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DEVELOPMENT OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC
SEPARATIONS FOR THE PURIFICATION AND ANALYSIS OF
PHOSPHOLIPIDS AND RELATED LIPONUCLEOTIDES -
A NEW CLASS OF ANTI-AIDS DRUGS

BY

JOHN V. AMARI

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
CHEMISTRY

UNIVERSITY OF RHODE ISLAND

1991

DOCTOR OF PHILOSOPHY DISSERTATION

OF

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UNIVERSITY OF RHODE ISLAND

1991

ABSTRACT

High performance liquid chromatographic methods were developed to isolate and purify naturally occurring phospholipids and synthetic experimental Anti-AIDS liponucleotides on a preparative scale. Separations were optimized on an analytical size method development column, packed with the same media used in the preparative separations. A phospholipid class separation, on a silica stationary phase, was developed for the isolation of lecithin in chicken egg yolk. The collected fractions were analyzed for purity by analytical HPLC with UV and light scattering detection. Liponucleotides have been synthesized and required HPLC for purification. The methodology was based upon conventional phospholipid species separations on reversed-phase supports. The isolated compounds were analyzed by analytical HPLC and were characterized by UV analysis. In addition, a laser light scattering detector was used to quantitate the major phospholipids in egg yolk and evaluate lecithin and liponucleotide fractions. Preliminary work was also conducted on the evaluation of a new interface for HPLC-Infrared Spectroscopy.

DEDICATION

To my Family.

ACKNOWLEDGEMENTS

I am deeply grateful to Professor Phyllis Brown for her support. Her guidance and drive has kept me going especially in "rough times". I would also like to thank her for all the opportunities she has given me. Words are not enough to express my many thanks.

I wish to thank Professor Joseph Turcotte for his support and to participate in the AIDS project. The fruitful discussions on liponucleotides helped to broaden my understanding of the biological roles of phospholipids.

I would like to thank Professor Force for serving on my committee and for the discussions we have had and Professor Stanley Barnett and Professor Cynthia Zoski for serving on my committee.

Many thanks are given to my fellow graduate students, Chemistry Department faculty and staff, especially Steve Gesualdo for his expertise in computers and electronics. In addition, I wish to thank the members of Professor Turcotte's group, especially Phil Pivarnik for the many helpful discussions and the Medicinal Chemistry Department, especially Professor Anne F. Cichy for all her help.

I acknowledge my parents, who have supported me throughout and the rest of my family, especially my aunt Anna who understood what I went through.

Many thanks to Cheryl, who put up with me especially in the last few weeks, thanks for the typing.

I wish to acknowledge all the people who have given or have loaned equipment, so that my research could continue. I am especially indebted to SepTech for all the financial support; thank you. I also thank Bob Cooley of YMC, Dr. Fred Rabel of EM Science, Professor Eli Grushka, Dr. I. Molnar, Mr. Thomas Finn and everyone else who has assisted me in my research.

The support from the U.S. National Institute of Allergy and Infectious Diseases (AI 25690) and the National Cooperative Drug Discovery Group for the Treatment of AIDS (NCDDG/HIV) is greatly appreciated.

PREFACE

The analytical and preparative high performance liquid chromatographic separations of naturally occurring phospholipids and synthetic liponucleotides are presented. This dissertation has been written in manuscript form. The material is presented in two sections. Section one contains five papers and section two is comprised the appendix.

The first manuscript is a comprehensive review of the HPLC methodology, used for the preparative isolation and analysis of phospholipids and liponucleotides. This paper will be submitted for publication.

The second manuscript is The Preparative Isolation and Purification of Lecithin. This paper has been published in Journal of Chromatography, 517 (1990) 219-228

The third manuscript is on The development of an HPLC analysis of an anti-HIV glycerophospholipid, 3'-Azido-3'-Deoxythymidine Monophosphate Diacylglycerol (AZT-MP-DG). This paper has been accepted for publication and will appear in Journal of Pharmaceutical and Biomedical Analysis.

The fourth manuscript describes the micro-preparative HPLC isolation of experimental anti-HIV glycerophosphatidic

acid:nucleoside analogues, AZT-MP-DG and 2',3'-dideoxycytidine monophosphate diglyceride, ddC-MP-DG. This paper has been submitted for publication in Journal of Chromatography.

The fifth manuscript is a comparison of various HPLC detectors for the detection of phospholipids and liponucleotides. The discussion includes the limitations of separation conditions as a function of the type of detector used. This paper will be submitted to American Laboratory for publication.

The second section of the dissertation contains the bibliography.

TABLE OF CONTENTS

SECTION ONE

I. HPLC of Glycerophospholipids and Liponucleotides.....	page 001
II. Isolation and Purification of Lecithin by Preparative High Performance Liquid Chromatography.....	page 140
III. High Performance Liquid Chromatographic Analysis of 3'-Azido-3'-Deoxythymidine Monophosphate Diacylglycerol, an Anti-HIV Glycerophospholipid.....	page 174
IV. Isolation of Experimental Anti-AIDS Glycerophospholipids by Micro-Preparative Reversed-Phase High Performance Liquid Chromatography.....	page 212
V. Detection for HPLC Separations of Glycerophospholipids.....	page 252

SECTION TWO

Appendix A Bibliography.....	page 307
---------------------------------	----------

LIST OF TABLES

Table	Page
I.I.....	018
I.II.....	096
II.I.....	151
II.II.....	162
II.III.....	169
III.I.....	189
III.II.....	197
III.III.....	208
IV.I.....	234
IV.II.....	244
V.I.....	267
V.II.....	269
V.III.....	274
V.IV.....	278
V.V.....	282
V.VI.....	292

LIST OF FIGURES

Figure	Page
I.1.....	006
I.1.....	008
I.1.....	012
I.1.....	015
I.2.....	026
I.3.....	032
I.4.....	034
I.5.....	037
I.6.....	043
I.7.....	051
I.8.....	055
I.9.....	058
I.10.....	061
I.11.....	066
I.12.....	069
I.13.....	075
I.14.....	079
I.15.....	092
I.16.....	096
I.17.....	099
I.18.....	101
I.19.....	113
I.20.....	117

II.1.....	114
II.2.....	146
II.3.....	148
II.4.....	150
II.5.....	152
II.6.....	154
III.1.....	172
III.2.....	174
III.3.....	176
III.4.....	178
III.5.....	180
III.6.....	183
III.7.....	185
III.8.....	188
III.9.....	190
IV.1.....	205
IV.2.....	207
IV.3.....	209
IV.4.....	211
IV.5.....	213
IV.6.....	215
IV.7.....	219
IV.8.....	221
IV.9.....	223
IV.10.....	225
IV.11.....	229

v.1.....	255
v.2.....	258
v.3.....	260
v.4.....	262
v.5.....	264
v.6.....	266
v.7.....	268
v.8.....	270
v.9.....	272
v.10.....	274

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

GLYCOPHOSPHOLIPIDS AND LIPONUCLEOTIDES

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TABLE OF CONTENTS

1. Introduction

2. High Performance Liquid Chromatography

a) Separative Methods

b) Glycophospholipids

c) Liponucleotides

d) Species

e) Liponucleotides

f) Analytical Methods

g) Glycophospholipids

h) Class

i) Subclass

j) Species

k) Liponucleotides

l) Introduction

m) Separative Methods

n) Analytical Methods

o) High Performance Liquid Chromatography

**HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF
GLYCEROPHOSPHOLIPIDS AND LIPONUCLEOTIDES**

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TABLE OF CONTENTS

I. Introduction

II. High Performance Liquid Chromatography

A) Preparative HPLC

1) Glycerophospholipids

a) Class

b) Species

2) Liponucleotides

B) Analytical HPLC

1) Glycerophospholipids

a) Class

b) Subclass

c) Species

2) Liponucleotides

III. Detection

1) Refractive Index

2) Ultraviolet

3) Laser Light Scattering

4) Flame Ionization

5) Mass Spectrometry

6) Infrared

glycerol 7) Derivatization

long-term 8) On-Line Phosphorus Analyzer

(sp- 9) Radioactivity

glycerol 10) Electrochemical

phosph IV. Conclusions

glycerol phospholipids phospholipids

alcohol phospholipids phospholipids

large phospholipids phospholipids

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phospholipids phospholipids

glycerol phospholipids phospholipids

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hydrolysis phospholipids phospholipids

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INTRODUCTION

(R)-Glycerophospholipids (phospholipids) are derived from glycerol. Two acyl fatty acid chains (R_1 and R_2) of varying lengths and degrees of unsaturation are esterified to the first (sn-1 position) and second (sn-2 position) hydroxy groups of glycerol. The third hydroxy group forms an ester bond with phosphoric acid to give the structurally specific glycerophospholipid, phosphatidic acid (PA). Additionally, alcohols which are esterified to the phosphoric acid comprise a large majority of glycerophospholipids, for example phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI). Structures of all the glycerophospholipids with their respective abbreviations discussed in this review are illustrated in Figure 1-3.

The fatty acyl substituted diglycerides are nonpolar and hydrophobic and the phosphorylated alcohol head groups are polar; thus glycerophospholipids are amphipathic. Glycerophospholipid classes, based upon the polar head group, can be further divided into subclasses, which are distinguished by the type of bonding at the sn-1 position. These include the common diester (diacylglycerols) mentioned above as well as alkenylacyl (plasmalogen) and alkylacyl (ether glycerophospholipid) molecular species. Each naturally occurring subclass usually consists of a number of individual molecular species. The distribution of glycerophospholipids at the class,

Figure 1. Glycerophospholipid Structures.

Phosphatidic Acid (PA)
Phosphatidylglycerol (PG)
Phosphatidylinositol (PI)
Phosphatidylserine (PS)
Cardiolipin (CL)

Phosphatidic Acid (PA)

Phosphatidylglycerol (PG)

Phosphatidylinositol (PI)

Phosphatidylserine (PS)

Cardiolipin (CL)

Figure 2. Glycerophospholipid Structures cont.

Phosphatidylcholine (PC)
Phosphatidylethanolamine (PE)
Lysophosphatidylcholine (LPC)
Lysophosphatidylethanolamine (LPE)
Sphingomyelin (SPH)

Phosphatidylcholine (PC)

Phosphatidylethanolamine (PE)

Lysophosphatidylcholine

Lysophosphatidylethanolamine

Sphingomyelin

Figure 3. Glycerophospholipid Structures cont.

Diacyl Subclass

Alkylacyl Subclass

Alkenylacyl Subclass

Platelet Activating Factor (PAF)

Diacyl

Alkylacyl

Alkenylacyl

Platelet Activating Factor (PAF)

subclass and molecular species levels varies for each derived natural source.

Glycerophospholipids are the principle components of cell membranes. The composition on a class basis and on the molecular level influence many metabolic and enzymatic reactions in addition to regulating transport systems. Membrane fluidity is also highly dependent upon the glycerophospholipid class and species formulation. Many diseases or metabolic disorders, such as fetal lung maturity and adult lung diseases, can be predicted by determining the glycerophospholipid profile. The glycerophospholipid content is also a function of stress, environment temperature and nutrition. Biological sources of glycerophospholipids include cell membranes, tissues, body fluids and organs.

Glycerophospholipids are also found in plants, bacteria and algae. Soy beans provide a rich source of polyunsaturated glycerophospholipids. The diacylglycerol (diglyceride) distribution between plant and animal derived glycerophospholipids are generally different. Typically, there are more polyunsaturated molecular species in plants than in animals. The glycerophospholipid profile, in soy beans, for example, is used as a marker to monitor pre-and post harvest damage. Glycerophospholipids are used as biomarkers for the identification of bacteria and algae.

Glycerophospholipids have been used to synthesize novel pharmaceuticals and to aid in drug delivery. They are used as

membrane models, natural emulsifiers, wetting agents, dietetics and cosmetics. Analysis of glycerophospholipids help predict or diagnose certain disease states and determine metabolic pathways.

Since glycerophospholipids play important roles in many biological functions, it is necessary to have efficient separation methods both on the analytical and preparative scale. Two chromatographic methods used are thin layer chromatography (TLC) and gas chromatography (GC). For TLC silica has been used extensively to isolate and quantify glycerophospholipid classes. To fractionate individual molecular species, however, reversed phase or silica modified with silver ion is required. TLC does not possess the high resolving power for a complex mixture of glycerophospholipids and detection methods are difficult. Glycerophospholipids, because of their polar head groups, are not readily amenable for separation by GC since they do not possess adequate volatility. For GC, the head group must be cleaved and replaced with a non-polar moiety, such as a methyl group. However, GC is one of the best methods to determine the fatty acid profiles. In this review glycerophospholipid separations by TLC or GC will not be discussed and the reader is referred to the following texts and reviews (1-6)

HPLC on the preparative scale provides the means to purify large quantities of glycerophospholipids in a short period of time while minimizing degradation. On the analytical scale, HPLC can be used for subsequent analysis of the separated

glycerophospholipids.

Liponucleotides, Figure 4, can be defined as phosphatidic acid-nucleoside analogues. Naturally occurring liponucleotides, cytidine diphosphate diglyceride (CDP-diglyceride) and deoxycytidine diphosphate diglyceride (dCDP-diglyceride) have essential roles as intermediates for the biosynthesis of glycerophospholipids (7-9). Synthetic liponucleotides have been shown to exhibit antitumor and antiviral cytotoxic activity (10-14).

Sample Preparation

The ideal sample introduction would be to inject the sample without any prior cleanup, but this in reality not possible. Some type of sample pretreatment is necessary. The basic extraction for lipids are the methods described of Folch, Lees and Sloane (15) and Bligh and Dyer (16). Due to their insolubility in acetone, glycerophospholipids can be precipitated out of solution (17). The Folch extraction and acetone precipitation procedures are described in reference (18). Once the analytes are isolated from non-glycerophospholipids, they are dissolved in a suitable solvent, filtered and injected. Glycerophospholipids removed from their natural environment are prone to oxidation, especially the polyunsaturated ones. Precautions should be taken to prevent oxidation while exposed to air and light. The most commonly used anti-oxidant is butylated hydroxytoluene (BHT). A small quantity

Figure 4. Liponucleotides.

3'-Azido-3'-deoxythymidine monophosphate diglyceride,
AZT-MP-DG
2',3'-dideoxycytidine monophosphate diglyceride,
ddC-MP-DG

'Good-Bye Zilly?'

TOM WHEELER outsteps a Brown tackler for a few yards.

URI Sailors on Top, Place Second and Fourth

by Skip Whyte

The U.R.I. sailing team sailed in two meets this weekend, taking a second and a fourth. On Saturday U.R.I. finished second to M.I.T. 13 - 19 in an invitational here on Salt Pond. Tom Dykstra, Skip Whyte, John Telfeyan and Henry Bossett each won at least one race. Other scores Saturday were Merrimack 30, Coast Guard 32, S.M.U. and Nichols 45, and Stonehill 50. Mike Fenton and Chris Wells crewed.

On Sunday the team sailed

at the Coast Guard Academy and finished fourth behind M.I.T., Coast Guard, and Harvard. There were three divisions sailing at all times, one of Ravens, one of K boats, and one of dingheys. U.R.I. used the same four skippers as the previous day. The racing was very close with M.I.T. and Coast Guard winding up tied with 94 points each after 5 races in each division. M.I.T. won by having more first place finishes. Other scores were Harvard 106, U.R.I. 109, Yale 125, Tufts 130, and Stevens 160.

Rick Davids of Sigma Nu Wins Intramural Run

Rick Davids of Sigma Nu retained his title as URI Intramural Cross Country champion in the annual race which was held in rainy weather last Friday. Rick's time was 9:46.5 which was well off the record of 9:33.5 which he set in 1967. Rick also won the championships in 1968, which adds up to a three year sweep of the event.

A field of 76 runners turned out for the race in spite of the drizzling rain and soaking wet course. The team title went to Sigma Nu with 57 points. Sigma Chi was second with 76 points and Sigma Phi Epsilon was third with 121 points.

Top ten finishers were: 1.

Football Coach, Ram Grid Squad Face Trouble

URI's football season certainly has not opened on a very happy note. Rhody fans who are not satisfied with the team's record for the past few years are chanting "Good Bye Zilly." Is it really the fault of the coach that the team is not as strong as we would like? Possibly it is, but it takes more than a coach to make or break a team. A team is just what the name implies — a team.

It takes many people to make a team successful. A good scouting staff is necessary in order to recruit the best talent available. Could it be that our scouting staff is inadequate? Without teamwork and a desire to win a team is not a team. Could it be that there is not enough teamwork between the members of our squad? There may be many reasons why the team is losing. It may not be the fault of the coach, but the reason why they are losing should be found out and eliminated.

In any case, things could be pretty rough for coach Zilly if the team does not take a turn for the better. Under the coaching of Jack Zilly the team has a seven year record of 21 wins, 40 losses and two ties. Last year's record was three wins and six losses. To top things off Coach Zilly's contract expires this year. If things don't improve someone may have to drop back ten yards and punt, and if the disappointed fans get their way it might well be Coach Zilly.

QUARTERBACK TOM FAY lets loose a pass over menacing Brown defender in Saturday's game.

Frosh Harriers Overrun Hope

Ramlett harriers defeated Hope High School in a dual meet here in Kingston on Friday by a score of 23 to 36. John Bessette of the Ramletts was the individual winner with a time of 14 minutes and 35 seconds. Dave Keaney was second only 12 seconds behind John in 14 minutes and 47 seconds.

The record for the 2.7 mile course is 13 minutes and 13.4 seconds set last year by Dunsky from UMASS.

The race was held just minutes before the annual Intramural Cross Country meet. Although there was no wind and the temperature was 70 degrees a drizzling rain hampered the runners and slowed down the times. The varsity and freshmen cross country teams will face Springfield at

John Bessette

home this afternoon and travel to New Hampshire Saturday.

Sports This Week

— SATURDAY —
(Homecoming)

Football—Maine at Home—1:30
Soccer—Maine at Home—10:30
Cross Country—at New Hampshire—2:00

Have

a nice
day

Rick Davids

Some men see
are and ask wh
I dream thin
were and say w

KINGSTON, I

Ba

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Larry Coryell group

can be added to the glycerophospholipid extracts without affecting the separation.

Column Conditioning

In order to obtain reproducibility and preserve column performance, the column must be carefully maintained. The stationary phase, whether it is bare silica or a bonded phase, is different from vendor to vendor and even lot to lot. The silica is more influenced by the mobile phase composition than are bonded phases (vide infra). Therefore the reproducibility and efficiency of a silica column may be affected. Column conditioning procedures have been developed for silica phases used for glycerophospholipid separations. General methods are listed in Table I (18).

CHROMATOGRAPHY

Preparative HPLC Class Separations

Preparative HPLC can be defined as any HPLC separation in which the effluent containing the solutes is collected and subsequently used as a reference standard, as a product, as a precursor or for other types of analysis. Therefore, preparative separations may be performed on virtually any column independent of the size. Columns range from the traditional analytical dimensions (15-30 cm x 0.20-0.46 cm i.d.) to preparative scale (15-200 cm x >15 cm i.d.). The column size, purity requirement

Table I. Silica column conditioning procedures. Reproduced from reference 18.

	<i>Conditioning solvent</i>
<i>After use of acetonitrile/water system</i>	Pass 30 ml of methanol/water 1/1, 30 ml of methanol, 30 ml of dichloromethane, store in n-hexane overnight.
<i>Before use of acetonitrile/water system</i>	Equilibrate with starting solvent for 1–5 h.
<i>After use of hexane/isopropanol/water gradient system</i>	Pass 100 ml of isopropanol, 100 ml of n-hexane, store in n-hexane overnight.
<i>Before use of hexane/isopropanol/water gradient system</i>	Equilibrate with hexane/isopropanol/water/ (H ₂ SO ₄) 75/24/0.9 (0.1) Solvent B 20 column volumes.
<i>Between gradient runs</i>	<ol style="list-style-type: none"> (1) 20 column volumes 50% isopropanol, 50% starting solvent. (2) Gradient over 20 column volumes to starting solvent. (3) 20 column volumes starting solvent (30 min total).

and complexity of separation are a few of the parameters determining how much material can be isolated per run. Quantities isolated may range from mg to multi-kilograms. For small scale preparative separations, conventional analytical instrumentation can be used. As column size increases, specially constructed HPLC equipment becomes necessary. The theory of preparative HPLC will not be discussed, unless specific examples warrant it; however the reader is referred to the following texts (19-21).

Naturally occurring glycerophospholipids do not exist as a single discrete compound, but are composed of multimolecular species of varying fatty acid chain lengths and degrees of unsaturation. Silica has been the sorbent most commonly used in preparative and analytical separations of glycerophospholipid classes. Separations of glycerophospholipids into classes by silica are based upon the polar head groups.

Fager et al. (22) achieved a large scale purification of PE, LPE and PC with 20-40 μ m silica packed into eight 1 meter x 1 cm (i.d.) columns connected in series. The glycerophospholipids were derived from egg yolks and 10 g were injected. The classes were eluted at 5 mL/min with an increasing concentration of methanol (MeOH) in a CHCl_3 -MeOH gradient. A total of 844 fractions were collected at three minute intervals ending at a mobile phase composition of 50% MeOH. To analyze the fractions, each was spotted on a TLC plate and assayed by phosphate and amino acid analysis. Based upon the TLC analysis,

the PE eluted at 25% MeOH, LPE at 40% MeOH and PC at 50% MeOH with baseline resolution. A rapid isocratic system was developed to purify large quantities of egg PC and PE with radially compressed silica columns (30 x 5.7 cm i.d.) (23). The mobile phases consisted of CHCl_3 -MeOH- H_2O (60:30:4) and (60:30:2) for PC and PE respectively. A refractive index detector was used to monitor the separation since an UV detector could not be used due to the chloroform. Glycerophospholipids absorb UV radiation at wavelengths less than 210 nm. Twenty six grams of crude egg phospholipids were injected of which 10 g of PC was recovered. A mixture containing neutral lipids and PE was separated in which 3.48 g of PE was recovered. Each separation was accomplished in less than 20 minutes, which does not include the time to flush the column of highly retained components and re-equilibration. Water was eliminated from the CHCl_3 -MeOH mobile phase (24) for egg yolk PC and synthetic PC and PE. A 50 x 5 cm i.d. column packed with silica gel was used. Five grams of egg PC was chromatographed, (60:40) with 4.5 g recovered, which was rechromatographed (70:30) resulting in a final recovery of 4.32 g. The synthetic PC had to be chromatographed two times, whereas the synthetic PE required one pass. After two passes, 3.12 g of PC were isolated from 3.8 g of crude and 1.10 g of PE isolated from 1.30 g of crude. Relatively high recoveries were obtained; unfortunately long separation times (3-4 hours) were necessary. Preparative HPLC, on a 25 x 2.2 cm i.d. column, was used as a final purification step for beef heart CL after precipitation

and column chromatography procedures (25). A neutral eluent consisting of isopropanol, IPA, IPA-cyclohexane-H₂O (45:50:5) with RI detection was used. Out of 1 kg of beef heart, 1.5-2.1 g of 99% CL were recovered.

Although the refractive index detectors had to be exclusively used with UV opaque mobile phases, the RI lacks sensitivity to monitor minor components or impurities. Analytical separations were developed with UV transparent mobile phases (26-28) which have been adapted to preparative separations. The more sensitive UV detector could be used to monitor preparative separations, however a limited number of solvents are applicable. Hurst et al. (29) used gradient elution for isolating PI, PE and PC. The mobile phases consisted of a constant amount of H₃PO₄ (1.2%), and varying proportions of acetonitrile (ACN) and MeOH (95:5, 85:15 and 70:30) for PI, PE and PC respectively. The solvents permitted detection at 205 nm. The maximum load of crude soy bean phospholipids was 180 mg on a 30 x 5.7 cm column packed with 55-105 μ m silica. The mobile phases containing ACN and MeOH have been modified with other solvents or salts. The ACN-MeOH mixture was modified with isopropanol (IPA) H₂O and Trifluoroacetic acid, TFA, with a final composition of ACN-IPA-MeOH-H₂O-TFA (135:20:10:6.7:0.85) (30). A column packed with 10 μ m silica was reported, indicating a highly efficient column was required to isolate PC from rat lung tissue and lung lavage. The isolated PC was assayed for inorganic phosphorus and by GC analysis. Recently, another

isocratic mobile phase consisting of 5 mM ammonium acetate (NH_4OAc) in ACN-IPA-MeOH- H_2O (80:13:5:12) was used to isolate PC from chicken egg yolk (31). The NH_4OAc sharpened peaks considerably thereby increasing the load injected per run. In addition, a user packed preparative A/ETM column (20 x 5.00 cm i.d.) was compared with a high pressure packed semi-preparative column (20 x 1.93 cm i.d.). Each column was packed with 15-30 μm silica. The maximum load of crude glycerophospholipids on the high pressure packed semi-preparative column was 35 mg with 25 mg of purified PC (99%) recovered. The load injected and flow-rates were directly scaled up to the A/ETM column, 240 mg of crude glycerophospholipids were injected and 130 mg of purified PC (99%) was recovered. The separations were virtually identical for each column and highly purified PC was processed. The performance of the A/ETM column, a user packed column, was as efficient as a high pressure packed column of smaller diameter.

Geurts Van Kessel et al. developed an analytical separation with UV transparent solvents, hexane (Hex), Hex-IPA- H_2O (6:8:2) (27). The conditions were proposed to be adaptable to purifications on the gram scale. Hax and Geurts Van Kessel (28) isocratically purified SPH from beef erythrocytes. Up to 10 mg could be injected onto a 5 μm , 25 x 0.9 cm i.d. column. The SPH was split into three peaks. Fatty acid analysis revealed the longer chains eluted in the first peak, while the shorter chains were more retained. This solvent system was also applied to isolate gram quantities of microsomal glycerophospholipids for

preparing model membranes (32). To decrease quantitation errors of the glycerophospholipids analyzed after separation, acids, bases, salts and buffers were not used in the Hex-IPA-H₂O mobile phase. In the linear gradient the water increased while the hexane and isopropanol were kept constant. All the glycerophospholipids PE, PI, PS, PC and SPH were well resolved (Figure 5) and up to 200 mg could be injected. A similar gradient was used to fractionate algal derived glycerophospholipids (33); however water was not present in the initial solvent A, which consisted of Hex-IPA (6:8). Throughout the gradient program, water was added while the Hex-IPA ratio was kept constant. The glycerophospholipids were baseline resolved with a load of 17.5 mg. In addition to solvent programming, flow programming can increase the column loadability (34). A study, on two types of silica, was conducted to determine the influence of water on the loadability, retention and peak shapes keeping the Hex-IPA ratio at 55:44. The water content in the mobile phase plays an important role in the degree of retention for phospholipids. Very high k' values were observed for PA, PC and PI if the water content was less than 5% with both types of silica, thus reducing the amount injected per run. On the other hand the k' value for PE was acceptable with less than 5% water. Therefore a step gradient was incorporated, with an Hex-IPA-H₂O (55:44:4) eluent which allowed the early eluting glycerophospholipids to be resolved. Then the solvent was changed to a ratio of 55:44:5.7. The step gradient was used

Figure 5. Elution of extracted microsomal phospholipids by preparative HPLC. Seventy mg of a phospholipids dissolved in 3 ml of CHCl_3 was pumped onto the column at 18 ml/min and separated by the preparative HPLC procedure. SF, solvent front. Reproduced from reference 32.

with timed increases in flow-rate. Only one type of silica gave adequate scaled up separations with the conditions developed and 100 mg load of soy bean glycerophospholipids were isolated.

In order to obtain complete separation for preparative isolations, two chromatographic methods were required (35). For milligram quantities of CL, PC, PE, PI, PS, LPC and SPH for additional analysis and membrane models, two HPLC methods were combined. Human heart glycerophospholipids, 7 mg, were separated with a IPA-Hex-H₂O gradient (54:41:5 to 52:32:9). All the glycerophospholipids were separated and collected, except PC and SPH which coeluted. The PC/SPH fraction was resolved by rechromatography with an isocratic mixture of ACN-MeOH-H₂O (71:21:8). The two sets of separations were completed within 90 minutes. Milligram quantities (1-2 mg) of PE, PI, PS and PC were isolated on an analytical column (25 x 0.46 cm i.d.) by gradient elution with a combination of the mobile phases Jungalwala et al. (26) and Geurts van Kessel et al. (27). Solvent A consisted of Hex-IPA-ACN-H₂O (364:486:94:56) and in solvent B there was no acetonitrile (394:526:80). This combination of two HPLC methods has recently been incorporated into a single system for the isolation of cod tissue derived glycerophospholipids (36). The initial mobile phase, solvent A, eluted PE and PI, followed by a switch to solvent B for PS and PC. Only 1-2 mg, which provided sufficient quantities for subsequent analysis, were loaded on the 25 x 0.46 cm i.d. column.

Glass (37,38) replaced hexane with isooctane for the

isolation of PC derived from soy beans. A semi-preparative separation of PC was achieved in 20 minutes with an isocratic eluent of isooctane-IPA-H₂O (40:53:7) at a flow-rate of 4 mL/min (37). As much as 1.25 mg of PC could be obtained per injection. The separations were accomplished on a 15 x 1.0 cm i.d. column packed with 5 μ m silica. The mobile phase was further modified by a reduction of isopropanol and an increase of water (40:51:9) along with a corresponding decrease in flow-rate (38). The optimum load was 5 mg with the 15 x 1.0 cm i.d. column. By a 2% increase in water, a 2% decrease in isopropanol and a decrease in flow-rate to 2 mL/min, the k' of PC was similar to that obtained with the original mobile phase composition (40:53:7) at 4 mL/min.

A rapid isocratic separation was developed to purify PAF and Lyso-PAF from human skin (39). A diol phase was used in a 25 x 0.46 cm i.d. column with a tert.-butylmethylether-MeOH-H₂O-NH₃ (200:100:10:0.02) mobile phase. From 1 g of human skin 20-200 ng of PAF-like material was recovered. Andrikopoulos *et al.* (40) reported that up to 2.5 mg of semisynthetic PAF and lyso-PAF could be separated per chromatographic run. The mobile phase was a mixture of ACN-MeOH-H₂O (300:150:35) and flow programming was used to reduce the separation time.

Species Preparative HPLC Separations

Within each glycerophospholipid class a number of molecular species exists due to the varying lengths and degrees of

unsaturation of the diglyceride chains. The separation of multi-molecular species differing in chain length or degrees of unsaturation is a formidable task, because as many as thirty or more species may be present. With many similar components present high efficiency packings are necessary and particle sizes of less than 12 μm are routinely used. Species separations are based upon the hydrophobicity and degree of unsaturation of the fatty acid chains with reversed-phase and/or argentation chromatography. Reversed-phase, C_{18} , supports have been exclusively used to resolve individual molecular species on a preparative scale (33,37,38,41), although there has been a report of partial species separation by silica (22). Molecular species are resolved on C_{18} supports primarily based upon the hydrophobic interactions of the diglyceride chains with the alkyl ligand of the stationary phase. Predications may be made on retention and the elution order of species. The retention progressively increases as the number of carbon atoms in the homologous series increases; however the retention decreases as the number of double bonds in the same fatty acid chains increase. Unfortunately reversed-phase can not resolve every molecular species.

Not only is the separation difficult, but sensitive detection is required. As of now UV detectors are the most sensitive, stable, rugged and easily used for preparative species separations. The UV response is primarily due to the double bonds in the diglyceride chains, but response is very low

for saturated diglyceride chains. In order to use UV detection the mobile phase must be transparent at the working wavelengths (200-210 nm). This requirement greatly limits the choice of solvents as it did for class separations.

A partial species resolution of PE, LPE and PC on silica has been reported (22). Although discrete peaks were not observed, the composition of the species were determined by GC analysis of the collected fractions. The separation profile was constructed based on glycerophospholipid concentration as determined by phosphate analysis and amino acid analysis versus fraction number. The profile revealed partially resolved peaks within the PE and PC peaks. High concentrations of polyunsaturated acyl side chains were present in the early fractions of PE, LPE and PC, while later fractions consisted of mono- and disaturated as well as saturated acyl chains.

Individual PC species were required for calibration factors in the quantitation by HPLC of human bile PC species (41). Egg yolk PC, 15 mg, was fractionated into 15 distinguishable peaks, (Figure 6), on a 30 x 4 cm i.d. column packed with 7 μ m C₁₈. Solvent A consisted of 100% MeOH and B was 20 mmol/L choline chloride in MeOH-H₂O-Acn (90:8:3) permitting UV detection at 205 nm. Each peak was composed of more than one molecular species. The fatty acid composition was determined by GC and HPLC analysis. Twenty three probable PC molecular species were identified. The most abundant species were found in peaks 6, 9 and 15 which comprised 25%, 21% and 22% respectively. The most

Figure 6. HPLC fractionation of 15 mg egg phosphatidylcholine (PC) on a LiChrosorb RP-18 preparative column, 7- μ m particles. The column was eluted as described in 'Materials and methods'. Detection was performed by absorption at 205 nm. Isolated peaks are numbered in sequence of elution. The identification of the probable PC molecular species present in each peak fraction is reported in Table I of reference 41. Reproduced from reference 41.

probable that

on GC analysis

and 18:1-18:2

peak 15: 18:2

PG, PI, PA.

separated into

Choline ester

(39.5:7:2.5)

glycerophospho

species are

of analysis

by HPLC

by HPLC

(12.24) and

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ACH (57.5

analysis

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probable fatty acid coupling within individual molecules, based on GC analysis, were as follows; peak 6: 16:0-20:4, 16:0-18:2 and 18:1-18:2 (1:95:1), peak 9: 16:0-18:1 and 16:0-16:0 (80:20), peak 15: 18:0-18:1 (100). Individual glycerophospholipids PE, PG, PI, PA, PS and PC derived from alga Chlorella kessleri were separated into species on a 5 μ m, 25 x 2.12 cm i.d. column (33). Choline chloride, 20 mM, was used to modify the MeOH-H₂O-ACN (90.5:7:2.5) solvent system. The maximum load for each glycerophospholipid was 5 mg in 100 μ L. Eighteen molecular species were well resolved for PC, (Figure 7). The distribution of molecular species for each class varied which was determined by HPLC and GC-MS. For PC, peaks 2, 7, 13 and 18 were identified by HPLC as 18:3-18:3 (12.4%), 16:0-16:1 (16.8%), 16:0-18:1 (12.2%) and 18:0-18:1 (3.5%) respectively.

Regioisomers of LPC were isocratically separated with a mobile phase comprised of 20 mM choline chloride in MeOH-H₂O-ACN (57:23:20) (42). A difference of 20-25% in retention was sufficient to separate 1-acyl-sn-glycero-3-phosphocholine and 2-acyl-sn-glycero-3-phosphocholine. The k' values were lower for LPC isomers when the acyl chain was in the sn-2 position versus the sn-1 position. Utilizing a 25 x 1 cm i.d. column, 20-30 mg of isomeric LPC species were isolated. Under similar chromatographic conditions, bovine heart and brain LPE and soy bean LPC were separated into molecular species to provide milligram quantities of starting material for subsequent use in the synthesis of homogeneous diracylphospholipid probes (43). Up

Figure 7. Preparative reversed-phase HPLC of PCs. For experimental conditions see Materials and Methods; for peak numbers see Table IV in reference 33. NL = Neutral lipids (mainly triacylglycerols, waxes, sterols, etc.). Reproduced from reference 33.

to 2 mg of the lysoglycerophospholipids could be injected without overloading the column.

In addition to choline chloride, NH_4OAc , has been successfully incorporated into the mobile phase (38,44). Typically, 100 mM of NH_4OAc has been used. The pH, although undefined in organic solvents, was reported to be 7.4 in the aqueous constituent. A semi-preparative separation of soy bean leaves derived PC species was reported with a mobile phase of 100 mM NH_4OAc in $\text{MeOH-H}_2\text{O}$ (95:5) (38). With 2.1 mg injected, five peaks were observed in the chromatogram (Figure 8). The highly unsaturated species, 18:3/18:3, eluted in peak 1, while 16:0/18:2 eluted in peak 5. Polyunsaturated glycerophospholipid species, 16:0/22:6 PC and PE were isolated along with the corresponding oxidation products with a 25 x 2.25 cm i.d. column (44). Up to 10 mg were purified isocratically with 100 mM NH_4OAc in 100% MeOH.

Liponucleotides

Experimental anti-AIDS liponucleotides, AZT-MP-DG and ddC-MP-DG (16:0/18:1), have been synthesized (13,14). Preparative HPLC has been used to isolate and purify these compounds for characterization by spectroscopic methods and to test for antiviral activity, consequently large amounts of high purity are required. Preparative HPLC was performed on reversed-phase supports (45). A column, 25 x 1.0 cm i.d., packed with 10 μm C_{18} was used and the eluent consisted of $\text{MeOH-1 mM KH}_2\text{PO}_4$, pH 2.4,

Figure 8. Chromatogram for the separation of soybean PC into its molecular species on a semipreparative Excello Ultra Pac ODS column (150 x 10 mm). UV detection was at 205 nm (0.08 AUFS). The mobile phase was methanol: 0.1 M ammonium acetate, pH 7.4 (95:5, v/v) delivered isocratically at 2.5 mL/min. Column pressure was 785 psi; chart speed, 0.25 cm/min. Chromatogram was obtained from an injection of 1.0 mL of soybean PC (1,000 μ g/mL) isolated on the semipreparative silica column. Reproduced from reference 38.

(93:7) for AZT-MP-DG and (95:5) for ddC-MP-DG. In order to obtain purities of greater than 99%, up to 25 mg of the crude synthetic reaction mixtures could be loaded onto the column.

Analytical Class Separations

Silica is one of the most commonly used adsorbents for the separation of glycerophospholipid classes. The mode of chromatography associated with silica packings is adsorption chromatography. In the classical sense adsorption chromatography, also called normal phase chromatography, is carried out with non-polar eluents, hexane, which can be modified with more polar solvents, such as chlorinated solvents and/or alcohols. The silica packing is polar in nature and retention is based upon the polar moiety(s) of the solutes (19,46,47). There are complex and substantial interactions between the silica stationary phase and the polar head groups of the glycerophospholipids. The non-polar diglyceride chains do not possess any significant attraction to the polar stationary phase. Since the attraction is very strong between the head groups and the silica, non-polar mobile phases have to be modified with polar solvents otherwise k' values would be too large. The large k' values increase analysis time and band broadening, thus reducing sensitivity.

The two most commonly used mobile phases for class separations consist of ACN-MeOH-H₂O, developed by Jungalwala et al. (26), and Hex-IPA-H₂O, developed by Geurts van Kessel et

al. and Hax et al. (27,28). These mobile phases which were developed to allow the separation to be monitored with an UV detector, have been modified to improve separations by incorporating gradient elution and/or with additional solvents, salts, acids, bases or buffers. The additional modifiers further complicate the understanding of the basis for separation because simple adsorption can not describe all interactions between the solutes, mobile and stationary phases. The water in the mobile phase acts like a liquid layer which deactivates the silica by coating the silanols via hydrogen bonding.

SILICA PHASE

Acetonitrile-Methanol-Water

With an isocratic mobile phase of ACN-MeOH-H₂O (65:21:14), and a 10 μ m column, PC and SPH in biological fluids were separated with baseline resolution (26). However the solvent peak tailed into the other glycerophospholipids (PE and PS) which eluted prior to PC and SPH. To improve resolution Chen and Kou (48) modified the mobile phase with the addition of a minor amount of 85% phosphoric acid (H₃PO₄). The presence of the acid suppressed ionization, thereby minimizing peak splitting and band broadening. An isocratic eluent of ACN-MeOH-85% H₃PO₄ (130:5:1.5) resolved the six major glycerophospholipids present in tissue extracts but the minor ones coeluted. In addition, off-line determination of lipid phosphorus could be inaccurate because of the presence of the H₃PO₄. The plasmalogens may also be degraded due to the acid. The plasmalogens are the

glycerophospholipids containing an α,β -unsaturated ether at C_1 . The H_3PO_4 was replaced with sulfuric acid (H_2SO_4) to allow better measurements of the lipid phosphorus measurements (49). The amount of acid in the ACN-MeOH- H_2SO_4 (100:3.0:0.5) was critical to the separation. As the acid content was reduced all the peaks broadened and the k' values of PC, PE and PS increased. The PC, PE and PS did not elute without the acid. Again the plasmalogens degraded because of the acid; the PE plasmalogens were degraded to LPE which coeluted with the PC. Despite the problems with the H_3PO_4 , Hurst and Martin used an ACN-MeOH-85% H_3PO_4 (780:10:9) mobile phase (50) to separate plant derived glycerophospholipids. In a recent report (51) a mobile phase consisting of ACN-MeOH-85% H_3PO_4 (100:10:1.8) was used to separate PS, PE, ethanolamine plasmalogen, PC and SPH within 8 minutes. The ethanol plasmalogen had to be degraded with HCl fumes prior to injection. However, with a mobile phase composition of 100:40:0.4 the ethanolamine plasmalogens were separated without degradation within 25 minutes. The investigator reported that the acid in the eluent did not degrade the plasmalogens.

An isocratic mobile phase without an acid modifier was described for the determination of polar glycerophospholipids in serum (52). An ACN-MeOH- H_2O (100:10:18) provided adequate resolution of a standard mixture consisting of PS, PE, LPE, PC, SPH and LPC (elution order). The PS was partially resolved from the solvent front and the authors commented that with gradient

elution the PS could have been better separated. Gradient elution was not used because of baseline drift at the low wavelength (200 nm) used to monitor the separation. The separation was complete within 20 minutes.

In order to resolve completely all the major glycerophospholipids; CL/PG, PI, PS, PE, LPE, PC, SPH and LPC (elution order) in a single injection, gradient elution was necessary (53). The initial solvent A consisted of ACN-H₂O (80:20) and solvent B 100% ACN. With a linear gradient from 87.5 to 25% B, glycerophospholipids derived from human semen were resolved. Peak splitting was observed for PS, PC and SPH due to a partial species separation or due to a subclass separation of the plasmalogens. An identical gradient was used to profile the glycerophospholipids in the adult human bronchoalveolar lavage (54). Molecular species of PC; dipalmitoyl PC (DPPC), dilinoleoyl PC (DLPC) and diarachidoyl PC (DAPC) were resolved, although the DAPC coeluted with the PE. Dethloff et al. combined two chromatographic methods (48,81) for the separation of pulmonary surfactant glycerophospholipids (55). Gradient elution coupled with flow programming separated seven classes. Mobile phase A consisted of ACN-85% H₃PO₄ (99:1) and B was modified with methanol; ACN-MeOH-85% H₃PO₄ (79:20:1). Good separation was observed but the PG eluted near the solvent front and SPH was a split peak. A simultaneous polarity and pH gradient was developed by Seewald and Eichinger (56) to separate major glycerophospholipid classes. The gradient was programmed in

several timed steps; initially ACN was used followed by ACN containing 0.2% H_3PO_4 and then changing to MeOH containing 0.2% H_3PO_4 . The conditions used, baseline resolved the following solutes; CL, PI, PS, PE, LPE, PC, SPH and LPC from a pig muscle extract within 60 minutes.

Deuterated solvents have been used to separate isocratically PE, PC and SPH in tissue extracts for IR detection (57). Chloroform was added to the mobile phase to increase the transparency of the mobile phase. For the PE and PC deuterium oxide (D_2O) was used and for SPH deuterated methanol (CH_3OD) in addition to D_2O . The PC and SPH peaks indicated a partial species separation.

Hexane-Isopropanol-Water

It has been reported that lipids have good solubility in Hex-IPA- H_2O (27). The polarity of the mobile phase can be adjusted with water, while keeping the Hex-IPA ratio constant. Glycerophospholipids extracted from human erythrocytes were separated in 40 minutes with gradient elution. The ratio of Hex-IPA was constant at 6:8. The initial H_2O content was 0.75 and final content was 1.4. The PE and LPE eluted in the gradient slope, while the PI, PS, PC/SPH, SPH, LPC eluted at the gradient plateau. A complete separation of PC and SPH was not achieved. Other modifications were made in the gradient program (28). A step gradient was developed for the glycerophospholipid separation of snail suboesophageal ganglia in which the proportion of Hex-IPA- H_2O (6:8:0.75) was stepped to (6:8:1.75).

PA (peak b), PI(peak d), PE(peak e,f) were eluted in the initial solvent while PS(peak g), PC(peak h) and LPC(peak j) were highly compressed (Figure 9). The break in the baseline was due to the abrupt change in water content.

Separations on silica were examined using neutral and acidic mobile phases (58). The neutral system contained 0.1 M ammonium chloride, NH_4Cl , which was used in place of water, and H_2SO_4 for the acidic eluent. The H_2SO_4 was used for ion suppression. The Hex-IPA ratio was varied while the NH_4Cl concentration was constant. Increasing the IPA content did not decrease k' , but as the IPA was decreased the retention was also decreased. The glycerophospholipids least affected were PG and PI since the phosphodiester was the only ionizable group, whereas the ones with two ionization sites, PE, PC and SPH, and the one with several ionization sites, PS, were the most affected. Phosphatidylserine, which has several ionizable groups, eluted as a broad peak due to the presence of a number of ionic species. Therefore, to suppress the ionization H_2SO_4 was added to improve the peak shapes of acidic glycerophospholipids. Experimentally it was shown that with the presence of 0.14% acid the plasmalogens were converted to the lyso forms. In the analysis of rat brain the peak shapes of PI and PS were much improved with the acidic mobile phase. In addition the elution order of some solutes was altered. To circumvent the degradation of the plasmalogens, acids were not used for the separation of cellular glycerophospholipids (59).

Figure 9. Separation of total lipid extracts from suboesophageal ganglia of the snail *Helix pomatia*. Sample load, 5 μg in 50 μL of solvent; room temperature; flow-rate, 1.1 mL/min; detection at 206 nm; column, Spherisorb S-10 W. The n-hexane-2-propanol-water gradient used is indicated. Reproduced from reference 28.

The gradient system used was previously described (27). Fairly broad peaks were observed and most of the solutes eluted as partially resolved pairs. The authors identified the peaks, however there was one discrepancy. The LPC was resolved near the solvent front, while LPC has been reported to be highly retained eluting last after the SPH (27,28,60). This change in elution order may be caused by the type of silica used or the LPC was incorrectly identified. β -Glycerolysophospholipids were synthesized enzymatically from the corresponding parent glycerophospholipid (60) and were separated with a mobile phase previously described (27). The retention behavior of the β -lyso derivatives were correlated with those of the intact glycerophospholipids and an average ratio, $t_R (\beta\text{-lyso})/t_R (\text{intact})$, of 1.51 was calculated, with a range between 2.2 (LPE/PE) and 1.1 (LPI/PI). Thus an estimate can be made for other β -lyso derivatives provided the elution profile is known for the intact glycerophospholipid. With a judicious choice of gradient conditions and flow-rates, Dugan et al. (61) were able to separate all major glycerophospholipid classes including ethanolamine and choline plasmalogens and most minor classes. Four gradient methods were developed consisting of various combinations of Hex-IPA-H₂O. The first two methods (I,II) were for general class separations. Method I was modified to method II to increase the separation between LPE and PS. A rapid separation, less than 15 minutes, of neutral, glycolipid and glycerophospholipid fractions was achieved with method III.

Method IV was used to separate the ethanolamine plasmalogens from the PE.

Soy beans provide a rich source of glycerophospholipids. The glycerophospholipid distribution plays an important role in the development of soy beans. The distribution of glycerophospholipids in soy beans (62), hexane extracted oil (63) and crude oil (64) were investigated. A step gradient of Hex-IPA-H₂O (57.8:39:3.2) to (52.6:42:5.4) was used to separate soy bean glycerophospholipids (62). The H₂O content was low in the initial eluent permitting good separation of the PE and PI. By increasing the water content the more retained PI, PA and PC eluted. If a higher initial H₂O content was used, the PE eluted with the glycolipids and the PI peak was broadened. A step-wise gradient (63) adapted from Geurts van Kessel (27) was used to separate hexane extracted soy bean oil. The initial eluent consisted of Hex-IPA-H₂O (6:8:0.3), the second eluent ratio was (6:8:0.75) and the final ratio was (6:8:1.4). Glycerophospholipids in crude oil were eluted with a continuous gradient, Hex-IPA-H₂O (56:42:2) to (38:51:11) (64).

Chloroform-Methanol-Water

Conventional TLC separations have also been adapted to HPLC methods. Mobile phases for TLC usually consisted of CHCl₃-MeOH-H₂O in varying proportions along with other modifiers such as salts, acids or bases. However, the presence of CHCl₃ precludes UV detection. Refractive index (RI) (65,66) provided isocratic elution is used, or laser light scattering (LLSD) (67-69)

detectors (vide infra) are conveniently used with UV opaque mobile phases.

Rhee et al. (65) separated PC in soy lecithins with a CHCl_3 -MeOH-acetate- H_2O (14:14:1:1 v/v) mobile phase. The PC was well resolved but broad and the PE and PI coeluted. Paton et al. (66) used a CHCl_3 -MeOH- H_2O eluent (178.5:64:5) to separate glycerophospholipids in amniotic fluid. The elution order for a standard solution was PE, PG, PI, PC and SPH. A negative solvent peak interfered with the PE and the SPH was broad due to splitting.

Although good separations from conventional TLC conditions were adapted to HPLC, these methods lack sensitivity with the RI detector. These UV opaque eluents can be readily used with detectors that volatilize the eluent. Recently, an isocratic mobile phase with light scattering detection was developed for the analysis of egg yolk glycerophospholipids (70). The mobile phase, CHCl_3 -MeOH- H_2O (60:30:4) was modified with 4 mM NH_4OH (0.025) to sharpen peaks, since the presence of acids had the opposite effect. The base may have neutralized the acidic stationary phase, thereby reducing tailing due to irreversible adsorption or ionic interactions. Each glycerophospholipid (elution order), PE, LPE, PC and SPH eluted as single sharp peaks, within 20 minutes, however due to a partial species separation the LPC peak was split but both peaks remained sharp. Although the egg yolk matrix was not complex, many biological samples are composed of lipids of widely varying structures and

polarities. For the separation it is usually necessary to employ step or continuous gradient elution with solvents of widely varying polarities. These solvents are not all compatible with all detectors nor are they all miscible.

A number of ternary gradient systems have been developed to optimize lipid separations with light scattering detection (67-69,71-73). The mobile phases consisted of three vessels; a low polarity eluent, a mediator eluent and a high polarity eluent. The low polarity eluent was used to elute the nonpolar lipids and the high polarity eluent eluted the polar glycerophospholipids. The mediator solvent was used to ensure miscibility between the nonpolar and polar solvents. Nonpolar solvents have consisted of isooctane-tetrahydrofuran, THF, (99:1) (67,73) and Hex-THF (99:1) (71); with mediators of IPA-CHCl₃ (4:1) and polar solvents of IPA-H₂O (1:1) (67,71,73).

It is not necessary to use complex ternary gradient systems when glycerophospholipid classes have been removed from the low polarity lipids. Breton et al. (74) used a CHCl₃-TFA (400:5) to CHCl₃-MeOH-Heptane-H₂O-TFA (100:400:50:15:5) step gradient system for the separation of a glycerophospholipid extract from rat brain hippocampus. The separation was complete in approximately 40 minutes. The MeOH was added to insure miscibility of the H₂O with the CHCl₃. A continuous gradient with CHCl₃-MeOH-NH₄OH (80:19.5:0.5) to CHCl₃-MeOH-H₂O-NH₄OH (60:30:5.5:0.5) was applied to separate commercial soy bean and pig brain glycerophospholipids (69). The separations were

completed in 16 minutes, with all the components baseline resolved.

The stationary phase discussed thus far has been silica. The silica is sensitive to the presence of water. In all the silica separations for glycerophospholipids a large amount of water comprises the mobile phase. Water deactivates the silica by being adsorbed onto strong adsorption sites. Additionally unmodified silica is difficult to re-equilibrate after gradient elution. Silica modified with ion exchange, amino, diol, and cyano functionalities offer different selectivities, improved resolution and faster re-equilibration times for class separations.

ION EXCHANGE PHASE

Glycerophospholipids containing primary or quaternary amino functional groups can be readily separated by ion exchange. At neutral pH the amino groups are positively charged and can be separated by cation exchange chromatography. The separation is based on the polar interactions due to the high organic content of the eluent and charge interactions based on the stationary phase. Acidic glycerophospholipids (PI, PA and PG) with the neutral conditions employed are not retained in any significant extent. Due to the complex amphipathic character of PS, it is coeluted with PE. Gross and Sobel (75) used a neutral ACN-MeOH-H₂O (400:100:34) mobile phase to completely resolve PE, LPE, PC, SPH and LPC (elution order) on a strong cation exchange column. The mono amino classes were least retained with respect to the

quaternary classes which was expected. The PI eluted in the void and PS could not be separated from the PE. Increases in the water content reduced all the retention times, whereas increases in ACN increased retention. The mobile phase developed by Gross and Sobel (75) was optimized to (300:150:35) and improved the resolution between semi-synthetic PAF and the LPC (40). The lyso-PAF, which exhibited highest retention, followed the usual pattern of elution after the intact PAF. Flow programming was used to decrease separation time. Rabbit lung lavage fluid derived PC was chromatographed on a strong cation exchange column with an ACN-H₂O (86.2:13.8) solvent system (76). The PC was well resolved from the other glycerophospholipids (Figure 10). The isocratic separation was rapid and the recovered PC was subsequently analyzed by FAB-MS.

AMINO PHASE

The amino phase requires less re-equilibration time for gradient elution separations and is not deactivated by water. The amino phases can act as a weak ion exchanger provided the pH of the eluent is less than 5.0 or as a partitioner with neutral eluents. Glycerophospholipids with choline head groups are less retained with respect to ethanolamine head groups. The presence of the three methyl groups in the choline head group may sterically hinder interaction with the amino groups thus eluting before the ethanolamine solutes. Without the presence of acid in the mobile phase the amino groups are not protonated, therefore the mode of separation is based upon the charge interactions

Figure 10. Magnified part of the cation-exchange HPLC separation of the PC fraction isolated from rabbit lung lavage fluid after Bligh and Dyer extraction (a) and after Bligh and Dyer extraction followed by TLC purification (b). The hatched parts were used for FAB-MS and GC-FID analysis. Chromatographic conditions as described in Experimental. Peaks: PC = phosphatidylcholine: SPH = sphingomyelin: LPC = lysophosphatidylcholine. Reproduced from reference 76.

between the positively charged glycerophospholipids and the electron rich stationary phase.

Separations of synthetic glycerophospholipids were optimized with a gradient system (77). Chloroform was the initial solvent which did not elute the glycerophospholipids. A MeOH-H₂O mixture (25:1 or 25:4) was introduced to elute the solutes. Various combinations of initial and final solvents were used. The water had the greatest influence on the retention. At high water contents the retention was decreased. A neutral gradient system was developed to separate egg yolk derived glycerophospholipids (78) and chloroplast polar lipids (79). The order of elution; PC, SPH, LPC, PE and LPE, with Hex-IPA (5.5:8) as solvent A and Hex-IPA-MeOH-H₂O (5.5:8:1:1.5) as solvent B, was different with respect to ion exchange separations. The glycerophospholipids, PG, PI and PS, did not elute under these conditions.

The synthesis of PC via the methylation pathway of PE was investigated (80). The glycerophospholipids and the two methylated PE, phosphatidylmonomethylethanolamine (PMME) and phosphatidyl dimethylethanolamine (PDME) forms were separated with an ACN-MeOH-H₂O mixture. The composition of the eluent, along with whether an isocratic or gradient elution was to be used, depended upon the condition of the column. New columns required gradient elution to resolve all the components, because the ethanolamine glycerophospholipids were highly retained and the choline compounds eluted with the solvent front. Therefore

a low polarity eluent resolved the choline solutes with an increase of polarity the ethanolamine solutes were resolved. Over a period of time the retention of the ethanolamine solutes decreased. The polarity of the eluent was decreased to the point where isocratic elution (13:7:1) was used. To explain the retention difference between a new column and one which had been used, the authors surmised that the amino groups were deactivated by peroxides, ketones and aldehydes or by the accumulation of contaminants.

DIOL PHASE

The amino phase could not elute all the glycerophospholipids without chloroform. A diol stationary phase, which is less polar than silica, consists of two -OH groups on adjacent carbon atoms in an aliphatic chain. To resolve all the major glycerophospholipids a silica guard column had to be placed before the diol column. The silica changed the elution order of PE which interfered with the PC and SPH peaks. Diol phases, along with silica guard columns, have been used to determine PC/SPH ratios in amniotic fluid (81,82). A gradient of ACN-H₂O (80:20) to ACN (100) resolved PG, PI, PS, PE, PC, SPH and LPC. Kuhnz et al. replaced the water in the ACN gradient system with 5 mM phosphate buffer at pH 5.0 (83). Sharper and more symmetrical peaks were observed, in addition to baseline separation of seven glycerophospholipids. The vessel containing the buffer was prepared fresh daily, because on standing the salts may precipitate. The acidic glycerophospholipids, PG, PI

and PS, were influenced by pH changes whereas the PC, SPH and LPC were not.

CYANO PHASE

Cyano stationary phases have been reported to increase resolution of glycerophospholipids and were less prone to equilibration problems (82,84). Andrews (82) compared (Figure 11) a cyano and diol column for the separation of the major glycerophospholipids in amniotic fluid. The order of elution was identical to the diol phase. Better resolution was observed with the cyano column. The cyano phase required a slightly less polar mobile phase and was easier to re-equilibrate than the diol. Substitution of the water with 5 mM sodium acetate (NaOAc) and two columns in series led to an improved separation (Figure 12) (84). The columns were thermostated at 60°C to increase the solubility of the glycerophospholipids in the presence of a minimal amount of water.

Analytical SubClass Separations

Glycerophospholipid classes can be broken down into 3 subclasses, which are based upon the type of linkage of the sn-1 diglyceride chain. The subclasses are composed of diacyl, alkyl/acyl and alkenyl/acyl-forms. The diacyl derivatives are the most common, while the others are present in smaller quantities. The PE and PC are the major sources of plasmalogens. The plasmalogens are present in preparative HPLC isolations and in the samples for analytical analysis, unless removed prior to

Figure 11. Comparison of the same gradient elution procedures, with two different columns (CN and DIOL), using similar solvents but containing different concentration of water in solvent B. The phospholipids separate in the same order but are better resolved on the CN column. (a) 5- μ m DIOL column (25 cm). Initial conditions: 25% solvent A and 75% solvent B. For peak identification, see Figure 3 in reference 82. Reproduced from reference 82.

Figure 12. HPLC elution profile showing the separation of a mix of arachidonic acid and authentic phospholipids standards. Arachidonic acid (6.7 μg) and 67 μg of each phospholipid standard were injected in a volume of 50 μl of chloroform. Detection was by UV at 206 nm. 0.5 AUFS. Reproduced from reference 84.

injection. An indirect method to determine the amount of plasmalogens is conversion to lyso derivatives with exposure to HCl fumes and analysis with HPLC.

The separation of subclasses is becoming increasingly important. The plasmalogens are important components of central nervous system membranes, blood cells and many other vertebrate tissues (85-87). The subclasses have to be converted to nonpolar derivatives prior HPLC fractionation. The polar head group(s) are cleaved off with Phospholipase C and then acetylated. Normal phase HPLC was used to fractionate bovine brain PE into subclasses for subsequent HPLC species analysis (85). The PE was isolated from the other glycerophospholipids by TLC and converted to acetylated derivatives prior to injection. The mobile phase, which permitted UV detection, was composed of cyclopentane-Hex-methyl-t-butyl ether-acetic acid (73:24:3:0.03). Baseline separation of the three subclasses was achieved within 16 minutes (Figure 13). Myher et al. (86,87) fractionated PE and PC derived from human erythrocytes and human plasma into subclasses after derivatization to trimethylsilyl ethers. The mobile phase was a binary mixture of Hex-IPA (99.7:0.3). The fatty acid profile of each collected subclass was determined by GC.

Analytical Species Separations

The separation of molecular species of glycerophospholipids has been a formidable and difficult task. Species separations

Figure 13. Separation of alkenylacyl (a), alkylacyl (b), and diacyl (c) analogues by normal-phase HPLC on a μ Porasil column. The solvent mixture was cyclopentane-hexane-methyl-t-butyl ether-acetic acid 73:24:3:0.8 (by vol) at a flow rate of 2 ml/min. Column temperature was 36°C. The sample (0.2 μ mol) was dissolved in 20 μ l of hexane and injected. Detection was by absorption at 205 nm. The full-scale absorbance was 0.64. Reproduced from reference 85.

are based upon the subtle differences of varying hydrocarbon lengths and degrees of unsaturation in the diglyceride chains. Reversed-phase, C_{18} , (88-91) and silver ion impregnated, Ag^+ , silica (92,93) supports are able to resolve a number of molecular species, although no one support can separate all molecular species.

Analytical Reversed-Phase Species Separations

Based on reversed-phase species separations of PC, studies involving structure retention interactions have shown general elution patterns of various molecular species (88-91). The separation mechanism proposed for reversed-phase is due to the hydrophobic interactions between the diglyceride chains and the stationary phase alkyl groups. The interactions are dependent upon the amount of contact area between the solute and the stationary phase. Thus the conformation of the solute, length of the hydrocarbon chains and number of double bonds determine the extent of retention. The order of elution corresponds with the number of carbons in the diglyceride chains and degrees of unsaturation. The retention increases as the number of carbon atoms increases in the hydrophobic chains of a homologous species. The presence of a single double bond reduces the retention, however introduction of a second double bond, in the same chain containing the first double bond, does not reduce the retention linearly with respect to one double bond. If, however, the second double bond is located in the other side chain then

the retention is decreased more than a second double bond in the same side chain, for example, 18:1/18:1 has a lower k' than 18:0/18:2. A semilogarithmic plot of log retention time versus total hydrophobic carbons depicts the retention trend graphically (89). In addition, a plot relating hydrophobic carbons to the number of double bonds indicates how much the hydrophobic interaction decreases as a function of double bonds. For example, one double bond decreases the hydrophobic interaction (retention) by the same amount as removing 1.8 carbon atoms from the chain if the double bond was not present (89).

Porter et al. used two types of columns to fractionate commercial egg yolk and synthetic PC with a MeOH-H₂O-CHCl₃ mobile phase (88). A C₁₈ column was used to separate a mixture of saturated PCs. The elution order of fully saturated species was according to the number of carbons; 14:0/14:0, 16:0/16:0, 16:0/18:0 and 18:0/18:0. Additionally, a phenyl or a Fatty Acid Analysis (FA) column separated the saturated PC species in the same order as the C₁₈. The resolution was better with the C₁₈ than the FA column. The saturated species were well separated on either column, except for a mixture of 16:0/18:0 and 18:0/16:0 which are isomers. Mixed chains consisting of double bonds, such as 16:0/18:1 and 18:0/18:1 were not adequately separated by the C₁₈ but were resolved with the FA column. Therefore by a combination of two columns all but one pair of mixed species were separated.

UV transparent isocratic mobile phases have been exclusively used to allow for more sensitive detection. Nakagawa and Waku (94) developed a separation of the molecular species of dimethylphosphatidic acid (DMPA) derived from PC. Prior to injection the PC was treated with phospholipase D, cleaving the $-\text{CH}_2-\text{CH}_2-\text{N}(\text{CH}_3)_3$ group, producing PA. The PA was subsequently treated with diazomethane to produce DMPA. The mobile phase of ACN-IPA-MeOH- H_2O (50:27:18:5) was able to resolve 12 peaks.

Mobile phases for reversed-phase separations are usually comprised of MeOH- H_2O and ACN- H_2O with either choline chloride, potassium dihydrogen phosphate (KH_2PO_4) or NH_4OAc . The salts are added to suppress ionization of the polar head groups. Additional solvents and modifiers have been added to improve separations. A MeOH-1 mM K_2PO_4 , pH 7.4, (95:5) mobile phase was used to separate PC species into 11-13 peaks (89). Egg PC was resolved into 13 peaks and 20 approximately different molecular species were identified which indicates that some peaks contain pairs of mixed species. The major species was 16:0/18:1. Bovine brain and porcine liver PC were fractionated into 12 and 13 peaks respectively. Increased broadening indicated that peaks contained pairs of mixed species. Rat liver PC, PE, and PI were separated into molecular species with a 20 mM choline chloride in MeOH- H_2O -ACN (90.5:7:2.5) and PS with 30 mM choline chloride in MeOH-25 mM KH_2PO_4 -ACN-acetic acid (90.5:7:2.5:0.8) (90). Twenty-six peaks for PC and PE and 28 peaks for PI and PS were detected (Figure 14) and 30 to 35 molecular species were

Figure 14. HPLC separation of the molecular species of rat liver PC, PE, PI, and PS. In the examples shown, 220 μg of PC, 224 μg of PE, 48 μg of PI, and 55 μg of PS were chromatographed on a C18 reverse phase column. The column was eluted as described in Materials and Methods. Detection was by absorption at 205 nm (0.2 absorbance units full scale). Peaks are numbered in sequence of elution and are identified in Table 3 in reference 90. PI and PS, peaks which were too small to be detected in the examples shown, were not numbered. Reproduced from reference 90.

identified within each phospholipid class.

Jungalwala et al. (91) reported the species separation of SPH from bovine brain and sheep and pig erythrocytes with CH_3OH -5 mM KH_2PO_4 , pH 7.4, (9:1). Although 10 peaks were observed for the bovine brain, 11 for the sheep and 12 for the pig erythrocytes not all the molecular species were completely separated because some species coeluted. The fatty acids were determined by collecting each peak and analyzing each fraction by GC.

Ion-pairing agents have been added to the mobile phase to improve species separations of soy bean PI (95). Several tetraalkylammonium phosphates were used, which differed in length of the alkyl group. The longer chained ion-pair reagents caused an increase in the k' values of the PI species. Also an increase in concentration of the ion-pair reagent produced an increase in k' .

Argentation Chromatography

Chromatography performed on a silver column is called argentation chromatography. A different type of interaction process takes place between the molecular species and the silver impregnated silica. The separation is based upon a complexation between the double bonds in the hydrocarbon chains and the silver, thus the separations are based upon degrees of unsaturation. There are few reported HPLC argentation separations for glycerophospholipids (92,93). A silver(I)-

loaded resin was packed into an analytical column and used to separate phosphatidyl acid dimethyl esters derived from egg PC (92). Molecular species with one double bond were not well separated from those with two but were baseline resolved from the species with three double bonds. The eluent for the separation was diethyl ether. This type of silver stationary phase has been recently reported for the fractionation of egg and soy bean PC (93). The stationary phase was synthesized and the column packed in house. A gradient consisting of MeOH (100%) to MeOH-ACN (75:25) resolved the PC species in one hour. The elution order based upon degree of unsaturation can be clearly seen in Figure 15 A,B. The resin based columns performed better than the silver nitrate silica gel columns. Over a period of time resolution degraded fortunately; the column was easily regenerated and exhibited no significant loss of silver ions.

A combination of reversed-phase and argentation chromatography was used for the multispecies fractionation of derivatized SPH for bovine brain (96). The reversed-phase system with a mobile phase consisting of MeOH-ACN-5 mM phosphate buffer, pH 5.4, (100:20:1) separated the species into a number of peaks, in which some contained mixed species (e.g. 24:0 and 26:1 coeluted). The silver column with a mobile phase of MeOH-IPA (8:2) separated the mixture into two peaks which were collected and subsequently chromatographed on the reversed-phase system. Based upon reversed-phase separation and GC analysis, the first peak from the silver column was composed of

Figure 15 A. Fractionation of soybean PCs. Sample size = 10 μ l of 10 mg PC/ml chloroform solution. Flow-rate = 1.0 ml/min programmed, after a 5-min hold, from 100% methanol to acetonitrile methanol (25:75). B. Fractionation of egg PCs. Sample size and conditions as in 13c in reference 93. Reproduced from reference 93.

A **B**

saturated fatty acid species and the second consisted of monounsaturated fatty acids.

POLYMER PHASE

Christie and Hunter (97) reported the species separation of rat liver PC on a polystyrene based polymer column, PLRP-S. The separation did not require inorganic salts in the ACN-MeOH-H₂O (70:15:15) mobile phase. Six distinct peaks were observed, but none contained a singular molecular species. The early fractions were composed of the 16:0 species, while the 18:0 species were in the later fractions.

Liponucleotides

Liponucleotides are a class of glycerophospholipids consisting of a polar nucleoside head group and two fatty acid chains. Rustow *et al.* (98) compared the species patterns of PA, CDP-diacylglycerol and diacylglycerol synthesized de novo in rat liver microsomes by HPLC. The CDP-diacylglycerol was cleaved with phosphodiesterase and methylated with diazomethane. Chromatographic conditions previously developed were used for the species separation with detection at 205 nm (94). Synthetic liponucleotides, 16:0/18:1 AZT-MP-DG and ddC-MP-DG (13,14) have been purified by micro-preparative HPLC (45). Analytical HPLC methods were developed to determine the purity of the liponucleotides isolated from preparative HPLC separations. The analytical HPLC separations have been based upon conventional glycerophospholipid species conditions (45,70) because

liponucleotide properties are more glycerophospholipid than nucleotide. Reversed-phase supports with MeOH-1 mM KH_2PO_4 , pH 2.4, (95:5) have been used to analyze for purity after micro-preparative isolation. The KH_2PO_4 could be substituted with NH_4OAc (99). The NH_4OAc at 1 mM gave the same k' values as those obtained with 1 mM KH_2PO_4 .

DETECTION

After chromatographic separation solutes must be monitored to obtain quantitative information. A number of on-line detectors are available for HPLC. The choice of detector is limited to the types of solutes being detected, the sensitivity required, availability and the compatibility of the eluent(s) with the mode of detection. This section will discuss various on-line HPLC detectors for the quantitative analysis, based upon calibration curves derived from standards of glycerophospholipids from a number of sources. The linear ranges and limit of detection (LOD) will be presented. There will be some reference to mobile phases and columns used, when quantitation is influenced.

HPLC-RI

One of the first on-line detectors used for HPLC is the refractive index detector (RI). The RI, a nondestructive bulk property detector, has been considered to be the "universal

detector" because it is capable of responding to all solutes. Therefore solutes not need to possess UV absorbing chromophores. The only criteria of response is the solutes must possess refractive indices that differ from the mobile phase, since it is the mobile phase which is constantly monitored. Good quantitation can be obtained, although sensitivity is moderate. Though isocratic elution must be used, the choice of solvents comprising the mobile phase is virtually unlimited. Solvents in which are opaque in the UV can be easily used. The RI is a simple detector but has many disadvantages. It is sensitive to temperature, flow-rate and pressure fluctuations. In addition gradient elution is from a practical point impossible. Long equilibration times are usually necessary to achieve baseline stability, especially at sensitive settings.

The RI has been used to monitor glycerophospholipid separations, especially on the preparative scale (22-24). Classical silica TLC glycerophospholipid separations, which incorporate chloroform in the mobile phase, have been adapted to HPLC systems. Chloroform precludes the use of UV detectors, since low wavelengths are necessary to monitor the glycerophospholipids.

Class Detection

On the analytical HPLC scale, the RI has been used to monitor class and molecular species separations. Response for the species separation was not limited to the degree of

unsaturation as in UV. Refractive index detection was utilized to monitor the isolation of hydrolysis products from PC (100).. The fatty acid chain from the sn-2 position was cleaved with phospholipase A₂. The free fatty acids (FFA), LPC and residual PC were isocratically separated with an ACN-MeOH-H₂O (50:45:6.5) and although the mobile phase was UV transparent, an RI detector was used. The isolated FFAs were converted to methyl esters and separated by GC for distribution analysis. Since relatively large amounts of the hydrolysis products were produced, the RI provided sufficient sensitivity. A similar mobile phase, ACN-MeOH-H₂O (50:45:2), was employed with RI detection for the analysis of glycerophospholipids in cow's milk (101). Each glycerophospholipid peak was collected for subsequent GC analysis. Recently a quantitative determination of high-purity egg yolk and soy bean PC by an HPLC-RI system has been reported (102). The HPLC mobile phase consisted of Hex-IPA-H₂O (1:4:1). This mobile allowed the RI detector to be compared an UV detector. In addition the RI was contrasted to the conventional TLC-Phosphate (TLC-P) method. Coefficient of variation (C.V.) of peak area measurements for standard solutions was less than 2.0% for the range, 100 µg to 20 µg. The HPLC-RI method displayed a much smaller C.V. than the TLC-P method. Some inconsistencies in purity arose when the HPLC-UV was compared to the HPLC-RI method. The inconsistencies resulted from the differences of UV response due to the fatty acid compositions, whereas RI response was not influenced by the fatty acids.

Species Detection

An isocratic molecular species separation for egg PC was described by Porter *et al.* (88). A reversed-phase and a fatty acid column was used with mobile phases consisting of MeOH-H₂O-CHCl₃ (100:10:10) and MeOH-H₂O-CHCl₃ (70:19:10) respectively. The composition of egg PC correlated well with other methods. An amount of 0.50 μ mole of PC could be readily detected. Egg yolk (103,104) and soy bean (104) PC was converted to phosphatidic acid dimethyl esters (PAMEs) and fractionated into molecular species. The soy bean PC was found to contain a greater number of esterified polyunsaturated fatty acids than egg yolk.

The profiling of glycerophospholipids in biological fluids can predict various abnormalities. Amniotic fluid glycerophospholipids were analyzed to predict fetal lung maturity (66). The HPLC method was adapted from and compared to the conventional 2-D TLC with phosphate analysis. Temperature changes caused baseline instability because the RI detector was set at a very sensitive attenuation. To circumvent the baseline drift the column, tubing to the detector and the refractometer had to be insulated from temperature fluctuations. Due to the extreme insensitivity of the RI, LPC was difficult to detect and PS could not be detected though a large volume of the amniotic fluid was extracted. The sensitivity, recovery, and precision was determined for each analyte. The accuracy of the HPLC method was compared to the TLC-phosphate method. Although the HPLC

method shortened the analysis time considerably, it suffered from inferior reliability due to the instability of the baseline and a relatively large sample had to be extracted.

Human lung and carcinoma tissue PC was converted to diacylglycerol acetates, which were separated into molecular species and quantified with RI and UV detectors (105). The unsaturated species produced a higher UV response with respect to the saturated species, whereas good RI response was obtained for the saturated species. The UV and RI chromatograms are illustrated in Figure 16. Peaks 10, 14 and 16 were disaturated species which were easily detected with the RI, but detected with difficulty by UV. The response of the acetates was linear in the range of 20 μg , the LOD, to 100 μg . In addition, the species distribution was determined with a scintillation counter which correlated well with the RI percent distribution.

Although sensitivity of RI detectors is low, new models have been thermostated to maintain a constant temperature and pressure fluctuations can be minimized with additional pulse dampers. Under micro-processor or computer control the detectors can be electronically set to maintain a stable baseline and to zero out automatically.

HPLC-UV

Ultraviolet detectors in comparison to RI detectors offer increased sensitivity, stability, ruggedness and gradient elution is readily used provided the solvents have little or no

Figure 16. High performance liquid chromatography separation of diacylglycerol acetates derived from rat lung phosphatidylcholine. Detection: upper chromatogram, UV absorption at 205 nm; lower chromatogram refractometry detection (RI x 8). Reproduced from reference 105.

absorption at the working wavelength. Glycerophospholipids, however are not easily detected because they lack strong UV absorbing chromophores. Glycerophospholipids have low molar absorptivities and must be monitored at low UV wavelengths, 190-210 nm; thus there are a number of constraints on the elution solvents and detector. A limited number of solvents are available which are transparent in this region. For class separations, the two most commonly used mobile phases consist of ACN-MeOH-H₂O (26) and Hex-IPA-H₂O (27,28). For molecular species separations a MeOH-H₂O mobile phase (41,106) is primarily used. These UV transparent solvents can be used in gradient elution modes; but baseline drift at low wavelengths may be a problem. More importantly direct quantitation is difficult because the UV response is predominantly a result of the degree of unsaturation. The response for synthetic and naturally occurring glycerophospholipids varies. A detection limit of 2.5 μ g and 0.2 μ g was reported (52) for synthetic and naturally occurring glycerophospholipids, respectively.

Apparent molar absorptivities were experimentally determined for individual molecular species of PC, which differ in the number of double bonds in the fatty acid chains (26). The absorptivity increased as the number of double bonds increased with little contribution from the carbonyl, carboxyl, phosphate and polar head moieties. For example, the UV response of PC derived from various sources can be different (55). Pulmonary lung surfactant PC absorbed 40% less UV radiation than PC

derived from egg yolk, which is illustrated in the calibration curves **Figure 17 A**. The egg PC contains a higher degree of unsaturation. The UV response of individual molecular species is also affected by the number of double bonds, **Figure 17 B** illustrates the response due to a molecular species containing one double bond, 16:0/18:1 PC, versus a fully saturated molecular species, 16:0/16:0 PC. Therefore, glycerophospholipids composed of multi-molecular species with unknown degrees of unsaturation are difficult to quantify. In order to obtain quantitative data with UV detection, standards consisting of similar diglyceride compositions should be used for calibration. Quantitation by integration should be based upon an internal standard.

Class Detection

In absorption spectra of PC and SPH, the maximum wavelength is approximately 203 nm with a significant decrease in response when approaching 210 nm (26). A linear response was observed for bovine PC of 1 and 60 μg , and for SPH of 1 and 40 μg . Each standard eluted isocratically with ACN-MeOH-H₂O. This mobile phase did not resolve the other glycerophospholipids. Approximately 1 nmole of PC containing at least one double bond was detected. Extracts from liver, heart and human erythrocytes were analyzed for both PC and SPH. The quantitation was based upon the standard curve. The amounts calculated did not correlate well with TLC and phosphorus analysis. The errors were

Figure 17 A. Standard curves of egg PC and surfactant-derived PC. Various amounts of egg PC and surfactant-derived PC were chromatographed as described under Experimental. Both phospholipids were injected in a 5- μ l volume chloroform-methanol (2:1) with the detector response set to 0.100 absorbance units full scale (a.u.f.s.).

B. Chromatogram of 1-palmitoyl-2-oleoylphosphatidylcholine. A 5- μ l volume of chloroform-methanol (2:1) containing 125 μ g of 1-palmitoyl-2-oleoylphosphatidylcholine was chromatographed as described under Experimental. b) Chromatogram of DPPC. DPPC (250 μ g) was injected into the chromatograph in a 10- μ l volume chloroform-methanol (2:1). Reproduced from reference 55.

attributed to the different fatty acid distributions between the samples and standards. To obtain a complete lung profile in amniotic fluid gradient separation was developed to improve the separation of other glycerophospholipids, in addition to improving the peak shapes of PC and SPH (81). Calibration curves of individual glycerophospholipids, from a number of sources, were constructed each exhibiting linearity between 2.5 μg and 20.0 μg . The HPLC method was compared to a conventional TLC procedure using charring and reflectance densitometry. A correlation coefficient of 0.860 was reported, indicating a deviation between the methods. An identical gradient program was used to analyze glycerophospholipids in human semen (53). The calibration curves were linear for the range 0.5 μg to 10 μg for each glycerophospholipid; CL, PI, PS, PE, LPE, PC, SPH and LPC. Quantitation was based upon the standard curves even though the standards were derived from other sources, which may differ in the fatty acid composition and thus in molar absorptivity. To determine how much the carbonyl and phosphate groups contribute to the UV response, two sources of PE, bovine and pig, were analyzed. Nearly identical UV absorbances were produced from the bovine and pig PE, thus the absorption was due not only by the double bonds. However the fatty acid distribution, as determined by GC, was not significantly different.

To improve the correlation between a conventional TLC and a HPLC method, a cyano column with a modified ACN-H₂O gradient was used for the analysis of amniotic fluid glycerophospholipids

(82). Response factors were calculated for each glycerophospholipid and used along with an internal standard. Correlation coefficients relating HPLC and TLC analysis were 0.94 (PG), 0.95 (PI) and 0.97 (PC/SPH). Kuhnz et al. (83) improved UV detection by substituting water with phosphate buffer in the gradient mobile phase. The minimal detectable amount of each compound was 0.5 μg and the linear response was 1.0 and 20.0 μg . Linear response has been extended from 0.5 μg to 20 μg for eight major glycerophospholipids in human lung (54). The standards and samples were eluted in the gradient mode. The HPLC results were very similar to the two dimensional TLC method. However, large deviations were reported for pulmonary lung surfactant glycerophospholipids, when the peaks were based upon direct integration and compared to TLC analysis (55). Calibration by commercial glycerophospholipid standards improved the results slightly. Results improved dramatically when the separated glycerophospholipids were referenced against surfactant PC. To obtain the most accurate results all reference standards should be derived from the analyte sources. In a recent report, glycerophospholipids were quantified by peak area integration (51). The glycerophospholipid standards, derived from bovine, produced a linear relationship in the range 0.01 $\mu\text{g}/10\mu\text{L}$. A detection limit, at 203 nm, of 5 ng was reported. The solutes were originally dissolved with CHCl_3 -MeOH (2:1), but a large solvent peak tailed into the early eluting analytes (PS and PE). Therefore to eliminate the large solvent peak the

solutes were dissolved with Hex-IPA (3:1). The composition of human erythrocyte ghost membranes, lymphocytes and thrombocytes were determined.

Species Detection

Individual molecular species are inherently more difficult to detect and quantify. Disaturated species (i.e. 16:0/16:0) virtually lack any UV absorption and thus may not be detected.

Isolated molecular species of egg derived PC were subsequently used as UV calibration factors for the analysis of biliary PC (41). The molar percent composition for egg yolk PC was determined by the HPLC method and compared favorably with the GC method. Biliary PC was resolved into ten molecular species. The molar percent composition was determined for each species based upon the calibration factors. Consistent and comparable results were obtained for the HPLC method with respect to the GC method. Absorbance ratios at 205 nm and 215 nm were used to determine calibration factors to quantify PC molecular species in biological samples (106). The ratios were used to evaluate the average degree of unsaturation of the molecular species eluted in each band. Calibration factors were calculated by the ratio of the molar amount of each molecular species and the area of the chromatographic peak. There was generally good agreement between the HPLC-UV and GC analysis.

Indirect UV Detection

Indirect UV detection has been primarily used to monitor PAF. The PAF, hexadecyl-[³H]acetyl-PAF has virtually no UV absorption at 200 nm. UV absorbing glycerophospholipids were used as reference markers for detecting the elution of PAF (107). The PAF was eluted between SPH and acylacetyl-PAF with the conditions used. The SPH and acylacetyl-PAF served as good UV absorbing reference peaks. The hexadecyl-[³H]acetyl-PAF was monitored by collecting the elution region of the radio labelled PAF and subsequent off-line scintillation counting.

HPLC-LLSD

The mass detector, based on light scattering principles, was described by Charlesworth (108) and can be considered as an "universal" detector for HPLC. The construction, principles of operation and evaluation have been thoroughly discussed (67,70,108-112). The laser light scattering detector (LLSD) has been commercially available for several years. For light scattering detection analytes need not possess UV or fluorescent chromophores but the following constraints must be met: molecular weights must be greater than 250 and solute volatility must be less than the mobile phase volatility. Glycerophospholipids (62,67-74,97,113) from a variety of sources, as well as triglycerides (112,115) and carbohydrates (116) meet these constraints for LLSD detection. Quantitation is based directly on the mass injected of a substance and not dependent on the molar absorptivity as with UV detectors. Mobile

phases, provided it is volatile, can be composed of virtually any solvent(s) with gradient elution readily employed. High concentrations of salts and buffers, which will precipitate in the nebulizer tubing and syringe, can not be incorporated in the mobile phases although low levels (0.5 to 5 mM) have been used (71). Interferences due to the solvent system is eliminated since it is vaporized. The solutes detected are not recoverable, although a stream splitter may be used for subsequent manipulation. Detection limits and dynamic range, for glycerophospholipids, approach or surpass those of UV detectors.

In order to obtain an adequate signal response and stable baseline, the LLSD must be optimized with respect to evaporation temperatures and gas flow-rate which are independently controlled (109,110,112). The temperature of the nebulizer is critical to providing a homogeneous analyte size distribution. The temperature of the drift tube controls the evaporation efficiency of the mobile phase, while the gas flow regulates the spreading of the solute droplets while being carried to the laser.

Class Detection

Linear (68,74,113) and power (69,73,117) fits have been described for LLSD calibration curves. The linear curves have been further modified to include log/log plots (67,70,109). Christie (68) readily adapted a ternary gradient system to separate nonpolar and polar lipids, from rat heart, erythrocytes

and plasma in a single chromatographic run. The detector response reported (integrator counts verses lipid mass) was approximately linear in the range 50 to 200 μg with deviations from linearity below 10 μg for PE and PC. The quantitative data correlated well to published values. Breton et al. (74) also reported a linear behavior ($R^2 > 0.99$) for PE and PC in addition to PG, PI and PS. The amounts injected ranged between 5 and 40 μg . Linear calibration curves of PC, PE, PI and PS were constructed with respect to an internal standard (i.s.) (113). The area ratios, glycerophospholipid area/i.s. area, were plotted against the weight ratios, glycerophospholipid weight/i.s. weight. Glycerophospholipids from rat liver and heart were quantitatively determined with the standard curves. When the amount of PE and PC obtained by the LLSD was compared to that obtained by a phosphorus assay, small variations were observed which were not significant.

The power function best fit glycerophospholipid standards (69) for a range of 50 $\mu\text{g/mL}$ to 300 $\mu\text{g/mL}$. A detection limit, at $2 \times S/N$, of 20 ng was reported for PC and PE. Commercial soja bean lecithin and pig brain extract were analyzed, the results were in accordance with literature data. Power curves were constructed for eight glycerophospholipids at eight concentrations, 200 ng to 50 μg (73). Band broadening resulted in decreased sensitivity for the glycerophospholipids, with respect to neutral lipids, although 200 ng were detected rivaling UV detection. Lipids from rat tissues were routinely

quantified with only 80 μ g of the extract injected. The calibration curve of seven glycerophospholipid classes (CL, PE, PI, PS, PC, SPH and LPC) were fitted into a quadratic equation (117). The conditions were based on Christie's ternary solvent system (65), which separated neutral as well as polar lipids. The %RSD were less than 1% for retention times and ranged from 0.97 to 12.1% for the area response. Minor components, such as PS and LPC, were detected from a lipid extract from 10 mg of rat liver.

Plots of log area versus log mass injected produce linear curves (67,70,109). Stolyhwo et al. conducted evaluation studies on the response of triglycerides (109) which were adapted to glycerophospholipids (67). The glycerophospholipids were linear from approximately 50 ng to 1000 ng with a detection limit, at 2 X S/N, of 3 ng for CL. However, CL is approximately 2 times greater in molecular weight than any of the glycerophospholipids used. This may account for the very low amount which was detectable since a general decrease in response is a function of decreasing molecular weight (109).

All of the applications discussed have incorporated gradient elution since most mixtures contain lipids of widely varying polarities. An isocratic mobile phase was developed to separate the five major glycerophospholipids in chicken egg yolk, PE, LPE, PC, SPH and LPC (70). The LLSD parameters were optimized and a linear log/log was obtained. The PE, LPE, PC and SPH ranged from 1.25 to 50 μ g, while the LPC ranged 5.0 to 50

μg . The detection limits were $0.25 \mu\text{g}$ (PE), $0.50 \mu\text{g}$ (LPE, PC and SPH) and $1.0 \mu\text{g}$ (LPC). Results from injections of extracted egg yolk glycerophospholipids compared favorably with literature values. In addition to the glycerophospholipid analysis the LLSD was used in conjunction with an UV detector to determine the purity of PC and AZT-MP-DG. The PC (31) and AZT-MP-DG (45) were isolated in separate preparative HPLC runs. Since no non-UV absorbing impurities were detected by the LLSD, the purities of each isolated compound was verified. Fractions of soy bean derived glycerophospholipid isolated by preparative HPLC were analyzed for purity with an LLSD (34) because an UV detector was unsuitable to obtain quantitative results.

The ternary gradient system developed by Christie (68) was modified to contain 1 to 5 mM ammonium chloride or NH_4OAc . The ammonium chloride caused a noisy baseline, which became more stable with the NH_4OAc . The ammonium salts were changed to 0.5 mM serine buffered to pH 7.5 with ethylamine which was applied to the analysis of rat kidney and rat brain (71) and cereals (72). The buffered mobile phase did not increase the background noise significantly and the back pressure was relatively constant.

Species Detection

The LLSD has been employed to monitor species separations of PC (97,114) and PE (114) but mobile phase modifiers, choline chloride and KH_2PO_4 , had to be eliminated. An UV detector was in

series with a LLSD to compare responses for PC and PE species (114). Although the UV detector was more sensitive than the LLSD, in that more components were detected, the LLSD provided a more representative analysis since peak size is a function mass but did not detect minor components. Some peaks with the highest UV intensity were recorded as minor components with the LLSD hence the authors suggested the use of both detectors.

HPLC-FID

Flame ionization detectors have been applied to monitor HPLC glycerophospholipid separations. The flame ionization detector for HPLC is a transport type detector in which the effluent, after chromatographic separation, containing the analytes is deposited onto a moving belt or moving wire. This is analogous to the moving belt interface for MS. The mobile phase is evaporated by a block heater and the remaining analytes are transported to the detecting flame. The analytes must possess methylene groups, because the FID signal is proportional to the number of $-CH_2-$ present. The response is directly related to the mass injected, eliminating the use of response factors. High boiling mobile phase solvents and many buffers, ion-pairing or salts can not be used. Since the mobile phase is evaporated, complex ternary gradients may be run. Although good sensitivity and linearity has been obtained, the analytes are not recoverable. A stream splitter placed before the FID allows a portion of the effluent to be collected for additional analysis.

Class Separations

In 1973, A. Stolyhwo and O.S. Privett (118) reported a gradient separation of rat red blood cells containing nonpolar and polar lipids with FID detection. The FID was also used to monitor the lipid composition of soybeans (119,120) with gradient elution. The FID, in one chromatographic run, provided a means in which glycerophospholipids could be monitored in a matrix containing other lipids which differed in polarity. A linear response, 50 to 250 μg for PS, PE and PC, was observed when the detector signal was plotted against the amount injected (77). The dependency of response with respect to flow-rate was also investigated and as the flow-rate increased, detector response decreased. Detection limits for glycerophospholipids, in Table II, were reported by M.L. Rainey and W.C. Purdy (121) with an isocratic separation. Lipids, from rats, were extracted from blood serum, liver and kidney tissue (122). Due to the complex mixtures, gradient elution was used. A curvilinear relationship, over extended ranges, was described for each lipid class, although the relationship was linear for limited ranges. Maxwell et al. (123) evaluated the response of the FID for PE and PC. Class separations with isocratic elution produced a linear response ($R^2 > 0.9999$) from 6 to 200 μg . For plant glycerophospholipids a linear range from 1 to 200 μg has been reported (124). A ternary gradient system, consisting of UV

Table II. Slopes and detection limits for phospholipids
 Reproduced from reference 121.

Slopes and detection limits for phospholipids

Phospholipid	Slope	Detection limit (μg)
PE	1.3×10^3	10
PS	1.7×10^3	10
PI	1.9×10^3	5
PC	1.1×10^3	15
SM	1.5×10^3	50
LPC	1.1×10^3	90
PA	9.1×10^1	20

transparent solvents, was developed so that an UV detector could be utilized. A chromatogram with FID and UV detection of lipid classes is illustrated in Figure 18 (124). The FID and UV had comparable sensitivities, but UV quantitation was more prone to errors due to the low response for saturated glycerophospholipids.

Species Separations

Egg yolk PC (103) was converted to phosphatidic acid dimethyl esters (PAMES) for the FID, with a stream splitter, three fractions were collected. The PAMES contained in the collected fractions were hydrogenated and reanalyzed with the FID. Four molecular species were identified; 16:0/18:0, 18:0/18:0, 16:0/20:0 and 18:0/20:0.

Plant glycerophospholipid molecular species have been isolated and quantified with FID. Phosphatidylglycerol, from Dunaliella salina, (125) was fractionated into four molecular species. The molecular species composition was determined with the FID and compared to GC analysis. The results obtained with the FID were in agreement with those obtained by GC. Detection with the FID precluded derivatization steps necessary for GC analysis. The quantitation limit for one molecular species was approximately 1.3 nmole. Spinach leaf PC was separated into molecular species (126). The molecular species distribution by HPLC-FID was well correlated with GC analysis. The LOD reported was approximately 1 nmole for a single species. The FID, which

Figure 18. Separation of lipid classes from potato leaves and comparison of various types of detection: (A) FID, (B) UV detection at 205 nm. Peak identification: 15) CL, 16) PE, 17) PG, 19) PI, 20) PA, 21) PS, 22) PC and 23) LPC. Reproduced from reference 124.

is commercially available, is well suited to monitor glycerophospholipid separations. It does not depend upon the presence of chromophores nor isocratic elution. Almost any solvent(s) can be used, except salts or buffers and unless a splitter is used the analytes are destroyed. Due to its complexity and difficulty of operating an FID, the FID has not enjoyed wide spread use as a detector for HPLC.

HPLC-MS

The joining of HPLC to a mass spectrometer (MS) has provided structural characterization as well as quantitation for nonvolatile and/or ionic solutes. The MS can be considered to be the universal detector for HPLC. Although sample introduction has been problematic, three types of interface methods have been used: 1) direct inlet, 2) moving belt and 3) thermospray. Ionization modes, chemical ionization and fast atom bombardment, have been chosen to produce simple fragments.

This hyphenated technique is suitable for the determination of the fatty acid distribution of glycerophospholipids from a number of sources. The MS has been operated in the chemical ionization mode, since it is considered a soft ionization technique, minimizing fragmentation. Pind et al. (127) using a direct liquid inlet with chemical ionization, distinguished mixed species since the fatty acyl groups at the sn-1 or sn-2 position are not lost at the same rate. The group at sn-2 is lost faster than the sn-1 group. Egg yolk, soy bean oil and rat

liver derived PC were converted to sn-1,2-diacylglycerols by phospholipase C and then derivatized to tertiary-butyldimethylsilyl ethers prior to separation for species determination. The effluent, which also was the reagent gas, was split prior to the mass spectrometer. With the aid of calibration factors determined for each species in rat liver, the fatty acid composition from other sources has been quantified. Eighteen species were identified and the linear response was estimated to be 10 nG to 2.5 μ G.

Jungalwala et al. (128,129) has extensively used the moving belt interface for obtaining mass spectra of separated intact glycerophospholipid classes in one chromatographic run. An aliquot containing 5 μ G of the individual standards: PE, PC, PI, SPH and PS, was spotted on the moving belt. The MS was operated in the positive and negative chemical ionization mode with NH_3 or methane (CH_4) as the reagent gas. The major characteristic fragment ions $[\text{M}+\text{H}]^+$ in the positive NH_3 mode were: 142(PC), 141(PE), 105(PS), 198(PI) and 142(SPH). In addition to the standards spotted, lung PC was analyzed in this manner in which nineteen molecular species were identified and quantified. Good agreement was between the MS analysis and GC was obtained. Better results were obtained in the positive ion mode with NH_3 as the reagent gas, thus these conditions were used in HPLC-MS. A standard phospholipid mixture (5 μ G each) was separated. The reconstructed total ion current (TIC) plot (Figure 19 a) illustrates the separation. Specific ions, indicative of the

Figure 19. Reconstructed plots of total ion current (a) and various specific ions (b-h) monitored after HPLC-CIMS of standard phospholipids Bovine brain PE, PC, PS, PI, and PS (5 μ g each) were injected on a Brownlee silica gel (5 μ m) cartridge HPLC column and eluted as described in the text. The eluate was applied to the moving belt of a Finnigan HPLC-MS interface. The solvent was removed by heating the belt at 330°C under vacuum and the phospholipid was introduced into the ion source (150°C) of the mass spectrometer. Positive ion mass spectra were continuously collected in the chemical ionization mode with ammonia as the reagent gas from m/z 100-900, every 7 seconds, under the control of Teknivent model 56K MS data system. The magnification factor (*) is given under each panel. Lyso PI (LPI) is an impurity in PI sample. Reproduced from reference 128.

solutes, may then be plotted (Figure 19 b-h). A partial species separation can be observed from the peak splitting. Eighty micrograms of rat brain glycerophospholipids were separated (Figure 20) and quantified. Although relatively large masses were injected, the authors predicated that with specific ion monitoring (SIM) the quantity of analyte used may be decreased. In addition difficulty arose when two or species had the same molecular weight. Therefore if each class was further fractionated into molecular species the fatty acids with the same molecular weight could be distinguished.

Kim et al. has used the filament-on thermospray technique for molecular species analysis of PC, PE, PI, PS, and SPH (130-132). The filament was required, because sufficient ionization did not occur with a high organic content in the mobile phase. The separations ranged from 5 minutes (PI) to 30 minutes (PC). A three species PE mixture is illustrated (Figure 21). A relatively simple fragmentation pattern is observed for the mass spectrum for the di-18:2 PE peak after HPLC separation. Relative intensities were dependent upon a number of parameters such as vaporizer tip temperature, distance between the tip and the sampling aperture, source temperature and the inner diameter of the capillary vaporizer. The diglyceride fragment was the base peak for PC, PE, PI, and PS, while the ceramide segment was the major molecular ion for SPH. Other peaks generated were due to monoglycerides and the characteristic polar head groups. Typically 20 to 50 μ G of each glycerophospholipid were injected.

Figure 20. Reconstructed plots of the total ion current (a) and various specific ions (b-h) monitored after HPLC-CIMS of rat brain phospholipids (80 μ g). HPLC-CIMS data were collected as described in the legend of Figure 19. Reproduced from reference 128.

Relative Intensity

Figure 21. (a) Reverse-phase chromatography of a standard mixture containing di-18:3-, di-18:2-, and di-18:1-phosphatidylethanolamine using a 3- μ m Ultrasphere-ODS column and 100:6:5 methanol/hexane/0.1 M ammonium acetate. The flow rate was 1 mL/min. The total ion chromatogram (120-820 amu) is presented along with the reconstructed ion chromatograms of respective diglyceride ions.

(b) Positive ion spectrum obtained from the di-18:2-phosphatidylethanolamine peak in the chromatogram shown in Figure 21 a. Water adducts of the glyceride ion B are observed in the spectrum. F1 and F2 in the spectrum represent the fatty acids released by hydrolysis at positions F1 and F2, respectively. Reproduced from reference 130.

a

b

With selected ion monitoring species could be detected in the range of tens of nanograms. Biological activity is dependent upon the fatty acid chain lengths at the sn-1 and sn-2 position of the platelet activating factor (PAF). Determination of the molecular species is important for the elucidation of various biological pathways. Twenty micrograms of beef heart PAF was separated within 4 minutes and five species identified. The species separation of derivatized aminophospholipids, PE and PS, using MS confirmed the molecular species assignments of GLC analysis (133). The PE and PS were derived from rat brain and human red blood cells. Good consistency was obtained between the GLC and the LC-MS results.

Glycerophospholipids from human erythrocytes were fractionated into subclasses (alk-1-enylacyl, alkylacyl and diacyl) with TLC and each subclass derivatized. The derivatives were separated into molecular species. The peaks were sent to a thermospray interface after a post column addition of MeOH-0.2 M NH_4OAc (50:50) (134). Simple fragmentation patterns of standards were obtained, with the ammonium adduct $[\text{M}+\text{NH}_4]^+$ as the base peak. For the derivatized erythrocyte glycerophospholipids, the sodium adducts and monoglyceride fragments were monitored. More than 100 different molecular species were identified.

The fast atom bombardment mass spectroscopy (FAB-MS) technique has recently been applied to the characterization of glycerophospholipids (76,135,136) although the analysis have

been off-line. FAB-MS also produces a relatively simple fragmentation pattern. Operation in the positive ion mode cleaves the phosphate/glycerol bond producing a neutral head group, leaving a positive charge on the acyl chains. The negative mode produces carboxylate anions (RCOO^-) derived from the sn-1 and sn-2 positions, leaving behind a neutral monoglyceride. Rabbit lung lavage fluid phosphatidylcholine was purified by HPLC for FAB-MS and GC analysis (76). The PC molecular ion species distribution correlated well with the GC analysis. Cole et. al (135) reported the direct determination of glycerophospholipid structures with FAB-MS-MS. Tandem MS yielded high selectivity and sensitivity. One picogram of PE extracted from E. coli, equivalent to 10 cells, was easily detected. Therefore, by introducing the effluent from a HPLC column to a mass spectrometer a wealth of structural, as well as quantitative, information can be accumulated. Tandem MS has the potential of extending detection limits to subpicograms.

HPLC-IR

Infrared spectroscopy (IR), in addition to providing quantitation, is helpful in elucidating structural formulas by identifying functional groups. Fourier Transform IR (FT-IR) provides a high scan speed and enhanced detection, since multiple spectra may be acquired with signal averaging. For HPLC-IR two types of couplings have been used; the on-line continuous mode (137) and the off-line mode (138,139). The use

of an IR as a detector for HPLC, especially in the on-line mode, has had its share of problems and constraints. One or more solvents comprising the mobile phase usually are not transparent throughout the entire IR region. However, the absorption due to the solvent may be spectrally subtracted or the analytes extracted from the mobile phase into a more IR transparent solvent. Gradient elution is possible but difficult. Therefore the on-line approach is somewhat limited. The off-line technique may be better suited for HPLC, whereby the mobile phase is vaporized. Although microbore separations have been employed (138,139), most separations are conducted on conventional columns (10-25 cm in length and 0.40-0.46 cm in diameter) with 1-2 mL/min flow-rates. The difficulty with the larger columns is that a great amount of solvent must be vaporized.

The IR could be useful for the monitoring of glycerophospholipids. Glycerophospholipids, in addition to the hydrocarbon fatty acid chain(s) absorbing at $3.40\text{ }\mu\text{m}$, possess carbonyl groups which strongly absorb at $5.7\text{ }\mu\text{m}$. Therefore the IR may be operated at a specific wavelength or can be programmed to acquire a entire spectrum. In an early report, an on-line method was applied to quantify free acids and mono-, di- and triglycerides (140). The eluate was monitored at $5.72\text{ }\mu\text{m}$, with an IR detector designed for liquid chromatography. Despite baseline disturbances, due to the gradient, good quantitation was achieved. Chen et al. (57) reported isocratic separations of PC, PE and SPH substituting D_2O for water and CH_3OD for

methanol. The deuterated solvents permitted detection at $5.75\text{ }\mu\text{m}$ for PC and PE, SPH at $6.15\text{ }\mu\text{m}$ for the amide group. Standard curves were constructed exhibiting a linear response between 30 and 250 nmole. Rat liver glycerophospholipids were analyzed and compared well to the phosphorus assay. Though expensive deuterated solvents were used and sensitivity was poor, the IR was specific in response and the peak areas reflected the amount of glycerophospholipid eluted.

To eliminate baseline drift, due to gradients, and solvent absorption, an off-line system has recently become commercially available (138,139). The effluent from the HPLC column enters a splitter which then flows to a nebulizer nozzle, located above a rotating sample disc. The speed of rotation is under microprocessor control. The heated nozzle rapidly evaporates the mobile phase, while depositing the solutes in a tightly focused path. The nozzle temperature can be programed to accommodate gradient elution. Once all the solutes have been deposited, the disc is placed in the reflectance module for IR analysis of the individual components. Interferences due to solvents are completely eliminated without the need to spectrally subtract the solvents. Four glycerophospholipids, PE, PC, SPH and LPC, were isocratically separated and FT-IR spectra were obtained (70). The spectra clearly display the absorption due hydrocarbon and carbonyl groups. The splitter allowed $1.6\text{ }\mu\text{g}$ of each glycerophospholipid to be deposited onto the disc. Lower amounts could be detected, which may rival UV detection: The spectra

obtained are free from any interferences such as Nujol, KBr or solvents which are commonly used to treat or dissolve the compounds. Unfortunately high concentrations of salts and buffers can not be used, because of potential clogging of the nebulizer.

The IR offers functional group characterization and quantitation in HPLC. The off-line method eliminates many of the problems associated with direct coupling. Chromatographic resolution is maintained as the solutes are deposited onto the rotating disc. IR detection shows good potential for following reactions and detecting closely related isomers. Detection limits can be improved with multiple scans.

DERIVATIZATION

To enhance detection using a UV or fluorescence detector, various UV chromophores and fluorophores consisting mainly of aromatic ring(s) have been coupled to glycerophospholipids. The response is not dependent on the degrees of unsaturation, as it is for underivatized glycerophospholipids. Therefore the peak area can be directly correlated to a calibration curve without determining response factors. Commonly, for derivatization, glycerophospholipids must be degraded to diglycerides with phospholipase C and then coupled with the chromophore.

Class Derivatizations

Jungalwala et.al. (141) had to derivatize

glycerophospholipids because detectors with low wavelengths were not readily available in 1976. A procedure for a sensitive analysis for the glycerophospholipids, PE, LPE and PS, containing a primary amino groups was developed in which biphenylcarbonyl was the derivatization agent. The biphenylcarbonyl-glycerophospholipids were monitored at 280 nm and exhibited extinction coefficients of approximately 20,000. The high wavelength allowed the use of halogenated solvents in the mobile phase. An isocratic solvent system consisting of methylene chloride-MeOH-15M NH_3 (92:8:1) for PE and (80:15:3) for LPE and PS was used. A linear response was obtained ranging from 10-500 nmol, with a limit of detection of 10-13 pmol (or 0.3-0.4 ng of phospholipid P). 1-Dimethylamino-naphthalene-5-sulfonyl chloride (Dns-Cl) has also been used to derivatize the amino-containing PE, LPE and PS, LPS (142), for fluorescence detection. An excitation of 342 nm and an emission of 500 nm was used. The separation was a modification of the method of Jungalwala (141) in that a gradient method was developed to separate all components in a single analysis. The calibration curve ranged from 50-500 ng, with a reported LOD of approximately 20 pmol. Good agreement was obtained for the analysis of rat brain PE, LPE and PS when compared to other methods. In this method sample preparation was simpler and no prior purification of the reaction mixture was necessary. Ethanolamine and serine containing glycerophospholipids were derivatized with succinimidyl 2-naphthoxyacetate and monitored

with a fluorescence detector (excitation $\lambda=228$ nm, emission $\lambda=342$) (143). The reaction time to reach maximal and constant response was within 1 hour. The analytes were separated by gradient elution with methylene chloride-MeOH-15 M NH_4OH (90:11:1.5) to (70:20:5). The response was linear between 0.5 nmole and 5 nmole with a LOD of 2 pmole.

Beef brain PE was benzoylated with benzoic anhydride producing diradylglycerobenzoates which were separated, by normal-phase HPLC into three subclasses (144). The subclasses were: alk-1-enylacylglycerobenzoates, alkylacylglycerobenzoates and diacylglycerobenzoates, each composed of multi-molecular species. For the subclasses an amount of 7.7 nmoles was routinely detected. The distribution of fatty acids, within each subclass, was then determined by reversed-phase HPLC. A wavelength of 230 nm was used for both separations. The HPLC method was developed to replace an older, less sensitive TLC method.

The derivatives described have involved the coupling of a chromophoric group chemically to the glycerophospholipid. Recently, a HPLC post-column method was developed for the analysis of hydroperoxy-glycerophospholipids, PC and PE, (145). The detection was monitored at 505 nm based upon the oxidation of Fe^{+2} to Fe^{+3} by hydroperoxides and the subsequent formation of an ironthiocyanate complex. A linear response was obtained at concentrations between 0.2 and 0.8 nmoles.

Species Separations

Derivatized and underivatized individual molecular species have been separated on reversed-phase packings for profiling the fatty acid distribution and for quantitation. However, the signal produced is weak or not present for underivatized glycerophospholipids composed of saturated mono and diglyceride chains. With derivatization, for example, glycerophospholipids with saturated fatty acids exhibit strong responses making quantitation possible. Hsieh *et.al.* (146) produced phosphatidic acid dimethyl esters (PAMES) from PC by an enzymatic hydrolysis with phospholipase D and treatment with diazomethane. Initially, a RI detector monitored the separation but was insensitive; therefore the dimethyl esters were replaced with dibenzyl esters. Egg yolk PC was converted to phosphatidic acid dibenzyl esters (PABEs) and separated on a reversed-phase column with UV detection at 254 nm. The PABE method was approximately 20 times more sensitive than the PAME method. To characterize PC molecular species of Gram negative bacteria, Batley *et al.* (147) generated 1,2- and 1,3- diglyceride p-nitrobenzoates. The 1,3-diglycerides were well separated from the corresponding 1,2-isomers and detected by UV at a wavelength of 254 nm. Relative peak areas of approximately 1 nmole were related directly to the molar ratios of the diglycerides present. Diacylglycerols of beef brain PE underwent benzylation and were fractionated into three subclasses (148). The derivatized subclasses were separated into twenty species, which were identified and

quantified at 230 nm.

Fluorescent derivatives of PE subclasses, diacyl-PE and lyso-PE, were esterified with 9-anthryldiazomethane (149). The derivatized subclasses were separated to determine the fatty acid composition, monitored at an excitation wavelength of 365 nm and an emission wavelength of 412 nm.

In addition to p-nitrobenzoate derivatives, glycerophospholipids after treatment with phospholipase C were reacted with 3,5-dinitrobenzoyl chloride (150-153). This procedure was modified from Batley et al. (147) to minimize the production of 1,3-diglycerides. Monoenoic and dienoic species of PC were separated (150), but the polyenoic species were not well resolved. Detection was at 254 nm and areas were directly related to the amount injected. A linear plot from approximately 20 to 500 pmol was obtained. Derivatized polyenoic PC and PE species were resolved (151). Human platelet PC and PE species were quantitatively analyzed (152). The molecular species composition of several glycerophospholipid classes: PC, PE, PI, PS and CL, from human umbilical artery and vein endothelial cells, were determined as dinitrobenzoyldiradylglycerols (153). Over twenty five species were identified within each class. In a study on the up-take of specific molecular species of PC, diacylglycerobenzoates were analyzed by HPLC (154). The amount of the increased species was easily determined in rat bile.

In addition to glycerophospholipids derived from animals and bacteria, plant glycerophospholipids have derivatized for

quantitation (155). Phosphatidyl glycerol was converted to p-anisoyl diacylglycerols. The separation was monitored at 250 nm and 200 nm. At 250 nm the absorbance was directly related to molar proportions. Additionally, relative response factors were calculated by 200 nm/250 nm wavelength ratios. The relative response factors were used to determine molar amounts of intact PG monitored at 200 nm. Saturated species were not detectable at 200 nm, which is a limitation of this method. Rye was grown in Europe and Japan to compare the glycerophospholipid molecular species distribution (156). Molecular species of PC, PE, and PI were derivatized to dinitrobenzoyl analogues. The major species of PC and PE were dilinolein, palmitoyllinolein and oleoyllinolein, whereas PI consisted mostly of palmitoyllinolein and dilinolein. For the rye grains grown in Europe and Japan the species distribution was similar for each glycerophospholipid.

Molecular species of beef brain sphingomyelin were benzoylated with benzoic anhydride forming 3-O-benzoylated sphingomyelin (96). A combination of argentation and reversed-phase HPLC, monitored at 230 nm, was used for the complete separation of derivatized molecular species. Cardiolipin, derived from bovine, was derivatized to 1,3-Bisphosphatidyl-2-benzoyl-sn-glycerol dimethyl ester and separated into 11 species (157). A reversed-phase gradient was developed with detection at 228 nm. Six molecular species were identified which accounted for 97 mol% of the mixture. The peak area was plotted against inorganic phosphorus. A correlation of 0.999 was obtained,

suggesting the HPLC method was quantitative. The molecular composition of CL was modified by catalytic hydrogenation producing longer retained saturated species, which required 3.5 hours for elution.

Fluorescence, as well as UV, detection was used to monitor separations, in the picomole range of diacylglycerols isolated from rat liver microsome PC as naphthylurethane derivatives (158). A detection limit of 100 pmol at 290 nm was obtained with UV and approximately 10 pmol with fluorescence.

Lyso glycerophospholipids are more difficult to monitor with UV, due to the absence of the fatty acid chains in one of the sn- positions. To increase the detectability, fluorescent fatty acids were used to derivatize lyso-platelet activity factor (lyso-PAF), lyso-PI and lyso-PE at the hydroxyl group at the 2-position in the glycerol backbone (159). The derivatization was carried out in a one pot procedure. The derivatized lyso glycerophospholipids could be monitored with an UV detector ($\lambda=253$ nm) or a fluorescence detector (excitation $\lambda=254$ nm and emission $\lambda=450$ nm). Six different fluorescing fatty acids were used. Smaller chain fatty acids provided a higher yield (>90%) of ester formation. Anthracenepropionyl derivatives produced the best response with detection down to 1 ng.

A detection limit of at least 1 pmole has been reported for naproxen fluorescent derivatives of glycerophospholipid molecular species (160). At 230 nm the molar absorption coefficient of the derivatives was $53,000 \text{ L mol}^{-1}\text{cm}^{-1}$. The

calibration curve was linear from 15 pmol to 10 nmol of a single molecular species. A chromatogram of the derivatives near the detection limit is shown in Figure 22. Postle (161) described a post column derivatization system for the analysis of rat liver and lung PC molecular species. The molecular species were separated isocratically and passed through an UV detector (200 nm) prior to derivatization. The species were derivatized with the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DHP). The optimum reaction temperature was determined to be 50° C. The post column response at 50° C was linear from 0.5 to 100 nmol for all species analyzed. The minimum detection limit per injection was 0.5-2 nmol. Rat liver contains a large majority of unsaturated species which were detectable with UV and easily quantified with derivatization. However, rat lung consists primarily of saturated species which were difficult if not impossible to detect with UV but was easily detected and quantified with fluorescence. The fluorescence response correlated well with lipid phosphorus measurements ($R=0.970$). The sensitivity could be improved by improving the design of the post column mixing chamber.

On-Line Phosphorus Analyzer

The determination of lipid phosphorus has been the classical method of analysis for glycerophospholipids. Glycerophospholipids are composed of approximately 4% phosphorus

Figure 22. High-performance liquid chromatograms of acetonitrile solution ($2\ \mu\text{M}$) of naproxen-diacylglycerols derived from rat cerebrum phosphatidylethanolamine. Operating conditions: column, 12.5 cm LiChrospher 100 RP-18 ($5\ \mu\text{m}$); mobile phase, acetonitrile-2 propanol (5:5); flow-rate, $2\ \text{ml min}^{-1}$ at room temperature. The sample 100 pmol of diacylglycerols was injected with a $50\text{-}\mu\text{l}$ loop. Fluorescence detector: excitation at 332 nm, emission at 352 nm. (1) 2.3 pmol 18:1/22:6; (2) 8.6 pmol 16:0/22:6; (3) 10 pmol 18:1/20:4; (4) 3.0 pmol 16:0/20:4; (5) 31.3 pmol 18:0/22:6; (6) 24.6 pmol 18:0/20:4; (7) 5.0 pmol 18:1/18:1; (8) 5.5 pmol 16:0/18:1; (9) 5.0 pmol 16:0/16:0; (10) 8.6 pmol 18:0/18:1. Reproduced from reference 160.

which, when converted to inorganic phosphorus, can be measured spectrophotometrically but is time consuming. An HPLC on-line phosphorus analyzer has been developed for glycerophospholipids (162). The mobile phases used for elution did not interfere with the analysis. A log/log plot produced a linear response for a range between 0.4 nmol and 200 nmol. The practical working range suggested was 1 nmol to 100 nmol. The relative standard deviation, 16.4%, was highest for the 0.4 nmol and 1.2% for 100 nmol. This on-line analyzer has not gained wide spread use.

Radioactivity

Glycerophospholipids have been labelled with ^{14}C , ^{32}P and ^3H for profiling metabolic or synthetic reactions. In many applications the separated radio labelled glycerophospholipids are collected and the radioactivity measured off-line by liquid scintillation counting. On-line radioactive flow detectors are commercially available for HPLC, eliminating the need to collect fractions for off-line measurement. Important use of radioactivity, in addition to quantitation, is the determination of recovery after chromatographic separation. The radioactive detector also allows the use of UV opaque solvents.

Class Detection

Alam et al. (163) used two isocratic mobile phases for the separation of radio labelled glycerophospholipids and PAF. The initial mobile phase consisted of IPA-ethyl acetate-benzene-

H₂O (130:80:30:20) which eluted PE and PI. After the elution of PI, the mobile phase was switched to IPA-toluene-acetic acid-H₂O (93:110:15:15) and PS, PC, SPH, PAF and LPC were eluted. Detection with UV could not be used with ethyl acetate, toluene and benzene. Recovery of radioactivity was reported to be greater than 90%. For the isolation of PAF and Lyso-PAF, Mallet et al. (39), monitored the elution of [¹⁴C] PAF and [¹⁴C]Lyso-PAF. The PAF and Lyso-PAF lacked any significant UV absorption but were easily monitored with radioactivity detection as illustrated in Figure 23. The analysis of rat alveolar macrophages by HPLC with radioactivity detection was compared with scintillation counting after TLC separation (84). Linear regression analysis demonstrated good correlation (0.95) between the HPLC and TLC methods.

Species Detection

Molecular species of ³²P labeled LPA species were separated by reversed-phase HPLC for studies on the formation and subsequent metabolism in biological model systems (164). The LPA were converted to dimethyl derivatives (DMLPA) by methylation of the phosphoric acid hydroxyl groups prior to separation. A good separation was obtained, except for the coelution of the 22:4 and 22:6 species. The authors commented that the sensitivity of the on-line detector was not as good as autoradiography of a band on a TLC plate followed by liquid scintillation counting.

Figure 23. HPLC traces for the separation of phosphorylcholines at 210 nm (solid line) and of [^{14}C]PAF and [^{14}C]lyso-PAF using an on-line radioactivity monitor (dashed line). Reproduced from reference 39.

Electrochemical Detection

Electrochemical detection has been reported for monitoring glycerophospholipid classes (52). The method was based on tensammetry with a mercury electrode. The glycerophospholipids possess strong adsorption to the electrode surface at potentials from -0.2 to -0.7 V and greater than -1.5 V. The optimum potential with the given conditions was -1.8 V. Under flow injection conditions the limit of detection was 0.4 μg per 20 μL for PC, PE and SPH. However, under chromatographic conditions sensitivity was reduced by more than 10 times. The reduction was caused due to interferences by organic compounds washed from the column. Detection of the analytes did not depend upon the degrees of unsaturation as in photometric detection.

Abbiati et al. (165) described an assay for L- α -glycerophosphorylcholine in pharmaceutical forms using a two-step enzymic conversion. The L- α -glycerophosphorylcholine was incubated with glycerophosphorylcholine phosphodiesterase producing choline and glycerophosphate. The choline produced was recovered and injected into the chromatograph. The mobile phase was 15 mM sodium hydrogenphosphate and 0.5 mM tetramethylammonium chloride at pH 7. After separation the solutes entered a post column reactor with immobilized choline oxidase. An electrochemical detector, with an oxidation potential of +0.5 V, measured the enzymatically produced hydrogen peroxide. The linearity of the assay ranged between 2-150 nmol/mL and the LOD was 2 pmol/20 μL . Repeated injections of

standards resulted in a %RSD of 1.15%. A relatively simple and sensitive procedure has been described for the enzymatic/electrochemical determination of L- α -glycerophosphorylcholine in pharmaceutical preparations. The method has been predicted to be useful for the analysis of glycerophospholipids in various samples.

Conclusions

Glycerophospholipids are a complex class of lipids. Their importance in many biological processes requires isolation and quantitation. Their amphipathic properties make chromatographic separations and subsequent detection a difficult task. In order to resolve glycerophospholipid classes many stationary phases are available and gradient elution is usually necessary. Molecular species, to be completely resolved, require multi-chromatographic steps. The mode of detection can place a constraint on the type of solvents and modifiers which can be used for the mobile phase. Many detectors are available each offers a unique method of detection. To fully isolate, purify, identify and quantify glycerophospholipids multiple separation and detection techniques will be necessary.

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ISOLATION AND PURIFICATION OF LECITHIN BY
PREPARATIVE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Mixed-chain, multispecies, egg yolk-derived lecithin was isolated and purified on a silica column with isocratic elution. A method development column (20 x 0.46 cm) packed with YMC 15 to 30- μ m 120 Å spherical silica and a mobile phase consisting of 5 mM ammonium acetate in acetonitrile-2-propanol-methanol-water (80:13:5:12) were used to separate the lecithin from other phospholipids. The mobile phase conditions for the method development system was adopted for two types of preparative HPLC systems: a Separations Technology SepTech NovaPrepTM 5000 system with a 20 x 1.93 cm column and a ST/800A system with a 20 x 5.00 cm Annular ExpansionTM (A/E) column. The

maximum load was 50 uL of crude solution (2mg) for the method development column, 0.90 mL (35mg) for the 20 x 1.93 cm column and 6.0 mL (240mg) for the 20 x 5.00 cm A/E column. The flow-rates were 2 mL/min, 35 mL/min and 235 mL/min, respectively. The fractions collected from the preparative systems were analyzed for purity by analytical-scale high performance liquid chromatography and by thin-layer chromatography with selective detection with molybdenum blue for phospholipids and detection of all organic compounds by sulfuric acid. Purity of the recovered lecithin was greater than 99%.

INTRODUCTION

Natural lecithin does not exist as a discrete compound but is composed of a choline polar head and multimolecular species of varying fatty acid chain lengths and degrees of unsaturation [1] (Fig. 1). Besides being a naturally occurring emulsifier and surfactant, lecithin is also of interest as starting material for the synthesis of novel anti-viral and anti-tumor drugs [2-6]. Since purified lecithin was needed for the synthesis of these drugs, we developed a method for the isolation and purification of lecithin from chicken egg yolk, where a large amount of lecithin is present.

Silica has been the sorbent most commonly used in analytical and preparative separations of phospholipids [7-26]. Patel and Sparrow [10] reported a high load when they separated lecithin from egg yolk on a preparative scale using silica

Figure 1. Structure of Lecithin.

**(R) - Phosphatidylcholine
(Lecithin)**

$(R_1)_n\text{CO}$ = sn-1 fatty acyl chains

$(R_2)_n\text{CO}$ = sn-2 fatty acyl chains

packed columns and a mobile phase of chloroform-methanol-water (60:30:4). One disadvantage of this method is that ultraviolet detection (UV) can not be used, since the chloroform has high absorption at low UV wavelengths. In addition, the large volumes of chlorinated solvents required for preparative separations are detrimental to the environment and to the health of laboratory workers. Analytical separations of lecithin from other sources were developed by Jungawala et al. [11] with a mobile phase of acetonitrile-methanol-water (65:21:14). In 1987 this mobile phase, to which 2-propanol and trifluoroacetic acid were added, was used for preparative work [20]. Gradient elution with a mobile phase of hexane-isopropanol-water has also been reported for analytical separations [16,17,21,22] and for preparative work [25]. However, isocratic elution is preferable if large quantities of lecithin are to be isolated routinely because of the ease of operation and cost savings. Ultraviolet detection is preferable to detection than by refractive index. Its higher sensitivity makes it possible to monitor the separation of lecithin from other phospholipids, which are present in trace amounts relative to the amount of lecithin. Since phospholipids absorb only in the region of 200 to 210 nm the mobile phase had to be transparent at 203 nm, the working wavelength of the separation.

EXPERIMENTAL

Sample Preparation

Crude phospholipids were extracted from one dozen, fresh chicken egg yolks by the method of Singleton et al. [27]. The combined mass of the yolks was approximately 242 grams. The membranous cuticle was removed by placing the yolk on a piece of screening on top of a 2 Liter beaker containing 600 mL of acetone. The yolk was broken and the fluid forced through the screen with a spatula. When the fluid contacted the acetone, a precipitate formed immediately. The mixture, which was allowed to stand at room temperature in the dark for one hour, was then vacuum filtered and the solid washed with 300 mL of cold acetone. The acetone extract, which contained most of the neutral fats and pigments, was discarded. The solid was suspended in 400 mL of 95% ethanol and stored in the dark for two hours. The mixture was vacuum filtered and the solids washed with 100 mL 95% ethanol. The ethanol extracts were combined and dried at 30-40°C. To dissolve the phospholipids, 2 (100 mL) portions of petroleum ether were added to the residue. The petroleum ether solutions were combined and reduced in volume with rotary vacuum distillation by a factor of 3 to approximately 70 mL. When the solution was poured into a beaker containing 400 mL of cold acetone, a precipitate formed immediately. The mixture was allowed to stand in the dark at room temperature overnight until the solution cleared. The clear solution was decanted and the yellow solid (11.9 g) was washed with cold acetone. The phospholipids were dissolved in methylene chloride, dried and stored in the freezer under nitrogen.

Materials

All solvents and reagents (Fisher Scientific Pittsburgh, PA U.S.A.) used for the extraction, method development and analytical analysis were of HPLC grade. The water was doubly distilled and deionized. Each solvent was filtered through a 0.45 μm Nylon-66 filter (AllTech Assoc., Derrfield, IL U.S.A.). For the preparative separations reagent grade solvents were used also from Fisher were used.

Standard egg yolk phospholipids, i.e. lecithin, lysophosphatidylethanolamine (LPE) and sphingomyelin (SPH) were obtained from Sigma Chemical Co. (St. Louis, MO U.S.A.); phosphatidylethanolamine (PE) and lysolecithin from Avanti Polar Lipids, Inc. (Birmingham, AL U.S.A.). These standards were used to characterize the phospholipids and determine retention times on the method development and analytical systems. The concentration of each standard was 1 mg/mL.

A stock solution of phospholipids from the egg yolks was prepared: 2.01 grams of the egg yolk phospholipids were dissolved in 5 mL of absolute ethanol and 4.9 mL of methanol. To the alcohol solution, 0.1 mL of 10% butylated hydroxytoluene (BHT) in methanol was added as an antioxidant. The stock solution was filtered through a 0.45 μm filter. From the stock solution, 3 mL were removed and diluted with 15 mL of methanol. This working solution contained 40.3 μg of phospholipids/ μL .

Method Development

The chromatographic system used for the method development

studies consisted of a Waters 6000A pump (Waters Division, Millipore, Milford, MA U.S.A.), a Rheodyne 7125 injector (Rheodyne, Berkeley, CA U.S.A.), a Knauer variable wavelength detector set at 203 nm with a sensitivity of 0.64 a.u.f.s. (Sonntek, Woodcliff Lake, NJ U.S.A.), and a method development column 20 x 0.46 cm, packed with YMC 15 to 30- μ m 120 Å spherical silica (Yamamura Chemical Co., Kyoto, Japan). The column was packed using a Haskel Pump (Haskel Inc., Burbank, CA U.S.A.). The isocratic mobile phase was 5 mM ammonium acetate in acetonitrile-2-propanol-methanol-water (80:13:5:12) at a flow-rate of 2.0 mL/min. The chromatograms were recorded on an HP 3393A integrator (Hewlett-Packard, Avondale, PA U.S.A.) at 0.2 cm/min, and an Omniscribe recorder (Houston Instruments, Austin, TX U.S.A.) at 0.5 cm/min. All separations were achieved at ambient temperature.

Preparative HPLC

Two preparative systems were used for the isolation of lecithin: a SepTech NovaPrepTM 5000 (NovaPrep) and a SepTech ST/800A (800A) (Separations Technology, Wakefield, RI U.S.A.). With the NovaPrep a ST/2001A column (20 X 1.93 cm) was used; with the 800A, a ST/3002B Annular ExpansionTM (A/E) column (20 X 5.00 cm). Each column was packed with YMC 15 to 30- μ m, 120 Å spherical silica. The NovaPrep column was packed under high pressure using a Haskel pump, while the A/E column was slurry packed.

The NovaPrep column was equilibrated with the mobile phase

at a flow-rate of 35 mL/min. A Knauer variable wavelength detector set at 203 nm, with a sensitivity of 0.64 a.u.f.s., was used to monitor the separation. The NovaPrep was under computer control, utilizing TurboPrepTM software (Separations Technology). The program was set for an equilibration time of 10 minutes, injection time of 0.3 minutes and a run time of 60 minutes. The working solution was manually injected with a syringe. Data were recorded on both a strip chart recorder and an HP 3393A integrator.

The 800A was operated under manual control and the A/E column equilibrated with the mobile phase for 20 minutes at a flow-rate of 235 mL/min (percent flow rate 56%). The Knauer detector with the same settings was used.

Analytical Analysis

Each fraction was analyzed for purity with a Waters 6000A pump, Rheodyne 7125 injector, a Schoeffel Spectro Flow Monitor SF 770 variable wavelength detector set at 203 nm, with a sensitivity of 0.1 a.u.f.s., (Schoeffel Instrument Division, Kratos, Inc. Westwood, NJ U.S.A.) and a 20 X 0.46 cm column containing 8 to 12- μ m Grace silica (W.R. Grace, Baltimore, Md U.S.A.). The flow-rate was 2.5 mL/min and the mobile phase consisted of acetonitrile-methanol-water (40:9:6).

Prior to HPLC analysis, the fractions were concentrated by rotary vacuum distillation at a temperature range of 30 to 40°C (Table I). One hundred microliters of each fraction were analyzed. The fractions containing purified lecithin were pooled

System

Methods

Table I. Volume of collected and concentrated fractions.

800A

System	Volume collected per fraction (mL)	Reduced Volume (mL)
Methods	4	0.5
Nova Prep	70	10
800A	230	40

RESULTS AND DISCUSSION

The results of the experiments described in this paper are summarized in Table I. The data show that the selectivity of the method for the separation of phospholipids from other components of the sample is high. The method is also suitable for the separation of phospholipids from other components of the sample. The results of the experiments described in this paper are summarized in Table I. The data show that the selectivity of the method for the separation of phospholipids from other components of the sample is high. The method is also suitable for the separation of phospholipids from other components of the sample.

and the remaining solvent was removed by lyophilization. A yellowish-white solid was left, which was a mixture of the purified lecithin and ammonium acetate. The lecithin was extracted with methylene chloride. The methylene chloride solution was reduced in volume to less than 10 mL. The final traces of the solvent were removed by a stream of nitrogen. The mass of the lecithin was then determined.

Each fraction was also analyzed with thin layer chromatography (TLC) since the spots could be detected visually. The mobile phase was composed of chloroform-methanol-water (60:30:4)[11]. The fractions were spotted on silica plates (Fisher) against the phospholipid standards and working solution. The plates were developed by two methods: molybdenum blue (Sigma, St. Louis, MO U.S.A.) which is specific for phospholipids, and sulfuric acid which visualizes all organic compounds.

RESULTS AND DISCUSSION

The mobile phase composition was optimized to provide a reasonable capacity factor for the lecithin and the best selectivity for the separation of lecithin from other phospholipids which are present in egg yolk. Since the mobile phase had little low end UV absorption, the separation of phospholipids could be monitored in the 200-206 nm region. A 1 ug/uL mixture of the standards was analyzed using the method development column. The PE and LPE, which had retention times

of 4.7 and 7.8 minutes respectively were eluted prior to lecithin (retention time of 15.6 minutes); the SPH was eluted afterwards at 23.8 minutes, as was the lysolecithin at 28.8 minutes (Fig. 2).

With the method development column a loading study was performed to determine the injection volume of the working solution which was optimal to obtain adequate selectivity and lecithin purity. Samples of 10, 25, 50, 75 and 100 μL were injected and fractions were collected across the lecithin peak.

The fractions were analyzed by analytical HPLC to determine the purity of the lecithin. Peaks were characterized by co-injections with the standards. The lecithin standard was eluted at 9.4 minutes (Fig. 3). In addition, two types of blanks were analyzed; the mobile phase before it entered the column (Before Column) and after the column (After Column) for each separation system. The fractions were also characterized by TLC using molybdenum blue spray to visualize selectively the phospholipids and sulfuric acid to detect all organic compounds.

The optimal injection volume was 50 μL (2mg) of the working solution (Fig. 4A). Nine fractions (2 minutes each) were collected, concentrated and analyzed. Of the 9 fractions, 3-7 contained only lecithin with the desired purity.

When the method development system was scaled-up for each of the preparative columns, the linear velocity was kept constant. In scaling-up the volumetric flow rate scales as the square of the column radius, the sample load scales as the

Figure 2. Method development chromatogram of phospholipid standard mixture, 100 μ L of 1 ug of each phospholipid/ μ L. Peak identification; 1=PE, 2=LPE, 3=Lecithin, 4=SPH and 5=Lysolecithin. Conditions given in Experimental Section.

Figure 3. Analytical chromatogram of 10 μL of 1 $\mu\text{g}/\mu\text{L}$ lecithin standard, t_R 9.4 min. Conditions given in Experimental Section.

Figure 4. Chromatograms of crude egg yolk phospholipids (A) method development chromatogram (2 mg), (B) Nova Prep 5000TM separation (35 mg) and (C) ST/800A separation (241 mg). Retention time of lecithin; 14.9, 17.9 and 18.0 respectively. conditions given in Experimental Section.

column volume, while the run time scales as the column length. The loading scale-up factors for the preparative systems were 17 for the NovaPrep and 118 for the 800A (Table II).

Nine hundred microliters (35 mg) of the working solution were manually injected into the NovaPrep column. The retention time of the lecithin was 17.9 minutes and the run was terminated at 40 minutes (Fig. 4B). Seven fractions of 70 mL each were collected and concentrated to 10 mL. Fractions 1-5 contained lecithin of 99+% purity. Fraction 6 was contaminated with SPH and fraction 7 contained only SPH. Representative analytical chromatograms of the fractions are shown in Fig. 5. Confirmation of purity was made using TLC with two staining methods. For each fraction there was only one spot whose R_f factor corresponded to that of the standard sample of lecithin. In the pooled fractions (1-5) 25 mg of pure lecithin was recovered (72% recovery).

When six milliliters (241 mg) of the working solution were manually injected directly onto the A/E column, the lecithin was eluted at 18.0 minutes and the run was terminated at 43 minutes (Fig. 4C). Fifteen fractions of 230 mL each, 1 minute in length, were manually collected. Each fraction was concentrated to 40 mL and an aliquot analyzed. Fraction 1 contained a minor component which was eluted prior to the lecithin. Fraction 13 contained only a very small amount of lecithin, whereas 14 and 15 did not have any detectable amounts of lecithin; therefore fractions 1, 13-15 were not included in the pooled fractions. Representative chromatograms of the analyzed fractions are shown

Column Number	Diameter mm	Length cm	Inner diameter mm	Flow Rate ml/min	Flow Time min	Flow Rate ml/min
57/2020	3.45	24.7	2.12	1.2	15.2	15.2
57/2001A	3.03	24.7	2.12	1.2	15.2	15.2
57/3002B	3.03	24.7	2.12	1.2	15.2	15.2

Table II. HPLC Scaleup

Column Number	Diameter cm	Length cm	Load g/run	Flow Rate mL/min	Run Time hours	S o l v e n t Usage L/run
ST/2020	0.46	20.00	0.002	2.0	0.83	0.10
ST/2001A	1.93	20.00	0.035	35.2	0.83	1.76
ST/3002B	5.00	20.00	0.236	236.3	0.83	11.81

Figure 5. Analytical HPLC chromatograms of lecithin containing fractions obtained from the Nova Prep 5000TM; (A) mobile phase (after column) blank, (B) fraction 1 and (C) fraction 3. For conditions see Experimental Section.

(Fig. 6). The amount of lecithin recovered was 130 mg (54% recovery). The lecithin from each preparative system was dissolved separately in 1 mL of absolute ethanol and aliquots of 10 μ L of each sample were reanalyzed. The lecithin both from the NovaPrep and the 800A had a purity of 99+%. Development of the TLC plate with molybdenum blue revealed that only one phospholipid, lecithin, was present; development with sulfuric acid also indicated no organic components other than lecithin.

In conclusion a rapid isocratic preparative HPLC method has been developed for the isolation and purification of natural lecithin. With both preparative systems virtually identical profiles were observed, giving credence to an accurate scale-up. The capacity factors and selectivity values for all three systems are given in Table III. High purity was obtained without the use of chlorinated solvents in the mobile phase. To increase the throughput this method can be automated. In addition, it is possible to adopt these conditions for the isolation and purification of lecithin from other natural and synthetic source or for the preparative HPLC of other phospholipids.

Figure 6. Analytical HPLC chromatograms of lecithin containing fractions obtained from the ST/800A; (A) fraction 2, (B) fraction 5 and (C) fraction 12. For conditions see Experimental Section.

System

Methods

Wave Freq

800A

Table III. Capacity factors (k') lecithin and SPH and alpha (α) values of SPH/lecithin.

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MANUSCRIPT III

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF
3'-AZIDO-3'-DEOXYTHYMIDINE MONOPHOSPHATE DIACYLGLYCEROL,
AN ANTI-HIV GLYCEROPHOSPHOLIPID

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SUMMARY

A reversed-phase chromatographic method is described for the analysis of an experimental anti-AIDS drug 3'-Azido-3'-deoxythymidine monophosphate diglyceride (AZT-MP-DG) [1], a phosphatidic acid derivative of AZT. Analytical conditions were based upon conventional separations of glycerophospholipid species. Where AZT-MP-DG was monitored by UV absorption, there were two wavelength maxima. The response was linear in the concentration range used in this study. The peak was characterized by absorbance ratios and peak purities were determined with a rapid scanning UV detector.

(Key Words: Reversed-phase chromatography, glycerophospholipid, phospholipid, liponucleotide, 3'-Azido-

3'-deoxythymidine monophosphate diglyceride, AZT-MP-DG)

INTRODUCTION

Several stages in the replicative cycle [2,3] of the human immunodeficiency virus (HIV) have been established or identified [4,5] as molecular targets or potential sites for drug intervention. At present the only FDA-approved drug for the treatment of AIDS/ARC and early asymptomatic HIV infection [6] is the antiretroviral analog of thymidine, 3'-Azido-2',3'-dideoxythymidine (Azidothymidine, AZT) which inhibits reverse transcription (polymerase). AZT and other clinical dideoxynucleosides (eg. ddI and ddC) exhibit dose-limiting toxicity [7,8] and drug resistant HIV are known. Further, clinical dideoxynucleosides exhibit less than ideal pharmacokinetics, as evidenced by short plasma half-lives of typically 30-60 minutes [8].

In an effort to decrease toxicity and increase efficacy, new experimental anti-AIDS drugs, stemming from earlier work on anti-cancer liponucleotides [9-12], have recently been developed [1] which are phosphatidic acid:nucleoside conjugates. One of these drugs is 3-Azido-3'-deoxythymidine-5'-phosphate diglyceride (AZT-MP-DG) in which AZT is coupled to 16:0/18:1w9 phosphatidic acid (Figure 1).

HPLC is a good technique for monitoring the synthesis of new pharmaceuticals and for assessing the purity of the final product. Therefore we are reporting an analytical reversed-

Figure 1. Structure of AZT-MP-DG.

phase HPLC method for the analysis of AZT-MP-DG. This method, which was optimized for reproducibility, precision, linearity and resolution was used to determine the purity of AZT-MP-DG. The AZT-MP-DG was purified by two different preparative chromatographic methods [13].

EXPERIMENTAL

Reagents. All solvents (Fisher Scientific, Pittsburgh, PA) were of HPLC grade. The water, filtered through 0.45 μm Nylon-66 filters (AllTech. Assoc., Derrfield, IL), was doubly distilled and deionized. The mobile phase was degassed with He before use.

AZT-MP-DG. The semi synthetic AZT-MP-DG (1) was obtained from the Department of Medicinal Chemistry, University of Rhode Island. The compound, a white powder, previously purified by open column adsorption (silica gel, 70-230 mesh) chromatography was considered to be an authentic standard. Purity was confirmed by TLC, NMR (^1H , ^{13}C , ^{31}P) and elemental analysis [1]. Ten milliliters of a stock solution of 20 $\mu\text{G}/\mu\text{L}$ was prepared. A mixture of $\text{CHCl}_3/\text{CH}_3\text{OH}$ (8:2 v/v) was used as solvent and 10 μL of 0.01% butylated hydroxytoluene (BHT) in methanol was added as an antioxidant. To prepare a 2 $\mu\text{G}/\mu\text{L}$ solution, a 2.5 mL aliquot of the stock solution was taken and diluted to 10 mL with $\text{CHCl}_3/\text{CH}_3\text{OH}$ (2:1) containing 25 μL of the BHT solution. For the following standards the solvent consisted of $\text{CHCl}_3/\text{CH}_3\text{OH}$ (1:2 v/v). A 1 $\mu\text{G}/\mu\text{L}$ standard was prepared by diluting 5 mL of 2

$\mu\text{G}/\mu\text{L}$ and 1 mL of the mobile phase to 10 mL with $\text{CHCl}_3/\text{CH}_3\text{OH}$ (1:2). Serial dilutions were made with the 1 $\mu\text{G}/\mu\text{L}$ solution for standards 1-4 in the following way: appropriate volumes of the 1 $\mu\text{G}/\mu\text{L}$ solution were diluted with 1 mL of the mobile phase and brought up to the 10 mL mark with $\text{CHCl}_3/\text{CH}_3\text{OH}$ (1:2). Subsequently, standards 5-7 were prepared in the same manner as 1-4, but with aliquots from the 0.1 $\mu\text{G}/\mu\text{L}$ solution. To each standard, 10 μL of 0.01% BHT was added. The standards spanned a range from 0.005 $\mu\text{G}/\mu\text{L}$ to 0.1 $\mu\text{G}/\mu\text{L}$.

Another batch of AZT-MP-DG was synthesized by the same synthetic route but purified by the reversed-phase mode of HPLC. Chromatography. The chromatographic system consisted of a Waters 6000 pump (Waters Chromatography Division, Millipore Corp. Milford, MA) a Rheodyne injector with 10 μL loop (Rheodyne, Berkeley, CA), a variable wavelength detector (Schoeffel Instrument Division, Kratos Inc. Westwood, NJ) and a rapid scanning UV detector (Barspec, Rehovot, Israel). For the linearity determination, the variable detector was used throughout at 267 nm at 0.02 a.u.f.s. The mobile phase, at a flow-rate of 1.5 mL/min, consisted of CH_3OH -1mM potassium dihydrogen phosphate (KH_2PO_4) (95:5 v/v) at pH 2.4. All separations were performed isocratically at ambient temperature on a 5- μm , 25 x 0.40 cm, LiChrospher^R 100 C_{18} column (EM Science, Gibbstown, NJ). All separations, except those monitored with the rapid scanning UV, were recorded on a HP 3393A integrator, 0.2 cm/min, (Hewlett-Packard, Avondale, PA) and an OminiScribe strip

chart recorder (Houston Instrument, Austin, TX).

RESULTS and DISCUSSION

Liponucleotides such as AZT-MP-DG are analogs of glycerophospholipids. Like naturally occurring glycerophospholipids, they have a polar head group and two non-polar fatty acyl chains esterified to the diglyceride moiety. The physical properties of the liponucleotides are more similar to glycerophospholipids than nucleotides. Thus, separation conditions were based on phospholipid separations in which reversed-phase supports have been used with a mobile phase composed of 90-95% CH_3OH and 5-10% of 1-2 mM KH_2PO_4 (14,15). This solvent system, which permits detection in the low UV range (200-210 nm), is required, since phospholipids do not contain strong UV absorbing chromophores. However, detection methods for AZT-MP-DG can be based on the nucleotide moiety of the molecule since the pyrimidine substituent absorbs strongly in the 250-280 nm region. Therefore, we were not limited in our choice of eluents to organic solvents that are transparent in the low UV region.

Method Development Studies

Since the AZT-MP-DG purified by open column adsorption chromatography was not completely soluble in the mobile phase, chloroform was needed to dissolve it. In the UV spectrum of AZT-MP-DG (Figure 2) which was obtained with the rapid scanning UV, two absorbance maxima are seen, one at 208 and the other at 267

Figure 2. UV spectrum obtained from the rapid scanning UV of purified AZT-MP-DG. Absorbance maxima: 208 and 267 nm. Chromatographic conditions given in Experimental.

183

nm. Either wavelength is suitable to monitor the separations. The wavelength maximum of 267 nm was used because of the strong absorption by the pyrimidine moiety at that wavelength. The UV spectrum of the AZT-MP-DG was identical to the nucleoside starting material, AZT, and the nucleoside intermediate, AZT-5'-MP. However, all three compounds have different retention times. With the rapid scanning UV, the separation was also monitored at 220 and 208 for the purposes of peak characterization and to detect impurities that do not have a chromophore that absorbs in the 250-280 nm region of the UV. Therefore the low UV region must be used to detect these impurities. However, the CHCl_3 used to solubilize the AZT-MP-DG produced a large solvent peak at low wavelengths as is shown in the 3-D plot in Figure 3. Thus, impurities eluting near the solvent front may be obscured; on the other hand at 267 nm there is little absorption due to the CHCl_3 as shown in the chromatogram and the 3-D plot.

Peak Splitting

Peak splitting at all concentrations was observed when the AZT-MP-DG was dissolved in $\text{CHCl}_3/\text{CH}_3\text{OH}$ due to the presence of the CHCl_3 . A higher salt content may have prevented the splitting, but the concentration of the KH_2PO_4 could not be increased, because it precipitates out of solution at concentrations greater than 2 mM. Since changing the salt to ammonium acetate did not solve the peak splitting problem,

Figure 3. 3-D plot of 10 μ g powdered AZT-MP-DG standard. Chromatographic conditions given in Experimental.

KH_2PO_4 was used throughout this study. The retention was not influenced by decreases in salt concentration, but at concentrations greater than 5mM, the retention increased. To eliminate splitting, each standard was "conditioned" with 1 mL of the mobile phase and a minimum amount of CHCl_3 was used for solubilization of the AZT-MP-DG.

UV Spectral Characterization

The absorbance ratios at two wavelengths provides a means of determining the purity of a compound. Usually, the ratio is compared to a literature value for confirmation, but reference values are not available for the AZT-MP-DG. The values of three sets of ratios (267/220, 267/208 and 220/208) are listed in Table I. The values were consistent for 5 samples of AZT-MP-DG. Since the relative standard deviation was lowest for the ratios 267/220 and 267/208, they are the ratios which should be used to characterize AZT-MP-DG.

In addition to absorbance ratios, the peak purity was determined with the rapid scanning detector. Three UV spectra, which are normalized, were taken at three points along the peak. The normalized spectra (Figure 4) were overlaid and the degree of the overlay was given a value. A value near 0.000 indicates with high confidence that no other component(s) were co-eluted with the solute. On the other hand, values approaching 1.000 or greater indicate that another component(s) is coeluting with the solute. The peak purity value of 0.215 we obtained for AZT-MP-

Table I. Absorbance ratios at 267 nm/220 nm, 267nm/208 nm and 220nm/208 nm of powdered AZT-MP-DG.

	(267/220)	(267/208)	(220/208)
	1.23	0.841	0.683
	1.28	0.846	0.660
	1.17	0.881	0.747
	1.25	0.825	0.663
	1.26	0.880	0.696
mean	1.24	0.855	0.690
sdev	0.0421	0.0249	0.0352
%RSD	3.40	2.91	5.10

Figure 4. Peak Purity UV spectrum of powdered AZT-MP-DG standard. Chromatographic conditions given in Experimental.

DG indicates that only a single component is present.

Purity Determination

In order to detect trace contaminants in pharmaceutical analyses injections of high concentrations [16,17] and the use of high sensitivity detectors [18] are required. For purity analysis, a high concentration of AZT-MP-DG was injected which produced a large peak at 267 nm. Minor components were detected, which eluted before and after the AZT-MP-DG. The impurities did not stem from the solvents used. The highest amount injected was 20 μ G. The levels of impurities present were 0.241% and 0.571% for injections of 10 and 20 μ G respectively. The 0.571% was normalized with respect to the 10 μ G injection, which resulted in a level of impurities of 0.286%. Thus the average percent of impurities present was 0.263%. The contaminants have not been identified.

Reproducibility and Precision

Various concentrations of AZT-MP-DG were injected to construct a calibration curve and determine the limit of detection. Each standard was injected five times, beginning with the lowest concentration. The calibration curve ranged from 55.0 pmol to 1100.0 pmol. Representative chromatograms are shown in Figure 5 A-B. Excellent reproducibility was obtained for retention times and the relative standard deviation ranged from 1.48 to 0.235. Constant temperature is required in order to

Figure 5. Representative powdered AZT-MP-DG chromatograms (267 nm), A) 0.005 $\mu\text{g}/\mu\text{L}$ (55 pmole) and B) 0.1 $\mu\text{g}/\mu\text{L}$ (1100 pmole). Chromatographic conditions given in Experimental.

obtain reproducible retention times as deviations were observed with fluctuations in temperature. Also, care must be taken when preparing the mobile phase; minor variations in either the organic or aqueous solvents changes retention times. Listed in Table II are the averaged retention times and areas for the standards. The relative standard deviation for standards of lower concentration are larger than for those of higher concentration. A calibration curve of area versus pmole injected is shown in Figure 6, $R^2 > 0.9988$. The intercept and slope were -433.6 and 2176.9, respectively. The absolute error, 17658 +/- 4959, is relatively constant throughout the working range. At approximately 4 times S/N, the limit of detection was 27 pmoles.

AZT-MP-DG Purified by Reversed-Phase HPLC

Fractions containing AZT-MP-DG which were collected during a semi-preparative RP-HPLC separation [13]. The fractions containing the AZT-MP-DG were not reduced to dryness and remained in the mobile phase used for the preparative separation. Therefore chloroform was not present. A 10 μ L aliquot of each collected fraction from the preparative separation was injected. Representative analytical chromatograms of fractions (Figure 7 A-B) show a degree of high purity. The purest fractions were pooled, concentrated and analyzed. Trace impurities, 0.104%, were detected in the pooled fractions. The pooled fractions were also monitored by the rapid scanning

peak

55

Table II. Reproducibility of AZT-MP-DG retention times and peak areas (n=5).

275

550

820

1100

pmole	t_R			AREA		
	Mean	Sdev	%RSD	Mean	Sdev	%RSD
55	8.29	0.12	1.48	118776	15971	13.4
82	8.59	0.071	0.83	200432	10510	5.2
110	8.82	0.046	0.53	269530	16338	6.1
275	8.70	0.047	0.54	575704	22652	3.9
550	8.65	0.074	0.85	1165700	13916	1.2
824	8.53	0.020	0.24	1719940	24685	1.4
1100	8.67	0.072	0.82	2462900	19537	0.8

Figure 6. Linear response (267 nm) of powdered AZT-MP-DG ($y=2176.9x-433.6$, $n=5$). Chromatographic conditions given in Experimental.

AZT-MP-DG Calibration Curve

Figure 7. Representative analytical chromatograms (267 nm) of AZT-MP-DG fractions collected during a semi-preparative separation [13]. (A) Fraction 3 and (B) fraction 15. Analytical chromatographic conditions given in Experimental.

detector as shown in the 3-D display (Figure 8). An important feature in Figure 8, is the absence of a large solvent peak in the low UV region, because the AZT-MP-DG in each fraction was in the preparative mobile phase which consisted of $\text{CH}_3\text{OH}/\text{H}_2\text{O}$. Therefore, it was possible to monitor the fractions at the low wavelengths without the interference from the solvent peak. A representative chromatogram of the pooled fractions at 208 nm is shown in Figure 9. It clearly illustrates the insignificant absorption due to the solvent. However at 208 nm, the noise levels and baseline drift are more pronounced; therefore, interpretation may be more difficult. For instance, small baseline fluctuations may be recorded as a peak, depending upon the sensitivity of the detector and the integration parameters used.

Absorbance ratios (Table III) were determined for the pooled fractions. The ratios were consistent with values obtained previously and the peak purity parameter, 0.225, agreed with values obtained with the AZT-MP-DG purified by adsorption chromatography.

In summary, an RP-HPLC method for the analysis of a new experimental anti-AIDS drug, AZT-MP-DG, has been described. The purity of AZT-MP-DG for the powdered form was greater than 99.7%. The AZT-MP-DG isolated from the reversed-phase separation was greater than 99.8%. The compound was characterized with a UV rapid scanning detector in which peak purities and wavelength ratios were determined. Although commercial reference standards

Figure 8. 3-D plot of pooled AZT-MP-DG fractions [13].
Analytical chromatographic conditions given in
Experimental.

Figure 9. Analytical chromatogram of pooled AZT-MP-DG fractions monitored at 208 nm. Chromatographic conditions given in Experimental.

ABS. [208 nm]

(267/220)

1.17

7.22

0.77

1.74

Table III. Absorbance ratios at 267 nm/220 nm, 267nm/208 nm and 220nm/208 nm of pooled AZT-MP-DG fractions from micropreparative HPLC.

mean 1.17

sd 0.16

msd 2.00

	(267/220)	(267/208)	(220/208)
	1.22	0.919	0.751
	1.29	0.899	0.696
	1.24	0.861	0.693
mean	1.25	0.893	0.713
sdev	0.0360	0.0295	0.0327
%RSD	2.88	3.30	4.58

are not available, our results were consistent when our methods were used on two different batches of AZT-MP-DG. The AZT-MP-DG behaved in a linear manner. The analytical method described can be used to analyze other synthenic liponucleotides [13]. This reversed-phase method has been scaled to a preparative separation for the purification of liponucleotides [13].

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ISOLATION OF EXPERIMENTAL ANTI-AIDS GLYCEROPHOSPHOLIPIDS
BY MICRO-PREPARATIVE REVERSED-PHASE
HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The experimental anti-AIDS glycerophosphatidic acid:nucleoside (sn-1/sn-2 diacylglycerol:dideoxynucleotide) drugs, 3'-azido-3'-deoxythymidine monophosphate diglyceride (AZT-MP-DG) [1] and 2',3'-dideoxycytidine monophosphate diglyceride (ddC-MP-DG) [2] were isolated and purified by the reversed-phase mode of high performance liquid chromatography (RPLC). The chromatographic separation was based upon the glycerophospholipid moiety of the drugs and the detection on the nucleoside component. The separations were optimized on method development columns packed with the stationary phase to be used in the micro-preparative column and monitored by a UV detector. Fractions were collected and analyzed for purity by analytical-scale high performance liquid chromatography [3] and by thin-layer chromatography [4].

purity of the recovered drugs based on UV and light scattering detection, and on TLC was greater than 99%. The purified compounds were isolated for studies on structure confirmation, physical, biophysical and formulation properties, and anti-HIV efficacy in culture [1,2].

INTRODUCTION

In the treatment of acquired immunodeficiency syndrome (AIDS), AIDS related complex (ARC) and early HIV infection, chemotherapeutic agents are designed to attack one or more stages of the replicative cycle of the human immunodeficiency virus (HIV) [5-8]. An antiretroviral analog of thymidine, 3'-azido-2',3'-dideoxythymidine (Azidothymidine, AZT) which inhibits reverse transcription (polymerase) is presently the only drug approved by the FDA for the treatment of AIDS/ARC and early asymptomatic HIV infection [9]. However, AZT and other dideoxynucleosides (eg. ddC and ddI) exhibit dose-limiting toxicity and have relatively short circulating lifetime [10,11]. In an effort to increase serum half-lives and decrease toxicity and consequently to increase efficacy, a new group of experimental liponucleotide anti-AIDS drugs, originating from earlier work on anti-cancer liponucleotides [12-15], has recently been synthesized [1,2]. These drugs include 16:0/18:1 ω 9 (sn-1/sn-2) phosphatidic acid:dideoxynucleoside or diacylglycerol:dideoxynucleotide conjugates, AZT-monophosphate-diglyceride, (AZT-MP-DG) and

dideoxycytidine-monophosphate-diglyceride (ddC-MP-DG) (Figure 1) [1,2]. To isolate and purify enough material for molecular confirmation, biophysical and anti-HIV (in culture) studies, a micro-preparative reversed-phase separation was developed.

Chromatographic purification techniques of naturally occurring liponucleotides have been accomplished by thin layer chromatography (TLC), normal phase column chromatography on a silica support and ion-exchange column chromatography on silica modified with diethylaminoethyl (DEAE) moieties [16-18]. Synthetic liponucleotides have been purified by TLC and adsorption chromatography [19-21] and DEAE chromatography [20,21].

Solvents used to elute the liponucleotides usually contain large amounts of chloroform. Since this halogenated solvent is a carcinogen, it is preferable not to use it for the preparative isolation and purification of a drug. In addition chloroform precludes monitoring by ultraviolet (UV) detection at wavelengths less than 240 nm. Since phosphatidic acid and other possible contaminants of the synthetic mixture absorb at wavelengths below 210 nm, UV transparent solvents must be used. To eliminate the use of chloroform, which is often used with silica columns to purify compounds, a reversed-phase HPLC method was developed utilizing a UV transparent solvent system. The solutes were monitored in the wavelength range of 260-280 nm and in the low end range of the UV. In addition, an analytical HPLC method with UV spectral characterization was applied to

Figure 1. Structure of AZT-MP-DG and ddC-MP-DG.

determine the purity of the fractions [3].

EXPERIMENTAL

Sample Preparation

AZT-MP-DG.

The AZT-MP-DG in the synthetic reaction mixture was first separated from the bulk of starting materials and side products by silica gel column chromatography. A solution containing approximately 50 $\mu\text{G}/\mu\text{L}$ of the AZT-MP-DG was prepared in chloroform-methanol (8:2) with 10 μL of 0.01% butylated hydroxytoluene (BHT) in methanol added as antioxidant.

ddC-MP-DG.

The gross impurities were separated from the ddC-MP-DG in the synthetic reaction mixture by the adsorption mode of HPLC [20]. The fractions were collected and pooled. The pooled fractions were taken to dryness with a stream of nitrogen, leaving a clear waxy solid of approximately 100 mg. To dissolve the solid, 2 mL of chloroform-methanol (3:2) was used along with 10 μL of 0.01% BHT in methanol. The concentration of the working solution was approximately 50 $\mu\text{G}/\mu\text{L}$.

Materials

All solvents and reagents (Fisher Scientific, Pittsburgh, PA) for the method development, micro-preparative separations and analytical analysis were of HPLC grade. The water was doubly distilled and deionized. Each solvent was filtered through a 0.45- μm Nylon-66 filter (AllTech Associates, Deerfield, IL). The

mobile phases contained 1-1.4 mM KH_2PO_4 adjusted to pH 2.4 with phosphoric acid.

Method Development

The chromatographic system used for the method development studies consisted of a M6000A pump (Waters Division, Millipore, Milford, MA), a Rheodyne 7125 injector with a 100 μL loop (Rheodyne, Berkeley, CA) and a Knauer variable wavelength UV detector (Sonntek, Woodcliff Lake, NJ). Method development columns were 25 x 0.46 cm, packed with YMC Prep-10 C_{18} (10- μm) silica (Yamamura Chemical Co., Kyoto, Japan). The columns were packed using a Haskel Pump (Haskel Inc., Burbank, CA). The separations were optimized on a small scale, thus reducing solvent consumption and sample.

The wavelengths used to monitor the separations were 267 nm for the AZT-MP-DG, and 280 nm for the ddC-MP-DG. Absorption at these wavelengths was due to the pyrimidine moieties.

The mobile phase consisted of methanol-1.4 mM KH_2PO_4 (93:7; v/v) at pH 2.4 for the AZT-MP-DG and methanol-1 mM KH_2PO_4 (95:5; v/v) at pH 2.4 for the ddC-MP-DG. After elution of the liponucleotides, the columns were flushed with 100% methanol to elute any strongly retained impurities. After the highly retained solutes were washed off with 100% methanol, the column was re-equilibrated with the working mobile phase. The optimum method development flow-rate was 2.8 mL/min for the AZT-MP-DG and 3.5 mL/min for the ddC-MP-DG. All separations were achieved at room temperature. The chromatograms were recorded on an HP

3394A integrator (Hewlett-Packard, Avondale, PA) at 0.2 cm/min and an Omniscribe recorder (Houston Instruments, Austin, TX) at 0.25 cm/min.

Micro-Preparative HPLC

Micro-Preparative separations were carried out on a 25 x 1.0 cm column, packed with the YMC Prep-10 C₁₈ (10- μ m) silica (Yamamura Chemical Co., Kyoto, Japan). The HPLC system was a SepTech NovaPrepTM 5000 (SepTech, Wakefield, RI) operated at a flow-rate of 10 mL/min for the AZT-MP-DG and 15 mL/min for the ddC-MP-DG. The NovaPrep was operated under computer control, utilizing TurboPrepTM software (SepTech). The variable wavelength detector was set at 267 nm with a sensitivity of 0.64 a.u.f.s. to monitor the AZT-MP-DG and at 280 nm for the ddC-MP-DG. The working solution was manually injected with a syringe. The NovaPrep contains two pumps: pump A delivered the methanol-KH₂PO₄ and pump B 100% methanol.

Analytical Analysis

Fractions collected during method development and micro-preparative separations were analyzed for purity. The analytical system was composed of a Waters M6000 pump, 10 μ L Rheodyne injector, Schoeffel Spectro Flow Monitor SF 770 variable wavelength detector (Schoeffel Instrument Division, Kratos, Westwood, NJ), a rapid scanning UV detector (Barspec, Rehovot, Israel) and an evaporative light scattering detector (Varex Corporation, Burtonsville, MD).

For the AZT-MP-DG analysis a 5- μ m, 25 x 0.40 cm

LiChrospher^R 100 C₁₈ column (EM Science, Gibbstown, NJ) with a mobile phase consisting of 95:5 methanol-1mM KH₂PO₄ (pH 2.4) was used at 1.5 mL/min. Sensitivity of 0.02 a.u.f.s. and wavelengths of 267 nm and 208 nm the two absorbance maxima of AZT-MP-DG (Figure 2a), were used to monitor the solute. The low wavelength was useful to detect impurities lacking strong UV chromophores such as phosphatidic acid or glycerol.

The ddC-MP-DG was analyzed on a 5- μ m, 25 x 0.46 cm Molnar C₁₈ column (I.Molnar, Berlin, Germany). A mobile phase of 95:5 methanol-1mM KH₂PO₄ (pH 2.4) was used at 2 mL/min. To monitor the solute, 280 nm and 208 nm were used since the ddC-MP-DG also displayed two absorbance maxima (Figure 2b).

For the liponucleotides the light scattering detector had the following conditions: nitrogen flow-rate 85 mm Hg with an exhaust temperature of 92.5 °C and heater temperature of 150.0°C. The mobile phase consisted of 95:5 methanol-1 mM ammonium hydroxide (pH 2.4) with a flow-rate of 1.2 mL/min.

Collected and pooled fractions were also analyzed by thin-layer chromatography on silica plates (Fisher Scientific, Pittsburgh, PA). The mobile phase consisted of chloroform-methanol-2-propanol-water-triethylamine (30:9:25:7:25) [4]. The plates were visualized by methods: molybdenum blue which is specific for phosphorus in the glycerophospholipids and sulfuric acid which is a general reagent for all organic compounds.

RESULTS AND DISCUSSION

Liponucleotides are analogues of glycerophospholipids. Like

Figure 2. (A) UV spectrum of purified AZT-MP-DG obtained with the rapid scanning detector. Scan rate 10 spectra per second. Absorbance maxima: 208 and 267 nm. Analytical chromatographic conditions: Methanol- 1 mM KH_2PO_4 (95:5), (pH 2.4), flow-rate 1.5 mL/min. and (B) UV spectrum of purified ddC-MP-DG obtained with the rapid scanning detector. Scan rate 10 spectra per second. Absorbance maxima: 208 and 280 nm. Analytical chromatographic conditions: same as in (a), flow-rate 2 mL/min.

naturally occurring glycerophospholipids, they are composed of a polar head group and two non-polar fatty acid chains esterified to the diglyceride moiety. The polar head groups of the experimental drugs AZT-MP-DG and ddC-MP-DG consist of the anti-viral nucleosides (AZT and ddC) coupled to 16:0/18:1 ω 9 (sn-1/sn-2) phosphatidic acid (PA) [1,2]. The physical properties of the liponucleotides resemble those of natural glycerophospholipids. Thus separations have been based upon glycerophospholipids which are generally separated by the normal phase mode. However, normal phase separations for glycerophospholipids require ternary mobile phases and/or gradient elution and with reversed phase liquid chromatography, a binary mobile phase with isocratic elution can be used which is preferable for preparative separations. Mobile phase compositions for the reversed phase separation of natural glycerophospholipids typically contain 90-95% methanol and 1-2mM KH_2PO_4 [22,23]. In addition to KH_2PO_4 , ammonium acetate (NH_4OAc) can be used as the salt in the mobile phase [24]. The NH_4OAc at 1 mM gave the same k' values as those obtained with 1 mM KH_2PO_4 .

For purity analysis, aliquots of the collected fractions were injected onto the analytical columns. Since commercial standards were not available for either compound, the liponucleotide peaks were characterized with UV detection using absorbance ratios and peak purity measurements. In addition to UV and TLC analysis, a light scattering detector was used [25].

AZT-MP-DG

The optimum mobile phase composition for the AZT-MP-DG was 93:7 methanol-1.4mM KH_2PO_4 (pH 2.4). The AZT-MP-DG solution was injected onto the analytical 5- μm column, to determine the number of components present (Figure 3). Impurities eluted near the solvent front and after the AZT-MP-DG peak.

Using the method development column a loading studying was performed to determine the highest load injectable, while maintaining a purity level of 99%. Various volumes of the AZT-MP-DG solution were injected and fractions collected. Each fraction was concentrated with a stream of nitrogen, to approximately 2 mL, and analyzed by HPLC. An aliquot of 100 μL of the 50 $\mu\text{g}/\mu\text{L}$ (5 mg) solution was optimal load.

The separation was scaled up to the micro-preparative column. For the micro-preparative separation a flow-rate of 10 mL/min provided adequate retention and resolution. A 400 μL (20 mg) aliquot was injected (Figure 4) which produced a split peak. Eighteen 5 mL fractions were collected across the split peak and each were concentrated by rotary vacuum distillation to approximately 2 mL. Each fraction was analyzed by analytical HPLC for purity. The k' values (3.5) of the AZT-MP-DG fractions across the split peak were identical. Thus the peak splitting may be attributed to the solvent effect due to the chloroform used as the sample diluent. The peak splitting phenomena has also been reported at the analytical level for the AZT-MP-DG [3]. Fractions 1 and 2 were discarded because of early eluting

Figure 3. Analytical HPLC chromatogram of crude AZT-MP-DG, 5 μ L of 50 μ g/ μ L (AUFS 0.10). Chromatographic conditions given in Experimental.

Figure 4. Micro-Preparative Chromatogram of (20 mg) AZT-MP-DG. Mobile phase: methanol- 1.4 mM KH_2PO_4 (93:7), (pH 2.4), flow-rate 10 mL/min, 267 nm, 0.64 AUFS. Fractions were collected between 9 and 20 minutes.

impurities. Fractions 3-17, containing AZT-MP-DG of 99% purity, were pooled. Fraction 18 contained a very minor amount of the product and an impurity after the product, thus it was not added to the pool. The pooled fractions were concentrated to approximately 2 mL with rotary vacuum distillation. Figure 5a illustrates the chromatogram of the pooled fractions. Based upon UV detection at 267 nm the purity of the AZT-MP-DG was 99.8%. The pooled fractions were also monitored at a wavelength of 208 nm to detect any impurities which did not absorb at 267 nm (figure 5b). However no additional impurities were detected at 208 nm. Peak splitting was not observed for the pooled fractions, because the AZT-MP-DG recovered was in a similar solvent mixture as the mobile phase used for the analytical HPLC.

The rapid scanning UV detector was used to obtain absorbance ratios and peak purity values. Three wavelengths at 267, 220 and 208 nm were used to determine the ratios. The ratios at 267/220, 267/208 and 220/208 were compared to ratios obtained on previously purified AZT-MP-DG. For multiple determinations the absorbance ratios were consistent, as shown in Table I. Peak purity values were determined by normalizing the UV spectra along three points of the chromatographic peak of interest. Peak purity values indicate if an impurity is co-eluting with the solute of interest. Low peak purity values, those less than 1.000, exhibit with high confidence that no other component(s) was co-eluted with the solute of interest

Figure 5. (A) Analytical HPLC chromatogram of pooled AZT-MP-DG fractions at 267 nm (0.02 AUFS). Chromatographic conditions given in Experimental and (B) Analytical HPLC chromatogram of pooled AZT-MP-DG fractions at 208 nm (0.02 AUFS). Chromatographic conditions given in Experimental.

ABS. [267 nm]

A

ABS. [208 nm]

B

Table I. Absorbance ratios of pooled AZT-MP-DG fractions from micro-preparative separation; 267 nm/220 nm, 267 nm/208 nm and 220 nm/208 nm.

	(267/220)	(267/208)	(220/208)
	1.22	0.92	0.75
	1.29	0.90	0.70
	1.24	0.86	0.69
mean	1.25	0.89	0.71
sdev	0.0360	0.03	0.033
%RSD	2.88	3.30	4.58

[26]. A peak purity value of 0.225 was determined for the AZT-MP-DG, which compared favorably to a value of which was 0.215 determined for a batch of AZT-MP-DG synthesized previously. To determine the mass of AZT-MP-DG recovered, the pooled fractions were evaporated to dryness with a stream of nitrogen and then extracted (3 times) with chloroform-methanol (8:2). The extraction solvent was evaporated with nitrogen, leaving 18 mg of purified AZT-MP-DG.

ddC-MP-DG

The ddC-MP-DG mixture was first chromatographed on an analytical column (Figure 6). In comparison to the AZT-MP-DG, more impurities were found. On the method development column an aliquot of 100 μ L of the working solution (5 mg) provided the optimum load. During the loading study, the column displayed overload when the k' values decreased with increasing mass injected. Flow-rate was directly scaled to 15 mL/min and 25 mg for the mass injected on the micro-preparative column. The chromatogram was similar to that of the method development separation (Figure 7). Nine fractions were collected across the ddC-MP-DG peak. Each fraction was concentrated by rotary vacuum distillation for HPLC analysis.

The ddC-MP-DG fractions were monitored at 280 and 208 nm and fractions 4-8 were pooled. Fractions 1-3 were not pooled because an impurity eluted prior to the ddC-MP-DG and in fraction 9 an impurity which eluted after the ddC-MP-DG was present. As in the case of the AZT-MP-DG, monitoring the ddC-

Figure 6. Analytical HPLC chromatogram of crude ddC-MP-DG, 5 μ L of 50 μ g/ μ L (AUFS 0.10). Chromatographic conditions given in Experimental.

Figure 7. Micro-Preparative Chromatogram of (25 mg) ddC-MP-DG. Mobile phase: methanol- 1 mM KH_2PO_4 (95:5), (pH 2.4), flow-rate 15 mL/min, 280 nm, 0.64 AUFS. Fractions were collected between 7 to 20 minutes.

ABS. [280 nm]

MP-DG at 208 nm did not reveal any additional impurities. Representative chromatograms of the pooled fractions at 280 nm and 208 nm are shown in figure 8a and 8b. Based upon UV absorption the purity of the pooled ddC-MP-DG was 99.8%. The pooled fractions were also monitored with the rapid scanning UV detector. Absorbance ratios, listed in Table II, at 280, 220 and 208 nm were determined. The ratios were consistent for multiple injections. A peak purity of 0.326 was obtained for the pooled fractions indicating a single component, ddC-MP-DG, was present. To determine the mass of ddC-MP-DG recovered, the pooled fractions were evaporated to dryness with a stream of nitrogen and then extracted (3 times) with chloroform-methanol (3:2). The extraction solvent was evaporated with nitrogen, leaving 13.4 mg of purified ddC-MP-DG.

Impurities

Impurities such as phosphatidic acid are difficult to detect with UV detectors even at low wavelengths. Thus evaporative light scattering detector, based on light scattering principles, was used for monitoring compounds that do not possess UV absorbing chromophores. Pooled fractions of AZT-MP-DG were analyzed with an evaporative light scattering detector to confirm purity (Figure 9). Based on the light scattering chromatogram the purity of AZT-MP-DG was greater than 99%.

Thin layer chromatography is very valuable to determine selectively phosphorus containing compounds using a molybdenum blue reagent and to monitor universally all carbon containing

Figure 8. (A) Analytical HPLC chromatogram of pooled ddC-MP-DG fractions at 280 nm (0.02 AUFS). Chromatographic conditions given in Experimental and (B) Analytical HPLC chromatogram of pooled ddC-MP-DG fractions at 208 nm (0.02 AUFS). Chromatographic conditions given in Experimental.

ABS. [280 nm]

ABS. [208 nm]

Table II. Absorbance ratios of pooled ddC-MP-DG fractions from micro-preparative separation; 280 nm/220 nm, 280 nm/208 nm and 220 nm/ 208 nm.

	(280/220)	(280/208)	(220/208)
	0.62	0.92	0.68
Figure 1	0.59	0.89	0.66
AAT-RF	0.59	0.89	0.66
mean	0.60	0.90	0.67
sdev	0.016	0.014	0.0072
%RSD	2.70	1.58	1.08

Figure 9. Evaporative light scattering chromatogram of pooled AZT-MP-DG fractions. Chromatographic conditions: methanol- 1 mM NH_4OH (95:5), (pH 2.4), flow-rate 1.2 mL/min. Detector parameters: Range (sensitivity) 5, heater and exhaust temperatures, 150.0°C and 92.5°C.

compounds by charring with sulfuric acid. With silica thin layer plates and detection with these two reagents, no impurities were detected in fractions 3-17 of AZT-MP-DG, in fractions 4-8 of ddC-MP-DG or in the pooled fractions of both compounds.

In summary a micro-preparative separation has been developed for the isolation and purification of new experimental anti-HIV glycerophospholipids. The conditions were based upon separations of naturally occurring glycerophospholipids on reversed-phase supports and detection based upon the nucleotide moiety. The eluent allows for low UV monitoring to detect impurities which lack strong UV chromophores. The AZT-MP-DG and ddC-MP-DG were obtained in purities greater than 99%. The purities were also confirmed by a evaporative light scattering detector and TLC. The optimized conditions for the micro-preparative column can be adopted to larger preparative columns when greater amounts of the liponucleotides are to be purified.

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MANUSCRIPT V

DETECTION FOR HPLC SEPARATIONS OF GLYCEROPHOSPHOLIPIDS

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Abstract

The performance of the evaporative light scattering detector (ELSD) for the monitoring of glycerophospholipids, separated by HPLC, was compared to that of the UV and RI detectors. The ELSD response was linear in a log/log plot in the range studied which was from 1.25 to 50.0 μg . The detection limit was approximately 0.5 μg and it approached the limit of the UV detector and surpassed that of the RI detector. Reproducibility of the peak areas ranged from 0.11 to 1.53% RSD.

Since the ELSD can be used to detect non-volatile solutes which do not have UV absorbing chromophores, it is well suited for the HPLC separation of glycerophospholipids. In addition, gradient elution and UV opaque mobile phases can be readily utilized. The ELSD is an universal transport detector, it can be used for the analysis of non-UV absorbing impurities and for determining the purity of a peak obtained from an analytical or preparative HPLC separation. In initial work,

the IR detector with an off-line IR TransformTM showed potential for functional group characterization as well as detection and quantitation.

Test compounds were the major egg yolk derived glycerophospholipids and purified fractions from preparative separations of a synthesized derivative of AZT, AZT-MP-DG.

Introduction

Glycerophospholipids, commonly known as phospholipids, are comprised of phosphate diester polar heads and two long chain hydrocarbon fatty acid tails esterified to glycerol backbones (Figure 1 A-E). Due to the varying lengths and degree of unsaturation in the fatty acids, naturally occurring phospholipids do not exist as a single discrete species, but as multispecies. These compounds are the principal components of cell membranes and used as precursors in the synthesis of novel anti-viral and anti-tumor drugs (1,2). Therefore rapid separation methods with accurate detection are required to quantify phospholipids and to determine the purity of products isolated and purified by preparative HPLC.

There are two common types of phospholipid separations; class separations, where the separation is based upon the polar head group, and species separations, which are based on the degree of saturation and length of the fatty acid chains. Silica is the most widely used sorbent for class separations, while species separations have been accomplished on reversed-phase and silver impregnated stationary phases (3). In this

Figure 1. (A-E) Generalized chemical structures of phospholipids. (A) Phosphatidylethanolamine (PE), (B) Phosphatidylcholine (PC), (C) Lysophosphatidylethanolamine (LPE), (D) Lysophosphatidylcholine (LPC) and (E) Sphingomyelin (SPH).
(F) Chemical structure of 3'-azido-3'-deoxythymidine monophosphate diglyceride, AZT-MP-DG.

A (R) - Phosphatidylethanolamine

B (R) - Phosphatidylcholine

C (R) - Lysophosphatidylethanolamine

D (R) - Lysophosphatidylcholine

(R₁)_nCO = sn-1 fatty acyl chains
(R₂)_nCO = sn-2 fatty acyl chains

E Sphingomyelin

F 16:0/18:1ω9 AZT-MP-DG

study naturally occurring phospholipids in egg yolk were used as model compounds and were separated on silica supports.

To monitor class separations on an analytical scale, refractive index (RI) (4,5) and ultraviolet (UV) (6-10) detectors have been commonly used. Phospholipids are difficult to quantify with either detector. The RI is a nondestructive bulk property detector. It is considered the "universal" detector for HPLC because it responds to all solutes provided the refractive indices of the solutes are different from the refractive index of the mobile phase. However, it has many disadvantages; it lacks sensitivity, it is dependent on temperature and flow rate, it requires a constant mobile phase composition and it needs long equilibration times for baseline stability. Thus with the RI detection, quantitation is difficult.

Ultraviolet absorption detectors, which are also nondestructive, are solute property detectors. The solutes must possess chromophoric groups capable of absorbing UV light at the working wavelength. Ultraviolet detectors are commonly employed in HPLC because of their high sensitivity and stability. Additionally gradient elution can readily be used. Although compounds which lack strong chromophoric groups are difficult to monitor without derivatization, it is possible to use low wavelengths (190-210 nm) provided the mobile phase has little or no absorption in that region of the UV. To monitor phospholipids the UV detector must be operated in the

200-210 nm region. Quantitation is difficult since the absorption is predominately due to the double bonds in the diglyceride moiety which have low extinction coefficients. In addition since the lipids have varying degrees of unsaturation in the fatty acids, each compound may have a different molar extinction coefficient. Furthermore few of HPLC mobile phases or the solvents commonly used to solubilize phospholipids are UV transparent at wavelengths lower than 215 nm. Although UV absorbing (11-13) or fluorescent (14) derivatives can be formed to enhance the detectability of phospholipids, derivatization requires additional handling which can cause sample loss and errors in quantitation.

Other detectors have also been used in the HPLC analysis of phospholipids. The flame ionization detector (FID) has been described for quantitation of soy bean and synthetic phospholipids (15,16) and multispecies quantitation of phospholipids (17,18); however it has not gained widespread use with HPLC. An electrochemical method based on tensammetry has recently been described for the determination of phospholipids in serum (8), but other components in the serum interfere with the analysis. Although with HPLC-Mass Spectrometry (HPLC-MS) good quantitation and structural information can be obtained (19,20), instrumentation is expensive and difficult to operate. Because phospholipids are non-volatile, it is difficult to separate them by gas chromatography (GC) or by GC-MS (21) unless they are

derivatized to volatile methyl esters. However, gas chromatography is still one of the best methods for determining the fatty acid distribution (22).

Various detection methods which can not be used with HPLC can be used with TLC to separate phospholipids (23-25). Spray reagents have been developed to act chemically with phospholipids producing a colored spot. In addition the unsprayed spots, containing the phospholipids, may be scraped and used in other types of analyses such as a phosphorus assay (26). However, inadequate resolution of closely eluting solutes, slow speed of analyses as well as poor reproducibility and quantitation limit the use of TLC for the phospholipids.

An evaporative light scattering detector (ELSD), which is an universal transport detector, has been reported for HPLC (27-29). This detector is based on light scattering principles and has many advantages. One of the major advantages is that it can be used to monitor high molecular weight, non-volatile compounds such as phospholipids (30-38), triglycerides (39) and carbohydrates (40). Quantitation is based directly on the mass of a substance injected. With the ELSD, solutes need not possess chromophoric groups but they must be non-volatile, a characteristic inherent in large molecules; however mobile phases must be volatile. In addition the eluent can be UV opaque and gradient elution is easily used. The limitations of the ELSD are that the solutes are not recoverable and the

mobile phase can not contain buffers or salts which will precipitate in the nebulizer syringe, although trace amounts of these ionic substances have been used (34).

Infrared (IR) spectroscopy has also been used to monitor phospholipids after HPLC separation (41). The IR offers functional group characterization and quantitation. Quantitation can be based on monitoring the absorbances at the wavelengths where the carbonyl and amide group(s) absorb. Since expensive deuterated solvents must be used in the mobile phase for direct coupling of the HPLC column to the detector, an off-line IR interface has been developed for HPLC, whereby deuterated solvents are not required (42,43).

Therefore the performance of the ELSD was evaluated and compared to other detectors for monitoring analytical separations of the major phospholipids of chicken egg yolk and to determine the purity of PC (44) and the 3'-azido-3'-deoxythymidine monophosphate diglyceride, AZT-MP-DG, (Figure 1 F) an anti-AIDS drug recently synthesized and purified by preparative HPLC (45). The conditions of the ELSD were optimized for linearity of response and reproducibility. An initial study with an off-line HPLC-IR interface was used to obtain spectra of phospholipids for identification and characterization.

Experimental

Reagents. All solvents (Fisher Scientific, Pittsburgh, PA), which were of HPLC grade, were filtered through 0.45 μ m

Nylon-66 filters (AllTech. Assoc., Derrfield, IL.) and degassed with He. The water was doubly distilled and deionized.

Standards. Multispecies phospholipids derived from chicken egg yolk, phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE), phosphatidylcholine (Lecithin, PC), sphingomyelin (SPH) and lysophosphatidylcholine (LPC) were obtained from Avanti Polar Lipids (Birmingham, AL). A stock solution of 2 $\mu\text{g}/\mu\text{L}$ in 9:1 chloroform and methanol of each individual phospholipid was prepared. However, the LPE stock solution consisted of 1 $\mu\text{g}/\mu\text{L}$ in 1:1 chloroform and methanol. Working solutions of 0.50 $\mu\text{g}/\mu\text{L}$, 0.25 $\mu\text{g}/\mu\text{L}$, 0.10 $\mu\text{g}/\mu\text{L}$, 0.050 $\mu\text{g}/\text{L}$ and 0.0125 $\mu\text{g}/\mu\text{L}$ of each phospholipid were prepared by diluting an appropriate aliquot to 10 mL with the chloroform/methanol (9:1) mixture. To each standard 10 μL of 0.01% butylated hydroxytoluene (BHT) in methanol was added. A crude mixture of phospholipids was isolated from chicken egg yolk by the method of Singleton et al. (46). In addition, PC fractions (44) and AZT-MP-DG fractions (45) from independent preparative separations were also analyzed by the ELSD and compared to UV detection to assess purity.

Apparatus. The chromatographic system consisted of a Waters 510 pump (Waters Chromatography Division, Millipore, Milford, MA) and a Rheodyne 7125 injector with a 100 μL sample loop (Rheodyne, Berkeley, CA). The following detectors were used a Knauer Refractive Index detector (Sonntek, Inc.

Woodcliff Lake, NJ), a Spectro Flow SF 770 variable wavelength detector (Schoeffel Instr. Division, Kratos Inc. Westwood, NJ), a rapid scanning UV (BarSpec, Rehovot, Israel), an evaporative light scattering detector (Varex Corporation, Burtonsville, MD) and an IR TransformTM (Lab Connections, Inc. Marlborough, MA) with an FTS 40 FTIR (BioRad, Cambridge, MA). The UV detector was set at a wavelength of 203 nm and a setting of 0.1 a.u.f.s. The ELSD had the following optimized settings; range 1, exhaust temperature 70.0°C, heater temperature 105.0°C and adjusted temperature of 109.0°C. Nitrogen gas was used as the carrier at 70 mm Hg. The flow-rate with the RI was 1.5 mL/min, the UV detector was 2.5 mL/min, the ELSD and the IR was 1.8 mL/min.

All phospholipid separations were performed isocratically at ambient temperature on a 25 x 0.46 cm column packed with 5 μ m SupelcosilTM LC-Si (Supelco, Inc. Bellefonte, PA). The mobile phases used were: for the ELSD, chloroform-methanol-water-14.8M ammonium hydroxide (60:30:4:0.025 v/v); for the IR, chloroform-methanol-water-Trifluoroacetic acid (TFA) (60:30:4:0.0027 v/v); for the RI, chloroform-methanol-water (60:40:4 v/v); and for the UV, acetonitrile-methanol-water (40:9:6 v/v). All separations were recorded on a HP 3393A integrator (Hewlett-Packard, Avondale, PA) at 0.2 cm/min.

For the AZT-MP-DG the ELSD nitrogen flow-rate was increased to 85 mm Hg with an exhaust temperature of 92.5°C, heater temperature 145.7°C and adjusted temperature of 150.0°C. The

mobile phase flow-rate was 1.2 mL/min. The column, 25 X 0.40 cm, was packed with 5 μ m LiChrospher^R 100 C₁₈ (EM Science, Gibbstown, NJ).

Results and Discussion

Optimization of Separation: Mobile Phase

Although phospholipids have similar structural characteristics and the fatty acid distribution is unique for each phospholipid class, the UV spectra of the phospholipids are similar, exhibiting wavelength maxima between 200 nm to 205 nm.

An isocratic mobile phase was used to separate the phospholipid classes. The separation was based upon the polar head group. With a ternary solvent mixture, the major phospholipids in egg yolk could be resolved, although gradient elution has previously been used to resolve more complex lipid mixtures with the ELSD (30,31,33,35,38). A mobile phase consisting predominantly of CHCl₃ was used with the ELSD because CHCl₃ is more volatile than mobile phases ordinarily used with UV detection. In addition phospholipids are more soluble in CHCl₃ rich solutions. However, the mobile phases commonly used with a UV detector can be utilized if the gas flow-rate and exhaust and heater temperatures are optimized for these eluents (47). It has been reported that increased resolution can be obtained by the addition of strong acids (48,49), but when sulfuric acid or TFA (5-10mM) was added to the mobile phase used with the ELSD, broad peaks and baseline instability resulted. For previous work ammonium acetate in the mobile phase sharpened

phospholipid peaks (44); however ammonium acetate could not be used with the ELSD because of precipitation of the acetate in the nebulizer tubing and syringe. Thus ammonium hydroxide was substituted (30). With 4mM ammonium hydroxide present in the mobile phase, each phospholipid eluted as a single sharp symmetrical peak except for the LPC which eluted as two peaks, probably due to a partial species separation.

The mobile phase for the AZT-MP-DG was previously developed for a reversed-phase system with UV detection (45). For the ELSD ammonium hydroxide was substituted for the potassium dihydrogen phosphate.

For the IR Transform a volatile eluent is also necessary. Therefore a mobile phase of chloroform-methanol-water was employed. Trifluoroacetic acid (TFA) which is very volatile, was added to decrease band broadening (48,49), however, the peaks were broadened instead of sharpened.

For UV detection, ternary low UV transparent solvents have been used as the mobile phase. Two types of mobile phases have been described; acetonitrile-methanol-water (10) and hexane-isopropanol-water (14). In this study, the mobile phase of Jungalwala et al. (10) was modified for the analysis of the PC in fractions from preparative HPLC separations (44).

The mobile phase of chloroform-methanol-water (60:40:4) which had been used with the ELSD detector was used with the RI detector.

Optimization of Detection

Evaporative Light Scattering Detection

For the ELSD the parameters which had to be optimized were mobile phase and nitrogen gas flow-rate, exhaust and heater temperatures. A fairly fast mobile phase flow-rate was used because of the high volatility of the chloroform. Consequently a high carrier gas flow-rate was required to insure adequate vaporization and minimize band broadening. The temperature settings of the heater and exhaust were critical. Low temperatures resulted in inadequate vaporization of the solvent causing baseline fluctuations and high temperatures vaporized the solvent too quickly. The temperature also affected the detector response by causing variations in solute droplet size (29). Therefore, during the optimizing process, the temperature and nitrogen gas flow-rate were adjusted with respect to the mobile phase flow-rate, in order to obtain the greatest response. Since the response of the ELSD does not depend upon chromophoric groups nor on the refractive index of the solutes, it is suitable for monitoring naturally occurring or synthetic phospholipids. The ELSD was operated at the most sensitive setting with negligible baseline noise even at negative attenuations on the integrator. Retention times and areas were very reproducible (Table I and Table II). The PE under these conditions eluted near the void volume; thus it could not be detected with the UV detector due to the large solvent peak but could be reproducibly detected with the ELSD. The response of the ELSD was non-linear and a logarithmic function best fit the

Table I. Retention times of individual standards. The values represent the mean of five injections at each mass. The %RSD was determined for each phospholipid from the means of the mass range injected.

ND=Not Detected. *The LPC was composed of two peaks.

Mass, μg	PE	LPE	PC	SPH	LPC*
50.0	2.04	4.20	4.57	8.94	15.41/16.35
25.0	2.01	4.10	4.54	8.94	15.65/16.48
10.0	1.99	3.94	4.52	8.92	15.90/16.79
5.0	2.04	4.19	4.62	9.41	15.99/16.94
1.25	2.09	4.00	4.69	9.71	ND
%RSD	2.67	2.79	1.53	3.91	1.68/1.64

Table II. Averaged Log Areas. The values represent the averaged of five injections of each standard. The %RSD ranged from 0.11 to 1.53.

Mass, μg	PE	LPE	PC	SPH	LPC
50.0	6.76	6.53	6.64	6.48	6.18
25.0	6.29	6.00	6.10	5.91	5.69
10.0	5.65	5.35	5.39	5.19	5.06
5.0	5.20	4.85	4.86	4.63	4.62
1.25	4.00	3.96	3.88	3.80	**

data (33). A linear log/log plot ($R^2 > 0.99$) was obtained with a non-zero intercept (Figure 2). A linear equation was derived for each phospholipid (Table III). The working range for this study was 0.0969 (1.25 μg) to 1.6990 (50 μg) and a detection limit of 0.25 μg (PE), 0.50 μg (LPE, PC and SPH) and 1.0 μg (LPC) was obtained. Lower detection limits with the ELSD have been reported with gradient elution (30,33).

A standard mixture of the five phospholipids, consisting of 10 μg of each compound, was injected. The phospholipids were well resolved with excellent peak shapes (Figure 3). The separation time was less than 20 minutes. The mass of each phospholipid in the mixture was calculated with the regression equations (Table IV). There was good agreement between the injected and the computed amount. The LPE had the largest deviation in the correlation between mass injected and mass calculated. It is postulated that the deviation was due to incomplete dissolution, since the LPE had the lowest solubility.

When a 1.18 $\mu\text{g}/\mu\text{L}$ solution of the crude egg yolk phospholipids was injected, all the major phospholipids were well resolved (Figure 4). The major phospholipids were PE and PC, while LPE, SPH and LPC were the minor ones. The percent of each phospholipid was calculated with the regression equations and there was good agreement with the literature values as shown in Table V (50,53). Since the phospholipid composition varies in different sources of eggs, the literature values must be considered to be general in nature.

Figure 2. Log/Log plot of individual standards of PE, LPE, PC, SPH and LPC. Each data point is the mean of 5 injections.

Table III. Linear regression equations.

Phospholipid	Slope	Y- intercept
PE	1.71	3.91
LPE	1.60	3.77
PC	1.72	3.69
SPH	1.68	3.55
LPC	1.56	3.52

Figure 3. 100 μL injection of a 0.1 $\mu\text{g}/\mu\text{L}$ standard mixture.
Detector: LPC. Detector: ELSD, Sensitivity: 1, Flow-rate: 1.8
 mL/min , Mobile Phase: CHCl_3 -MeOH- H_2O -4 mM NH_4OH (60:30:4:0.025
v/v). Elution order: PE(1), LPE(2), PC(3), SPH(4) and LPC(5).

Table IV. Calculated mass. The values represent the calculated mass from the linear equations for a standard mixture containing 10 μ g of each phospholipid (n=3 injections).

Phospholipid	Mass, μg
PE	9.41
LPE	13.0
PC	11.2
SPH	10.4
LPC	9.00

Figure 4. Analysis of crude egg yolk derived phospholipids. 100 μL injection of 1.18 $\mu\text{g}/\mu\text{L}$. Detector: ELSD. See Figure 3 for conditions. Elution order: PE(1), LPE(2), PC(3), SPH(4) and LPC(5).

Table V. Calculated amounts of phospholipids in percent from crude egg yolk. Literature values are empirical in nature.

*Column 1 from Ref. 50

**Column 2 from Ref. 53

Phospholipid	Experimental	Literature	
		(1)*	(2)**
PE	19.0	15.2	15.0
LPE	1.0	2.0	5.8
PC	70.0	74.2	73.0
SPH	2.6	2.5	2.5
LPC	2.2	6.1	5.8

After a preparative procedure for the isolation and purification of PC (44), the collected fractions were analyzed for purity. A representative HPLC-ELSD chromatogram shown in Figure 5 illustrates that only PC is present. The high peak purity was also confirmed with UV detection.

The ELSD was also useful in confirming the purity of AZT-MP-DG (Figure 6). The ELSD conditions, nitrogen gas flow-rate and temperatures were adjusted to vaporize adequately the mobile phase which has been developed for the HPLC separation with UV detection, to maintain a stable baseline and to exhibit good response. The high methanol content (95%) was easily evaporated, although sensitivity was decreased due baseline drift. The compound had not been removed from the micro-preparative eluent which was methanol-1mM KH_2PO_4 (95:5). Although a trace amount of KH_2PO_4 was present in the aliquot injected, the response and pressure remained constant during the analysis indicating that the salt did not precipitate. No other solutes were detected demonstrating the high purity of the compound. The high purity was also found with the UV analysis (45,54). However, with UV detection, impurities which do not have a chromophore can not be detected.

Infrared Detection

Infrared spectroscopy provides functional group characterization as well as quantitation. Direct coupling to an HPLC has been difficult, because of engineering problems and the limitations imposed by solvent absorption. An alternative off-

Figure 5. Purity analysis of egg yolk derived PC isolated from preparative HPLC (44). Detector: ELSD. Inj.Vol.: 100 μ L of pooled PC fractions. See figure 3 for conditions.

Figure 6. Purity analysis of pooled AZT-MP-DG fractions isolated from micro-preparative HPLC (45). Detector: ELSD, Mobile phase: MeOH-1 mM NH_4OH (95:5), pH 2.4, Flow-rate: 1.2 mL/min, sensitivity (range): 5, Injection vol.: 100 μL .

line system has been developed in which the effluent does not enter the IR sample compartment, thus eliminating many of the problems associated with direct coupling (42,43). A 1:25 splitter was used to divide the effluent. At a flow-rate of 1.8 mL/min, 0.072 mL/min was delivered to the IR Transform and nebulized. The mobile phase was evaporated and the solutes deposited through a syringe tip onto a rotating Germanium reflective disk. The rotation angle of the disk at any given time was known and the speed of rotation controlled. Once all the solutes had been deposited onto the disk, it was placed in a reflectance accessory in the FT-IR sample chamber. The reflectance accessory also rotated the disk to any angle where a solute has been deposited and an IR spectrum was taken.

An injection of a mixture containing 40 μ G each of PE, PC, SPH and LPC resulted in 1.6 μ g of each analyte being deposited on the disk. The IR spectra (Figure 7 A-D) clearly show functional group absorptions; for example the carbonyl, C=O, stretch at 1750 cm^{-1} to 1650 cm^{-1} . Other absorptions are due to the C-H stretch in the fatty acid chains at 3000 cm^{-1} to 2800 cm^{-1} , the phosphate group, P=O, at 1300 cm^{-1} to 1250 cm^{-1} and the covalent phosphate, P-O-C, at 1050 cm^{-1} to 970 cm^{-1} (51). The wave numbers for specific functional groups are in Table VI. For quantitative analysis the FT-IR can also monitor a particular functional group wavelength, which is analogous to a UV detector set at a specific wavelength. During a separation the ELSD was coupled in parallel to the IR Transform by connecting the waste

Figure 7. IR spectra of chromatographed mixture of phospholipids obtained with the IR Transform; (A) PC, (B) LPC, (C) PE and (D) SPH, 1.6 μg of each phospholipid deposited on to the rotating disk. Each spectra is a composite of 256 scans with a resolution of 4.

Table VI. Phospholipid absorption bands and their assignments. Each IR spectra is a composite of 256 scans with a resolution of 4.

Phospholipid	Functional Group		
	C=O, cm^{-1}	P=O, cm^{-1}	P-O-C, cm^{-1}
PE	1737/1730	1271	1011
PC	1737/1727	1274	1072
SPH	1729	1273	1041
LPC	1730	1273	980

line from the splitter to the inlet of the ELSD detector. Thus the time of deposition can be visually monitored and more importantly the exact deposition angle of the disk was known. With the angle known, the disk when placed in the accessory in the FT-IR sample chamber was rotated to the exact angle where deposition occurred. The coupling with the ELSD eliminated the need to rotate the disk every few degrees and take a spectrum.

Ultraviolet Detection

Although ultraviolet detectors are very sensitive, the sensitivity for phospholipids is primarily dependent on the number of double bonds in the diglyceride moiety. Moreover, the distribution of the fatty acid moieties differ within each phospholipid class. Consequently, direct quantitation by UV of phospholipids with an unknown number of double bonds is difficult. At lower wavelengths which are nearer the wavelength maximum, high sensitivity is obtained but at the expense of high background due to the mobile phase. A reported limit of detection (8) was $0.2 \mu\text{g}$ for natural occurring phospholipids with a linear response ranging from 0.2 to $15 \mu\text{g}$, whereas the limit was $2.5 \mu\text{g}$ for synthetic phospholipids. The lower detection limit for the naturally occurring phospholipids is due to the presence of a larger number of unsaturated fatty acids. The lyso-derived phospholipids, LPE and LPC, are inherently more difficult to detect by UV because they lack a fatty acid chain (Figure 1 C and D). Since the detector "sees" half the compound

with respect to PE and PC, the UV response of the lyso derivatives is lower than for PE and PC, (52).

A solvent peak, due to the chloroform-methanol solution used to solubilize the phospholipids, was very pronounced at low UV wavelengths. The tail of the peak obscured the PE and LPE peaks which eluted near the solvent front. A solvent peak is usually absent with the ELSD since the mobile phase is volatilized.

Detection by UV has been primarily used to determine the purity of PC (44) and AZT-MP-DG (45) isolated from preparative HPLC separations. Figure 8 is a 3-D chromatogram of the pooled fractions containing AZT-MP-DG in the highest purity (>99%) from a preparative HPLC separation.

Refractive Index Detection

Phosphatidylcholine from egg yolk and soy bean has been monitored with a RI detector. The RI detector was compared with a UV detector and TLC for purity determination of PC (5). A major advantage of the RI detector is that the response is not dependent upon the degree of unsaturation as in UV. However the response is affected by lack of sensitivity and temperature and baseline instability. Refractive Index calibration curves of PC have been reported to be linear in the range of 20 μg to 100 μg (5). Initially, the RI detector was used to monitor PC analyses, but due to its lack of sensitivity small quantities of other phospholipids were not detected. The amount of PC relative to

Figure 8. 3-D UV chromatogram of pooled AZT-MP-DG fractions. UV maxima: 267 and 208 nm. Inj. Vol: 100 μ L. Scan rate: 10 spectra per second. Mobile phase: 95:5 Methanol/1 mM KH_2PO_4 (pH 2.4), Flow-rate: 1.5 mL/min.

the total phospholipids content can vary from 65-85% in egg yolk (50,53), while less than a few percent of other phospholipids may be present. A HPLC-RI chromatogram of crude egg yolk phospholipids, illustrates the lack of sensitivity of the RI detector for the minor phospholipids (Figure 9). Although the PC was well detected, the PE and LPC were obscured by the solvent peaks and the SPH and LPC could not be detected.

Figure 9. RI chromatogram of 1.18 $\mu\text{g}/\mu\text{L}$ crude egg yolk phospholipids. Range (sensitivity) 16. Inj. Vol.: 100 μL . Flow-rate: 1.5 mL/min. $t_R(\text{PC}) = 8.756$ min.

Summary

The ELSD is a valuable means of detection in the HPLC of glycerophospholipids. The glycerophospholipids are non-volatile high molecular weight compounds which meet the detector requirements. The eluent must be volatile and can contain only trace amounts of salts or buffers. Good quantitative analysis can be obtained with the ELSD. A log/log plot of the individual standards produced a linear behavior and the linear working and detection limits approached those of UV detection. The sensitivity of the ELSD was set to the maximum with little baseline fluctuations, even at negative attenuations on the integrator. In addition, the ELSD was useful as a complementary detector to confirm the purity of fractions of PC and AZT-MP-DG isolated by preparative HPLC. Although the solutes are not recoverable, a stream splitter may be used for collection of a portion of the solutes prior to the effluent entering the detector.

By using the IR Transform, FT-IR can be used for functional group characterization glycerophospholipids as well as for detection and quantitation.

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APPENDIX A

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