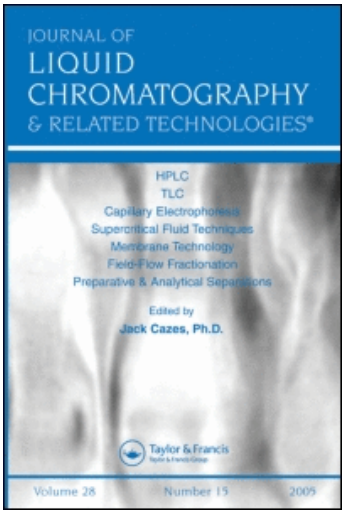

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DETERMINATION OF SERUM PHOSPHOLIPID METABOLIC PROFILES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A method for the separation of serum phospholipid molecular species by reversed-phase high-performance liquid chromatography is described. Total serum phospholipid extracts were hydrolyzed with phospholipase C and benzoylated, and the resulting monoglyceride, diglyceride, and ceramide derivatives were separated by using gradient elution with detection at 230nm. Both human and rat serum phospholipid extracts were examined, and the use of this method for the evaluation of serum phospholipid metabolic alterations accompanying exposure to an environmental toxicant (polybrominated biphenyls) is described.

INTRODUCTION

Multicomponent chromatographic analyses of physiological fluids (metabolic profiles) may provide valuable information in correlating specific chromatographic patterns with subtle

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metabolic alterations, such as those resulting from exposure to environmental toxicants. Metabolic profiling has perhaps been most thoroughly explored in evaluating urinary organic acid patterns associated with certain genetic disorders, but studies relating abnormal metabolic profiles to specific diseases - particularly diabetes - have also been reported (1-4). Although urine has commonly been used for these studies, mainly because of the technical advantages afforded by its low protein content (3), plasma or serum analyses are often preferable because of the higher degree of physiological regulation of blood metabolite levels. In this regard, blood lipid profiles have an advantage, since efficient procedures for isolating lipids from associated proteins and other polar components are already well established.

Although plasma lipid profiling has been approached through a variety of chromatographic techniques, an optimum method has not been developed. Thin-layer chromatography has been successfully employed for the rapid separation of major plasma lipid classes, but this technique does not readily lend itself to detailed molecular species separations or to reliable quantitation. An approach that offers considerable promise is the analysis of intact and/or partially degraded lipids by high-temperature gas-liquid chromatography (GLC) (5-7). The relative involatility of most intact lipids, however, necessitates the use of high oven temperatures and carrier flow rates; thus, this technique is generally restricted to short, nonpolar columns. As a result, resolution is predominately based on differences in chain length (carbon number), and considerable information relating to the degree of fatty acid unsaturation is lost. The instability of some lipids at elevated temperatures may further complicate these analyses.

In recent years, a growing interest has developed in the application of high-performance liquid chromatography

(HPLC) to the determination of metabolic profiles (8). HPLC is well suited to the analysis of lipid profiles, although it is limited by the absence of an optimum detector. In this paper, we describe the determination of serum phospholipid profiles by reversed-phase HPLC of their benzoylated derivatives, using ultraviolet (UV) detection, and demonstrate the potential application of these analyses to the elucidation of rat serum phospholipid metabolic alterations accompanying exposure to an environmental toxicant (FireMaster BP-6, a mixture of of polybrominated biphenyls.

MATERIALS AND METHODS

Materials

Lipid chromatographic standards were obtained from the Sigma Chemical Company, St. Louis, MO 63178, or from Nu-Chek-Prep, Elysian, MN 56028. Sphingosine, benzoic anhydride, 4-dimethylaminopyridine, and phospholipase C (C. perfringens, Type I) were also purchased from Sigma. Unisil was a product of the Clarkson Chemical Company, Williamsport, PA 17701. All solvents were HPLC-grade and were obtained from Burdick and Jackson Labs., Inc., Muskegon, MI 49442. Ceramide standards were synthesized from sphingosine and the appropriate fatty acid hydroxysuccinimide ester by the procedure of Ong and Brady (9). The hydroxysuccinimide esters were prepared as described by Lapidot et al. (10).

Sample Preparation

Serum extractions and the initial lipid class separations on a Unisil column were performed as previously described (11). After the neutral lipids had been eluted with 5 mL of chloroform, the column was then eluted with 10 mL of methanol:water (95:5, v/v) to recover the phospholipids. The phospholipid fraction was dried under N_2 and then dephos-

phorylated by the procedure of Kuksis et al. (6). One mL of diethyl ether was added to the dried residue, followed by 4 mL of 17.5 mM Tris buffer (pH 7.3) containing 300 µg of phospholipase C. After 1.3 mL of 1% $CaCl_2$ was added, the mixture was incubated and continuously mixed at 37° C for 2 hrs. The resulting diglycerides and ceramides were extracted with 10 mL of chloroform:methanol (2:1, v/v).

Benzoylation

The lipid extract obtained above was thoroughly dried under a stream of N_2 and then under a vacuum. The dried residue was subsequently derivatized with 10% benzoic anhydride in pyridine containing 4-dimethylaminopyridine (50 mg/mL) as a catalyst (12). After the samples were incubated for 2 hrs at 56° C, the solvent was removed under a stream of N_2, and the residue was dissolved in hexane and purified by the procedures of Ullman and McCluer (13). The final, dried residue was dissolved in 100 µL of isopropanol for analysis by HPLC.

HPLC Procedure

The benzoylated phospholipid derivatives were analyzed on a 4.6- x 250-mm 5 µ Ultrasphere-ODS column (Altex Scientific, Inc., Berkeley, CA 97410), which was eluted with 30% isopropanol in acetonitrile at 1.2 mL min^{-1}. After 10 min, a linear gradient of 30-60% isopropanol in acetonitrile was begun at 2% per min. Detection was at 230 nm with a Schoeffel SF770s UV detector (Kratos, Inc., Westwood, NJ 07675). The detector output was coupled to a HP 3390A recording integrator (Hewlett-Packard, Avondale, PA 19311). Two Altex model 100 pumps, a model 400 mixer, and a model 420 system controller were used for this assay. The system was automated by adding an autosampler (WISP 710B, Waters Associates, Milford, MA 01757), which activated both the system controller and the integrator at injection.

Peak identities were determined by the use of standards, and also by the collection and further analysis of individual peaks in the chromatogram. In this case the phospholipid groups were analyzed individually after a preliminary separation by thin-layer chromatography on 250 μ silica gel G plates (Analtech Inc., Newark, DE 19711), which were developed with chloroform:methanol:water (65:25:4, v/v). A portion of each collected peak from HPLC was transesterified, and the fatty acid methyl ester composition was determined by capillary GLC (14) and, in some cases, by GC/MS. An additional aliquot of the collected material from HPLC was analyzed directly by GLC to determine the carbon number of individual molecular species. The latter determinations were carried out on a 3-mm x 0.6-m stainless steel column packed with 1% Dexsil 300 on 100/120 Supelcoport and mounted in a Hewlett-Packard 5840A gas chromatograph equipped with a flame-ionization detector. After an initial 1-min interval at 170° C, the temperature was programmed from 170° to 350° C at 5° /min. On-column injection was used, with an injector temperature of 300° C and a detector temperature of 350° C. Helium was the carrier at a flow rate of 100 mL per min. The system was calibrated with appropriate benzoylated monoglyceride and diglyceride standards.

Animals and Treatment

Seven-month-old male Sherman strain rats (15) were used in this study. The "dosed" group received a single oral dose of FireMaster BP-6, lot #5143 (500 mg/kg), by gavage as a 5% solution in corn oil. Control animals received a single dose of corn oil. Dosed and control animals were killed with CO_2 1 to 8 weeks after being dosed, and blood was drawn immediately by cardiac puncture and allowed to clot; the serum was separated and stored at -55°C until analyzed.

Profiles from both dosed and control animals showed little variation as a function of time after they were dosed within the 1- to 8-week recovery period; therefore, the data were combined over all time intervals. Diet and caging conditions were as previously described (14).

Calculations

Peak areas were converted to component weights through the use of standard response factors, and the Weight % contribution was then calculated for the indicated analytes. Monoglyceride, diglyceride, and ceramide response factors in these analyses were estimated from the appropriate benzoylated palmitic acid derivatives. All statistical evaluations were performed with the aid of the SAS package (SAS Institute, Cary, NC 27511) implemented on an IBM 3083 computer.

RESULTS AND DISCUSSION

The direct analysis of phospholipids by reversed-phase HPLC is complicated by the ionic and amphiphilic nature of these compounds. Therefore, an indirect approach was used in which we first hydrolyzed the serum phospholipid fraction with phospholipase C (6) and then benzoylated the resulting partial glycerides and ceramides. This improved the chromatographic resolution and also provided for the introduction of a chromophore. A similar approach was used by Bateley et al. (16) for the resolution of phosphatidylcholine molecular species.

Representative chromatograms for the analysis of ceramide and partial glyceride standards and for human serum phospholipids are given in Figure 1. Despite the use of a high efficiency column, only partial resolution of all the phospholipid molecular species was obtained. In Figure 1 (B), peaks 3 through 7 and peaks 9 and 10 are derived mainly from phospho-

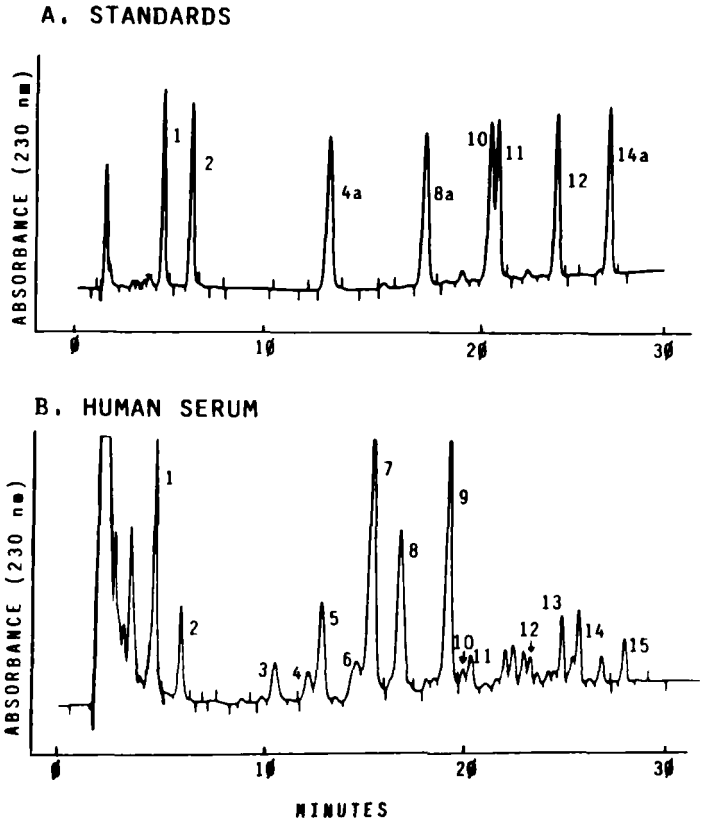

FIGURE 1. Phospholipid profiles. Identification of the predominant component in the numbered peaks is as follows: (1) 16:0-MG; (2) 18:0-MG; (3) 16:0/22:6-DG; (4) 18:0/22:6-DG; (4a) 14:0/14:0-DG; (5) 16:0/20:4-DG; (6) 18:1/18:2-DG; (7) 16:0/18:2-DG; (8) 16:0-CER + 18:0/20:4-DG; (8a) 16:0-CER; (9) 16:0/18:1-DG + 18:0/18:2-DG; (10) 16:0/16:0-DG; (11) 18:0-CER; (12) 20:0-CER; (13) 24:1-CER; (14) 22:0-CER; (14a) 18:0/18:0-DG; (15) 24:0-CER. MG = Monoglyceride; DG = Diglyceride; CER = Ceramide.

glycerides, with the predominant fatty acid composition in each case given in the Figure's legend. It should be emphasized that the composition indicated is that of the apparent major component. In some cases the peaks appeared to be mixtures with additional minor components contributing to the overall response. Peak 8 was a mixture of both a diglyceride containing stearic and arachidonic acid and the major serum ceramide containing palmitate. Peak 9 was also complex and appeared to contain two major diglyceride species, one comprised of palmitate and oleate and another containing stearate and linoleate.

The remaining major peaks in the latter part of the chromatogram were all derived from sphingomyelin. Palmitic acid is the predominant fatty acid in human serum sphingomyelin, and significant amounts of stearic, arachidic, behenic, lignoceric, and nervonic acids are also present (17). These six acids generally account for approximately 90% of the total sphingomyelin fatty acids (17,18). Resolution of sphingomyelin molecular species as ceramides presents additional problems, however, because of the heterogeneity of the associated long-chain base (17). Because of this complexity, not all of the peaks derived from sphingomyelin were identified, but through the analysis of ceramide standards and collected peaks, the major components noted above were tentatively identified as indicated in the legend to Figure 1.

As expected, the log of the elution volume of both diglyceride and ceramide standards increased linearly with the increase in diglyceride carbon number or in ceramide fatty acid chain length, respectively, when analyzed under isocratic conditions (Figure 2). Resolution was also influenced by fatty acid unsaturation as indicated by the location of unsaturated analogues in these plots. The nervonic acid derivative (24:1) had an effective chain length of 21.3 in

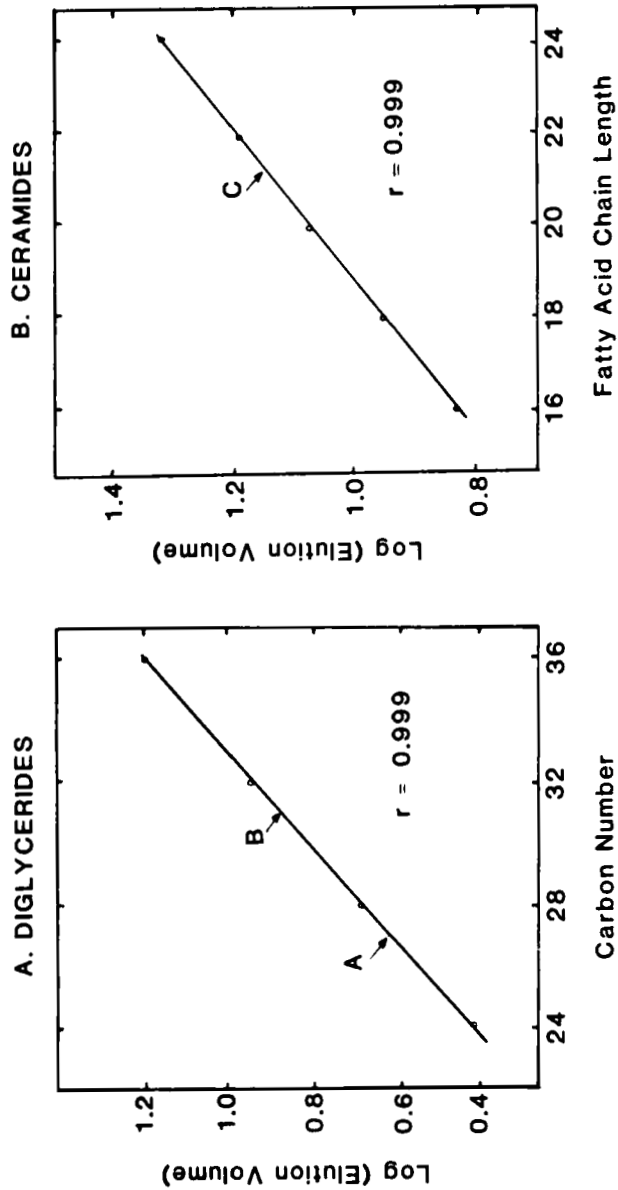

FIGURE 2. Effect of fatty acid chain length on the elution volume. Standards were analyzed isocratically with 45% isopropanol in acetonitrile. In the diglyceride plot, dilinolein (18:2/18:2-DG) eluted at (A) and diolein (18:1/18:1-DG) eluted at (B). In the ceramide plot, N-nervonyl sphingosine (24:1-CER) eluted at (C).

the ceramide series, whereas diolein and dilinolein gave effective carbon numbers of 30.9 and 27, respectively. Thus, the average effect of a double bond in these analyses was approximately equivalent to the removal of 2.5 methylene groups from the chain.

Analysis of monopalmitin, dipalmitin, and palmityl sphingosine standards demonstrated linearity of peak areas with component weights within the analytical range (Figure 3). The reproducibility of these analyses was examined by the extraction, derivatization, and analysis of six aliquots of a frozen human serum pool over a 1-month interval. Despite the extensive sample preparation procedures required, the results as summarized in Table 1 indicated that reproducible

TABLE 1

Analytical Precision of Repetitive Phospholipid Profile Determinations on a Pooled Human Serum Sample

	Wt %	
Peak Number	$\bar{X} \pm$ S.D.	C.V. (%)
1	5.9 ± 0.27	4.6
2	2.0 ± 0.12	5.8
3	3.3 ± 0.12	3.6
4	2.6 ± 0.22	8.6
5	8.0 ± 0.12	1.5
6	3.2 ± 0.12	3.8
7	25.0 ± 0.34	1.4
8	14.7 ± 0.64	4.4
9	20.4 ± 0.30	1.5
10	0.9 ± 0.10	11.1
11	2.3 ± 0.12	5.3
12	2.0 ± 0.33	16.4
13	3.7 ± 0.15	4.0
14	4.1 ± 0.13	3.3
15	2.4 ± 0.08	3.4
	N = 6	

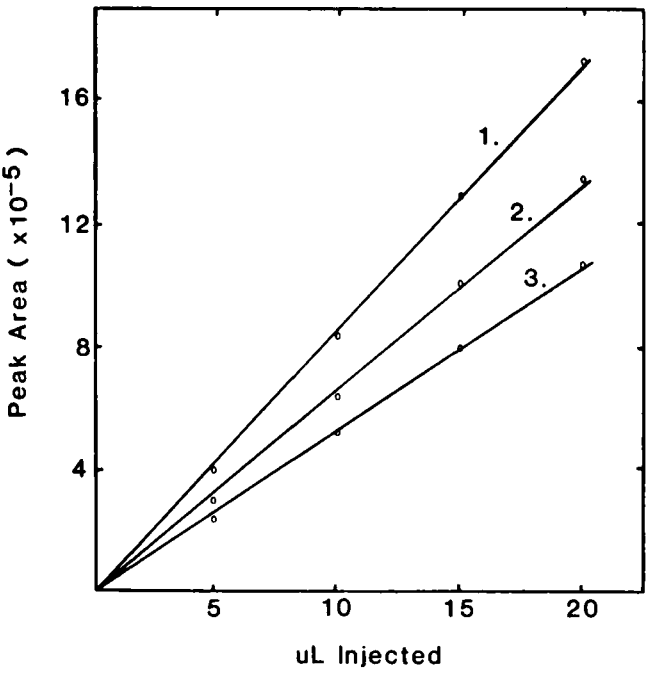

FIGURE 3. Linearity of peak areas with the amount of benzoylated standard injected. Various aliquots of a standard mixture of benzoylated dipalmitin (1), palmityl sphingosine (2), and monopalmitin (3) were analyzed under standard conditions.

patterns were obtained in these assays. With the exception of peaks 4, 10 and 12, all of which represented relatively minor components in the profile, the individual components had relative standard deviations of less than 6%. Of the three major peaks, peak 8 displayed the largest variability with a CV of 4.4%.

We have previously reported that dosing rats with polybrominated biphenyls (PBBs) leads to an alteration in their hepatic lipids within 1 week after they are dosed (14). We thus wanted to determine whether alterations in lipid profiles could be detected in the more readily accessible serum lipid fraction, since it is well known that serum

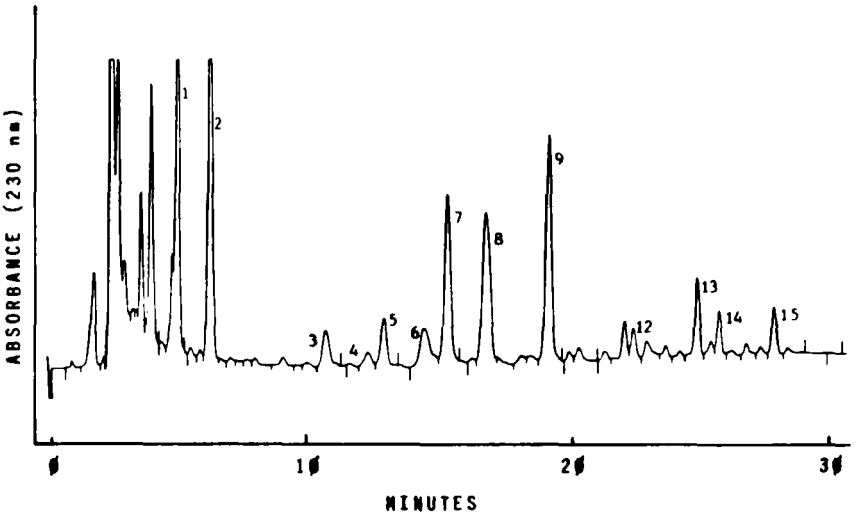

FIGURE 4. Rat serum phospholipid profile. Conditions of analysis and the identity of the numbered peaks are as in Figure 1.

phospholipids are derived predominately from the liver. As indicated in Figure 4, the phospholipid profiles of rat serum samples were generally similar to those obtained with human samples, although the relative contribution of individual peaks differed in many cases. For example, the monoglycerides and certain of the ceramide derivatives were more prominent in the rat serum phospholipid profiles. However, in general, the resolution of the rat serum lipid profiles was comparable to that obtained with human serum samples.

Evaluation of dosed and control samples indicated that characteristic alterations in serum phospholipid HPLC profiles could be demonstrated in the FireMaster-dosed animals. Table 2 summarizes the mean response of the 13 calibrated peaks from PBB-dosed rats (n=20) vs. the mean profile of the control group (n=6). In general, substantial increases in peaks 2 and 8 and in most of the ceramides were noted in the dosed

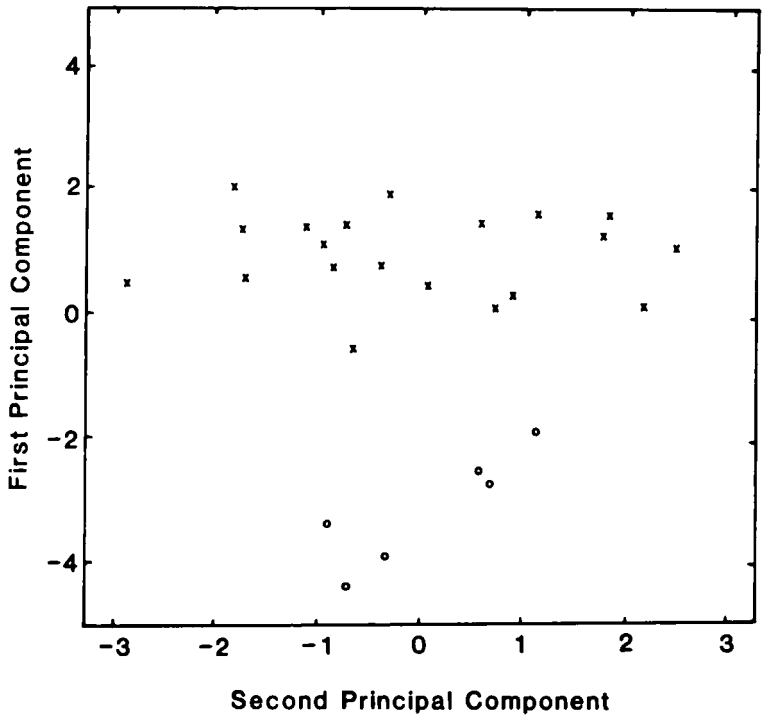

FIGURE 5. Plot of the first vs. the second principal components. Dosed animals are indicated by an "x"; "o" = controls.

group, in conjunction with declines in peaks 3, 5 and 7. The general increase in stearate-containing components and decrease in those containing palmitate in the dosed group is in agreement with the same shift observed in the liver phospholipid fatty acid in dosed animals (14), and with the results of total serum phospholipid fatty acid analyses (Table 3). A further degree of selectivity relative to individual molecular species is, however, also suggested by these results. A stepwise discriminant analysis of the data in Table 2 selected 8 of the 13 variables as sufficient for separation of the two groups. When this reduced variable list was examined by a principal component analysis and the first two principal

TABLE 2

Control and PBB-Dosed Rat Serum Phospholipid Profiles

Wt % ($\bar{X} \pm$ SEM)

Peak Number [1]	Control	Dosed	P [2]
1	13.4 ± 0.74	10.6 ± 0.33	.0008
2	8.6 ± 0.62	13.6 ± 0.28	.0001
3	5.5 ± 0.31	3.0 ± 0.17	.0001
4	2.0 ± 0.27	1.5 ± 0.05	.0088
5	9.0 ± 0.46	5.9 ± 0.31	.0001
6	5.8 ± 0.21	4.9 ± 0.27	NS [3]
7	16.7 ± 0.68	11.8 ± 0.40	.0001
8	14.4 ± 0.57	21.8 ± 0.48	.0001
9	18.4 ± 0.61	16.5 ± 0.43	.0339
12	1.6 ± 0.10	2.4 ± 0.11	.0019
13	1.8 ± 0.17	4.1 ± 0.85	.0001
14	1.0 ± 0.11	1.9 ± 0.11	.0003
15	2.3 ± 0.22	2.4 ± 0.08	NS
	N = 6	N = 20	

1. Peak numbers are as given in Figure 4
2. Student's t test
3. Not significant

components were plotted against each other, a clear separation between the dosed and control groups was demonstrated (Figure 5).

Two independent studies are represented in the data summarized in Table 2. The experiments were conducted in an identical manner but on different occasions separated by several months. When the data from the first series (3 control and 15 dosed animals) were used as a "training set" for discriminant analysis, each of the remaining samples was correctly classified as representing either a control (n=3) or dosed (n=5) pattern. Thus, under these conditions, it was possible to discriminate between the dosed and control animals solely on the basis of their serum phospholipid patterns.

TABLE 3

Serum Phospholipid Fatty Acid Composition in Control and PBB-Dosed Rats

Wt % ($\bar{X}$ ± SEM)

Fatty Acid	Control	Dosed
14:0	0.2 ± 0.03	0.2 ± 0.01
16:0	23.6 ± 0.92	19.0 ± 0.38
16:1 (n-7)	1.0 ± 0.09	0.6 ± 0.05
18:0	23.3 ± 0.86	30.3 ± 0.48
18:1 (n-9)	5.7 ± 0.25	6.2 ± 0.19
18:1 (n-7)	3.9 ± 0.41	2.4 ± 0.09
18:2 (n-6)	21.0 ± 0.76	18.0 ± 0.47
20:3 (n-6)	0.9 ± 0.15	0.7 ± 0.03
20:4 (n-6)	18.6 ± 1.26	19.7 ± 0.42
22:6 (n-3)	2.1 ± 0.33	2.2 ± 0.08
	N = 9	N = 19

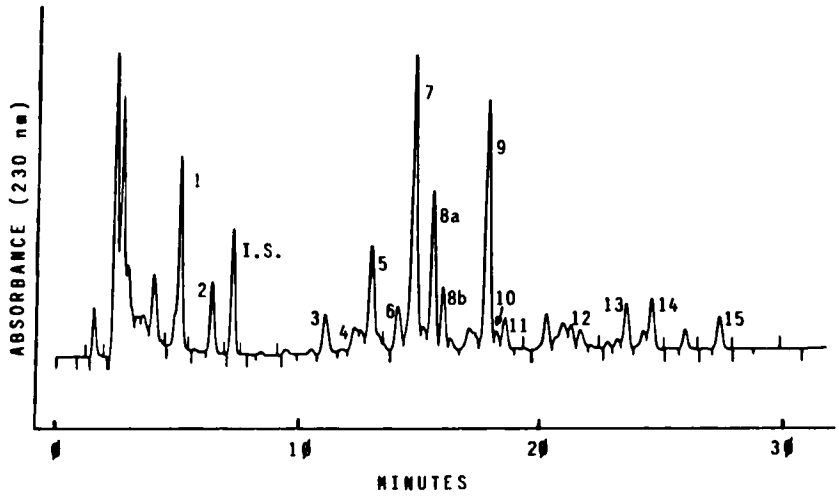

FIGURE 6. Human serum phospholipid profile. Conditions of analysis and peak identification were the same as those in Figure 1 except that solvent "A" was 10% methanol in acetonitrile, and a 20-min gradient of 30-60% isopropanol in solvent "A" was begun at 5 min. Peak "I.S." is derived from the internal standard (L-α-dilaurylphosphatidylcholine); peaks 8(a) and 8(b) are palmityl sphingosine and 18:0/20:4-DG; respectively.

In all cases, the serum phospholipid profiles were substantially altered in a characteristic way in the PBB-dosed animals, and these alterations were maintained for at least 2 months after the animals were dosed.

Recently, we have further modified this procedure by replacing acetonitrile with 10% methanol in acetonitrile as solvent A and slightly altering the gradient, and by adding L-α-dilaurylphosphatidylcholine to the serum sample as an internal standard. A representative chromatogram obtained under these conditions is given in Figure 6. The solvent modification enables the resolution of palmitoyl sphingosine from the diglyceride that formerly coeluted with it in peak 8, whereas the use of an internal standard should enable the quantitation of individual components in these analyses. The diglyceride critical pair in peak 9, however, remained unresolved in the modified system (Figure 6).

CONCLUSION

The complete resolution of all serum lipid molecular species is a difficult problem which has not been solved by any technique (19). Although resolution of all the serum phospholipid molecular species was not achieved in this study, we were able to obtain reproducible patterns in the analyses, and the results suggest that qualitative serum lipid profiles determined by simple HPLC techniques might be of value in detecting metabolic alterations, such as those accompanying exposure to environmental toxicants. Considerable evidence exists for the influence of many toxicants on hepatic and serum lipids in both experimental animals and in humans, and the complex interaction among multiple variables that may be monitored by chromatographic profiling techniques provides the potential for detailed discrimination and classifi-

cation among individuals and groups, perhaps even at the subclinical or preclinical stage. Further work will be required, however, to determine the extent to which this approach can be applied to a more heterogeneous human population.

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Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

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