradient UC separation of drug-containing lipoprotein-deficient plasmas, the majority of free and liposomal Nys (Table 3) and ^{3}H-CSA (Table 4) were recovered in the most dense fraction ($>$1.210 g/ml) with total recovery greater than 96%. Unlike the sequential UC method, the distribution profile of these compounds when incubated in lipoprotein-deficient plasma is significantly different from their distribution profile in human plasma when separation was performed using step-gradient UC (Tables 3 & 4). These findings suggest that drug recovery within each of the lipoprotein density fractions may be a result of its association with that specific lipoprotein and not because of its formulation density.

An explanation for the discrepancies between the step-gradient and sequential density UC methods may be a result of how the different density fractions are prepared and utilized. In sequential UC the density of drug-containing plasma is adjusted to 1.006-g/ml (representing VLDL and chylomicrons) with a sodium bromide solution (assuming plasma has a density close to 1.00-g/ml). This results in a one phase sodium bromide-plasma solution in which drug from the plasma is solubilized and homogeneously dispersed throughout the solution [The aqueous solubility of Nys and CSA are 1 mg/ml and 0.1 mg/ml respectively (Wasan et al.; unpublished findings)]. Theoretically, following centrifugation only particles with a density less than 1.006-g/ml [i.e., chylomicrons and VLDL] within this solution would rise to the top (14). Subsequently this layer is removed and the remaining solution was readjusted to a new density, and centrifuged to float the next lipoprotein fraction to the top. Since the drug was homogeneously dispersed within this solution after each subsequent spin a proportion of drug was removed independent of lipoprotein association (Tables 3 & 4). However in step-gradient UC, drug-containing plasma is placed on the bottom of the centrifuge tube and the density is readjusted to 1.25-g/ml with solid sodium bromide. Unlike the method of sequential UC the drug-containing plasma is not redispersed to form a homogenous sodium bromide-plasma solution. The different density fractions are layered on top of the plasma so that following a single spin, particles of different densities rise to their respective density fraction (7,8). Therefore, as demonstrated with the lipoprotein-deficient plasma distribution profiles (Tables 3 & 4), the recovery of drug within each of these density fractions appears to be due to its association with lipoproteins and not a result of its formulation density.

Additional aspects of these two methods as they relate to the different results obtained need to be considered. Conceptually, step gradient UC is less arduous in terms of technique manipulation than sequential density UC (i.e. decreased UC time, less volume of sodium bromide density solutions required), which is an advantage. Furthermore, stability of lipoproteins and plasma proteins (i.e. albumin and alpha-1 glycoprotein) is a potential issue with the sequential method due to the prolonged period of time required for completion of the centrifugation. The centrifugation temperatures are different for the two methods as well. This could possibly further account for differences between the distribution studies obtained by the two methods. Although neither method is performed at a physiological temperature, UC is performed at temperatures below the transition temperature of lipoproteins, which is between 27°C–34°C (15). During the transition, cholesteryl esters within the lipoprotein core exist as an isotropic solution, whereas below this temperature they form disordered liquid

Table 3. Distribution of Free and Liposomal Nystatin at 20 μg Nystatin/ml Within Human Plasma and Lipoprotein-Deficient Human Plasma Following Incubation for 60 Minutes at 37°C

	<1.006 g/ml fraction %[a]	1.006–1.063 g/ml fraction %	1.063–1.210 g/ml fraction %	>1.210 g/ml fraction %	Percent recovery %[b]
Free Nystatin					
I. Human plasma					
Step-gradient UC	3.7 ± 2.4	6.0 ± 2.8	31.4 ± 10.7	58.7 ± 3.4	99.8 ± 10.8
Sequential UC	17.1 ± 2.3*	21.1 ± 1.3*	18.4 ± 0.1*	51.8 ± 4.6	108.4 ± 8.0
II. Lipoprotein-deficient plasma					
Step-gradient UC	ND	ND	14.5 ± 2.3	84.6 ± 6.1	99.1 ± 8.2
Sequential UC	22.6 ± 1.9	14.5 ± 1.5	9.1 ± 0.5*	36.2 ± 3.3*	82.4 ± 7.2*
Liposomal nystatin					
I. Human plasma					
Step-gradient UC	1.5 ± 1.2	1.5 ± 2.7	45.8 ± 3.8	39.6 ± 2.6	88.4 ± 10.3
Sequential UC	14.9 ± 1.3*	14.9 ± 2.0*	25.5 ± 0.7*	51.5 ± 5.0*	106.8 ± 9.2
II. Lipoprotein-deficient plasma					
Step-gradient UC	ND	ND	12.0 ± 2.0	85.0 ± 5.1	97.0 ± 7.1
Sequential UC	14.2 ± 3.2	11.3 ± 1.8	19.8 ± 4.0*	41.9 ± 5.2*	87.2 ± 14.2

Note: After incubation samples were partitioned into different densities using step-gradient versus sequential ultracentrifugation (UC) and each fraction was assayed for nystatin by high pressure liquid chromatography. Data expressed as mean ± standard deviation.
* $p < 0.05$ vs. step-gradient ultracentrifugation, ($n = 6$).
[a] percent of the initial amount of nystatin incubated in human plasma.
[b] percent of initial drug incubated; ND, not detectable. In human plasma the <1.006 g/ml fraction represents very low density lipoproteins and chylomicrons, the 1.006–1.063 g/ml fraction represents intermediate and low-density lipoproteins, 1.063–1.210 g/ml fraction represents high-density lipoproteins and the >1.210 g/ml fraction represents albumin and alpha-1 glycoprotein.

crystals (15). In this liquid crystalline state the lipoproteins are easily separated from one another. However, when the lipoproteins exist as an isotropic solution you may have cross-contamination between density layers. In addition, preliminary work has been done by our laboratory which demonstrates that drug does not redistribute into different lipoprotein fractions at 4°C and 15°C (5). Therefore, following the incubation of the drug at 37°C the plasma is cooled down to at least 15°C to prevent any drug redistribution prior to UC.

The importance of these findings can be illustrated by the following example. A number of different research groups have

Table 4. Distribution of Free and Liposomal ^{3}H-Cyclosporine (^{3}H-CSA) at 1 μg CSA/ml Within Plasma and Lipoprotein-Deficient Human Plasma Following Incubation for 60 Minutes at 37°C

	<1.006 g/ml fraction %[a]	1.006–1.063 g/ml fraction %	1.063–1.210 g/ml fraction %	>1.210 g/ml fraction %	Percent recovery %[b]
^{3}H-CSA					
I. Human plasma					
Step-gradient UC	11.2 ± 1.0	29.7 ± 1.0	47.4 ± 3.5	4.5 ± 0.8	92.9 ± 4.1
Sequential UC	11.6 ± 0.6	25.3 ± 0.3*	44.1 ± 0.8	5.0 ± 0.9	86.0 ± 1.4*
II. Lipoprotein-deficient plasma					
Step-gradient UC	5.3 ± 0.7	8.0 ± 0.3	2.3 ± 1.5	84.7 ± 3.9	100.3 ± 4.7
Sequential UC	14.1 ± 0.3*	23.6 ± 0.4*	14.7 ± 3.3*	33.7 ± 5.2*	86.1 ± 7.8*
Liposomal ^{3}H-CSA					
I. Human plasma					
Step-gradient UC	41.8 ± 3.4	17.6 ± 0.2	30.2 ± 0.6	2.8 ± 0.3	94.3 ± 3.6
Sequential UC	22.1 ± 4.2*	19.4 ± 1.8	50.1 ± 4.5*	7.9 ± 3.6*	99.5 ± 7.8
II. Lipoprotein-deficient plasma					
Step-gradient UC	7.6 ± 0.9	4.2 ± 0.3	9.9 ± 0.9	75.0 ± 5.0	96.7 ± 6.0
Sequential UC	15.6 ± 0.6*	21.8 ± 0.6*	22.2 ± 1.9*	38.1 ± 4.8*	97.7 ± 3.3

Note: After incubation samples were partitioned into different densities using step gradient ultracentrifugation versus sequential ultracentrifugation (UC) and each fraction was assayed for ^{3}H-CSA by radioactivity. Data expressed as mean ± standard deviation.
* $p < 0.05$ vs. step-gradient UC, ($n = 6$).
[a] percent of the initial amount of ^{3}H-CSA incubated in human and lipoprotein-deficient plasma.
[b] percent of initial drug incubated; ND, not detectable. In human plasma the <1.006 g/ml fraction represents very low density lipoproteins and chylomicrons, the 1.006–1.063 g/ml fraction represents intermediate and low-density lipoproteins, 1.063–1.210 g/ml fraction represents high-density lipoproteins and the >1.210 g/ml fraction represents albumin and alpha-1 glycoprotein.

in human plasma (5,9–11). However, the relative percentage of association to specific lipoproteins reported varies from study to study. This may have to do with the varying lipid content of the plasma used from one study to the next (9). Alternatively, this may also be a result of indiscriminate use of different ultracentrifugation techniques (i.e., sequential ultracentrifugation was used in studies reported in reference 10, while step-gradient ultracentrifugation was used in studies reported in references 9 and 11). The consequences of erroneously reporting differences in CSA association with different lipoprotein fractions could have implications in interpreting CSA's therapeutic or toxic affects. This concern may be justified by recent studies that observed decreases in CSA activity within patients that have elevated plasma triglyceride levels and increases in CSA toxicity in those with low plasma cholesterol levels (5,9).

In conclusion, these findings suggest that incubating hydrophobic compounds in human and lipoprotein-deficient plasma and separating the lipoprotein and lipoprotein-deficient fractions by step gradient and sequential density UC may yield different drug distribution profiles. These differences are important to consider when comparing results from different studies.

ACKNOWLEDGMENTS

Funding for this project was provided by Aronex Pharmaceuticals Inc. and the Medical Research Council of Canada (Grant #MT-14484 to KMW). The authors would like to thank Dr. T.M. Madden for generously providing us the liposomal cyclosporine formulation used in these studies.

REFERENCES

1. R. A. Davis and J. E. Vance. Structure, assembly and secretion of lipoproteins. In: D. E. Vance and J. E. Vance (eds.), *Biochemistry of lipids, lipoproteins and membranes*. Elsevier, New York, 1996, pp. 473–493.
2. J. A. K. Harmony, A. L. Aleson, and B. M. McCarthy. Lipoprotein structure. In: A. M. Scanu and A. A. Spector (eds), *Biochemistry and biology of plasma lipoproteins*. Marcel Dekker Inc., New York, 1986, pp. 403–430.
3. A. Mbewu and P. N. Durrington. Lipoprotein (a): Structure, properties and possible involvement in thrombogenesis and atherogenesis. *Atherosclerosis* **85**:1–14 (1990).
4. P. N. Durrington. Lipoprotein assembly. In: P. N. Durrington (ed.) Lipoproteins and lipids. Wright, London, 1989, pp. 255–277.
5. K. M. Wasan and S. M. Cassidy. Role of plasma lipoproteins in modifying the biological activity of hydrophobic drugs. *J. Pharm. Sci.* **87**:411–424 (1998).
6. K. M. Wasan. Modifications in plasma lipoprotein concentration and lipid composition regulate the biological activity of hydrophobic drugs. *J. Pharmacol. Toxicol. Methods.* **36**:1–11 (1996).
7. M. I. Mackness and P. N. Durrington. Lipoprotein separation and analysis for clinical studies. In: C. A. Converse and E. R. Skinner (eds.). Lipoprotein Analysis: A Practical Approach, Oxford University Press, New York, 1992, pp. 11–43.
8. V. N. Schumaker and D. L. Puppione. Lipoprotein analysis. In: J. P. Segrest and J. J. Albers (eds.), *Methods of Enzymology*, Academic Press, London, 1986, 128, pp. 155–174.
9. K. M. Wasan, P. H. Pritchard, M. Ramaswamy, W. Wong, E. M. Donnachie, and L. J. Brunner. Differences in lipoprotein lipid concentration and composition modify the plasma distribution of cyclosporine. *Pharm. Res.* **14**:1613–1620 (1997).
10. R. J. Andrade, M. I. Lucena, J. A. Gonzalez-Correa, C. Garcia-Arias, and P. Gonzalez-Santos. Short-term effect of various doses of cyclosporin A on plasma lipoproteins and its distribution in blood: an experimental study. *Human Exp. Toxicol.* **12**:141–146 (1993).
11. A. M. Gardier, D. Mathe, X Guedeney, *et al.* Effects of plasma lipid levels on blood distribution and pharmacokinetics of cyclosporin A. *Ther. Drug Monitoring* **15**:274–280 (1993).
12. S. M. Cassidy, F. W. Strobel, and K. M. Wasan. Plasma Lipoprotein Distribution of Liposomal Nystatin is influenced by protein content of high-density lipoproteins. *Antimicrob. Agents Chemother.* **42**:1878–1888 (1998).
13. C. Ouyang, E. Choice, J. Holland, M. Meloche, and T. D. Madden. Liposomal cyclosporine-characterization of drug incorporation and interbilayer exchange. *Transplantation* **60**:999–1006 (1995).
14. R. J. Havel, H. A. Eder, and J. H. Bragdon. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34**:1345–1353 (1955).
15. R. J. Cushley, W. D. Treleaven, Y. I. Parmar, R. S. Chana, and D. B. Fenske. Surface diffusion in human serum lipoproteins. *Biochem. Biophys. Res. Commun.* **146**:1139–1145 (1987).