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Application of ^{31}P NMR Spectroscopy and Chemical Derivatization for Metabolite Profiling of Lipophilic Compounds in Human Serum

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Abstract

New methods for obtaining metabolic fingerprints of biological samples with improved resolution and sensitivity are highly sought for early disease detection, studies of human health and pathophysiology, and for better understanding systems biology. Considering the complexity of biological samples, interest in biochemical class selection through the use of chemoselective probes for improved resolution and quantitation is increasing. Considering the role of lipids in the pathogenesis of a number of diseases, in this study fingerprinting of lipid metabolites was achieved by ^{31}P labeling using the derivatizing agent 2-chloro-4,4,5,5-tetramethyldioxaphospholane. Lipids containing hydroxyl, aldehyde and carboxyl groups were selectively tagged with ^{31}P and then detected with good resolution using ^{31}P NMR by exploiting the 100% natural abundance and wide chemical shift range of ^{31}P. After standardizing the reaction conditions using representative compounds, the derivatization approach was used to profile lipids in human serum. The results show that the ^{31}P derivatization approach is simple, reproducible and highly quantitative, and has the potential to profile a number of important lipids in complex biological samples.

Keywords

NMR; ^{31}P; metabolite profiling; metabolomics; chemical derivatization; lipids

Introduction

The analysis of lipid constituents in biological samples is a vital part of metabolomics studies due to the important role of lipids in disease diagnosis and human health risk assessment.[1] Investigations have shown that alterations in the lipid profile are the result in a number of pathological conditions such as cardiovascular disease,[2] certain cancers,[3, 4] Alzheimer's disease[5] and some inborn errors of metabolism.[6]

Mass spectrometry is a commonly used methodology for lipid analysis because hyphenated techniques, such as liquid chromatography- (LC), gas chromatography- (GC) and capillary electrophoresis- (CE) mass spectrometry provide comprehensive profiles of the sample in addition to the potential for relative or absolute quantitation of the components. However,

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the reproducibility of the mass spectrometry-based metabolite profiling approach can be challenging in complex samples such as biofluids. NMR spectroscopy has also been applied in metabolic profiling studies of a wide range of complex samples such as urine, serum, plasma and tissue, both in *in-vivo* and *in-vitro* studies. These studies take advantage of NMR's experimental versatility, high resolution, reproducibility, and quantitative ability, as well as the fact that it can provide a high degree of information on the biological system of interest, often with limited sample preparation. [7–12] Applications of NMR to the analysis of lipids have been relatively sparse, although a number of lipids such as cholesterol, cholesterol derivatives, glycerophopsholipids and cholines can readily be detected by ^{1}H NMR. The NMR spectral resolution is somewhat diminished in highly viscous lipophilic compounds due to the line broadening that results from restricted molecular motion and a high degree of non-resolved J-couplings among the many methylene protons present. Close structural similarity amongst different lipid categories, combined with their varied concentrations in biological samples, further add to the difficulties in the analysis of lipids by NMR.

An alternative to NMR analysis of the whole biological sample involves a more selective experiment such as class selection. Metabolite class selection has been shown to be a powerful tool in metabolite profiling and biomarker discovery, particularly because it greatly alleviates the problem of peak overlap in biological samples.[13–15] In addition, this approach provides the potential for fast and efficient methods which enhance the sensitivity of certain classes of metabolites. Metabolite class selection by chemical derivatization is relatively common in MS and GC analysis.[13, 14, 16] However, despite a number of early studies,[17] chemical derivatization is less commonly utilized in NMR, especially for metabolomics applications. Recently, we reported a successful chemical derivatization based NMR methodology for metabolomics applications.[18] Derivatization of amines and amino acids using ^{13}C labeled acetic anhydride yielded approximately 100-fold enhanced sensitivity for analysis by ^{13}C or heteronuclear 2D NMR. This simple, one-step chemical derivatization reaction is fast, cost effective and requires minimal sample preparation. The spectrum is simplified because only a single peak is detected from the derivatizing agent or tag that reacts with the metabolite of interest. We have recently extended this approach to detect carboxylic acid containing metabolites by tagging them with ^{15}N labeled ethanolamine.[19]

In this study, as a continuation of exploring classes of metabolites in biological systems, we report a simple chemical derivatization procedure for selectively analyzing lipid metabolites with hydroxyl, carboxyl or aldehyde groups in a complex biological fluid, serum. Metabolites with these functionalities have been shown to be altered in a number of diseases. For example, accumulation of free fatty aldehydes and alcohols in the human body is a clinical indication of Sjögren-Larsson syndrome that is due to a genetic enzyme deficiency of fatty aldehyde dehydrogenerase.[20] The elevation of total free fatty acids in serum has been reported to be an indication of acute pancreatitis (AP), and more generally, free fatty levels provide an important measure of the physiologic state.[21, 22] The ^{31}P containing reagent, 2-chloro-4,4,5,5-tetramethyldioxaphospholane (CTMDP) was used to derivatize the lipid metabolites (Scheme 1). Derivatized metabolites were then detected with enhanced resolution using ^{31}P NMR. Previously, chemical derivatization using this reagent has been performed to quantify fatty acids and glycerides in olive oil for quality control purposes.[23, 24] These investigations report CTMDP to be an excellent derivatizing reagent for lipids with hydroxyl, carboxyl or aldehyde functional groups. We find this derivatization approach to be relatively fast and simple; the method requires only a modest amount of sample preparation and can be used to identify a number of lipophilic functionalities in one experiment, without the use of chromatography.

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Experimental

Chemicals and samples

Lyso lipids, cholesterol, phosphatidyl glycerol were purchased from Avanti polar lipids (Alabaster, AL) and 1,2 dipalmitoyl glycerol was purchased from Sygena (Switzerland). All other compounds (octanoic acid, palmitic acid, oleic acid, 1-hexanol, 1-octanol, spingomyelin, inositol, chromium acetoacetate) including solvents and 2-chloro-4,4,5,5-tetramethyldioxaphospholane, were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. Deuterated solvents were purchased from Cambridge isotope (Andover, MA). Human serum samples were obtained from Innovative Research (Novi, MI).

Lipid extraction

A 0.5 ml sample of serum was mixed with 1.0 ml of methanol, vortexed and kept at −20 °C for 30 min to precipitate serum lipids and proteins. The mixture was subsequently centrifuged at 5000 rpm for 5 min and the supernatant containing aqueous soluble metabolites was separated and discarded. 1.0 ml methanol and 0.5 ml deionized water were then added to the precipitate, vortexed and centrifuged at 5000 rpm for 5 min. The supernatant was again discarded and the residue was treated with 1.0 ml methanol and sonicated (Sonifier cell disruptor w-350, pulse mode, output control 3) for a total of 6 min, with delays of about 15 sec after every 2 min. During sonication the sample container was kept in an ice bath to avoid sample heating. The sample was then treated with an additional 3.0 ml methanol, mixed well and sonicated for another 6 min similarly, and then stored at −4 °C overnight. The sample was sonicated again for 6 min and then passed through a 0.45 μm cellulose filter (Thermo Scientific, Waltham, MA), which had been previously washed multiple times with a methanol/chloroform mixture using a glass syringe. Chloroform and methanol were removed using a dry nitrogen stream, and then the lipid sample was further dried using a speed vac system (Thermo scientific, Waltham, MA).

Chemical derivatization

The chemical derivatization procedure was adapted from the previously described method.[23] A stock solution consisting of 6.5 ml pyridine and 3.5 ml deuterated chloroform was mixed in a vial containing molecular sieves to protect the solution from moisture. Cyclohexanol (2 μl) was added as an internal standard and a catalytic amount of chromium acetylacetonate was added as a NMR relaxation agent. The stock solution was kept sealed to limit its exposure to moisture. For standardization and chemical shift calibration, 1–3 mg each (depending on the molecular mass) of a number of representative compounds were dissolved in 500 μl of the stock solution. Subsequently, 60 μl of CTMDP was added. The reaction was allowed to run for 30 min prior to NMR acquisition. For the serum experiments, the dried lipid extract was reconstituted in 500 μl stock solution before chemical derivatization was performed. In a different protocol, aliquots of serum samples were lyophilized to dryness and reconstituted in stock solution before derivatization with CTMDP. The sample was spun down to remove any residual solids prior to transfer to the NMR tube. Duplicate reactions and analyses on a split serum sample were also performed. To assess the quantitative yield and reproducibility, the derivatization reaction was performed in triplicate for a set of representative compounds (palmitic acid, cholesterol, 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate, 1,2-dipalmitoyl glycerol, hexanal and 1-octanol).

NMR acquisition

Measured volumes (500 μl) of the derivatized (serum extract or model compounds) were placed in 5 mm NMR tubes and a concentric capillary insert containing

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methylenediphosphonic acid (50 μl of 0.1 M or 50 μl of 9.3 mM for the serum experiments) as a separate internal standard was placed in the NMR tube. All NMR experiments were carried out at 25 °C on a Bruker DRX 500 MHz spectrometer equipped with multinuclear broadband observe (BBO) probe. One-dimensional ^{31}P NMR spectra were recorded with inverse-gated proton decoupling using the WALTZ-16 sequence. A total of 1024 (for lipid extracts) or 64 (for model compounds) transients were averaged and 64 K data points acquired for each sample. Appodization corresponding to a line broadening of 1.0 Hz was applied prior to Fourier transformation. A recycle delay of 10 sec was used to analyze the derivatized serum extracts, while 30 or 50 sec was used for the quantification and reproducibility studies.

Results and Discussion

The NMR spectrum of serum lipids typically shows a number of reasonably well resolved peaks in chloroform solution. The ^{1}H NMR spectrum (Figure 1) of a reconstituted serum lipid extract 8 was obtained prior to derivatization for comparison with features post-derivatization. Table 1 lists the observed peaks and their assignments based on literature reports[6, 12, 25] and NMR databases;[26, 27] assignments were subsequently confirmed via COSY (data not shown). Typically, the ^{1}H NMR spectrum of serum is complex as a consequence of severe spectral overlap from numerous low mass metabolites and macromolecular lipophilic compounds and proteins. However, as is apparent in Figure 1, exclusion of the proteins and hydrophilic low mass metabolites, especially the highly abundant glucose, glycogen and amino acids in serum, as well as the use of deuterated chloroform, leads to improved resolution and good line shape and to an unambiguous identification of individual compounds. The spectrum shows intense resonances from aliphatic fatty chains as well as cholesterol, cholesterol derivatives, cholines, phospholipids, glycerides, spingomyelin, etc. Aliphatic fatty chain resonances can be assigned to sub-groups such as methyl, methylene, olefin, and allylic features but it is not possible to identify individual lipophilic components. For example, it is not possible to distinguish between monacyl- and diacyl phospholipids. Despite the detection of 25 resonances, many of these are from the same category of lipid, and thus only 6 or so lipid categories are identified in the spectrum.

To expand on the number of identifiable and quantifiable lipid components, we examined the reactions of representative lipophilic compounds with CTMDP to ascertain whether these compounds could be differentiated in the ^{31}P NMR spectrum. Model compounds such as mono- and diacylglycerides, long chain fatty acids, fatty alkanols, lyso lipids, sphingomyelin, and steroidal compounds were derivatized to examine the applicability of this methodology and to build a reference library of chemical shift values. These compounds were chosen because they mimic a set of biologically relevant compounds found in human serum. ^{31}P NMR chemical shift values for 16 derivatized compounds are summarized in Table 2. Cyclohexanol was used as an internal standard as it readily reacts with CTMDP to produce a derivatized species that appears at 145.2 ppm.[28] In addition, a capillary containing methylenediphosphonic acid (17.9 ppm) was used as a separate internal reference in order to ensure that the signals produced by cyclohexanol and other derivatized metabolites were accurate. The derivatization reaction yields, evaluated using the ^{31}P peak integrals of the representative lipids reacted in triplicate were highly reproducible. A mixture of six different compounds carrying labile hydrogens (palmitic acid, cholesterol, 1-octanol, hexanal, 1,2-dipalmitoyl glycerol and 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine) was used to evaluate the reproducibility and reaction yield individually and in a mixture. The yields were quite reproducible, with coefficients of variation varying between 4 and 9% for the different chemical species. Reaction yields varied by class: long chain alcohols showed the highest reactivity, producing reaction yields over 95%; glycerides and cholesterol yielded over 90%

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product; lyso lipids and fatty acids produced ~65% and ~75% reaction yields; while aldehydes gave the lowest yield, 40%. These reaction yields for pure compounds and mixtures are similar.

The ^{31}P chemical shift values obtained in this study for mono- and diglycerides, free fatty acids and cholesterol are comparable to those previously reported in two studies focused on food analysis.[23, 24] The assignments were verified by running proton coupled experiments in which triplets and doublets were observed for the derivatized primary and secondary hydroxyl groups, respectively. Derivatized long chain fatty acids appear at 134.78 ppm; not surprisingly, there appears to be little or no chemical shift sensitivity to the length of carbon chain and degree of unsaturation. Chemical shifts for derivatized octanoic acid (8:0), palmitic acid (16:0) and oleic acid (18:1 9Z) are exactly the same separately and in a mixture of all three, indicating that the 10 structural similarity at the reactive carboxylic acid head is dominant in determining the ^{31}P chemical shift value. The straight-chain fatty alcohols 1-octanol and 1-hexanol also showed the same chemical shifts (147.12 ppm).

Cholesterol, an important constituent in human serum, appeared at 144.93 ppm after CTMDP derivatization. As there are several similar forms of cholesterol found in human serum, such as free and esterified cholesterol and cholestenol, we compared the derivatization of free cholesterol to 5-cholesten-3β-ol-7-one. Both derivatized cholesterol and 5-cholesten-3β-ol-7-one gave the same peak, suggesting that free cholesterol and structurally similar compounds will show essentially the same ^{31}P chemical shift. As esterified forms of cholesterol will not be derivatized by CTMDP, the total non-esterified cholesterol can be evaluated by this methodology.

We also examined several lyso lipids with different chain lengths, unsaturation and head groups: 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine, 1-behenoyl-2-hydroxy-*sn*-glycero-3-phosphocholine, 1-1auroyl-2-hydroxy-*sn*-glycero-3-phosphocholine and 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate. All of these derivatized lyso lipids had the same ^{31}P chemical shift and produced two peaks at 146.68 ppm and 146.74 ppm. This is likely due to the chirality of the sn-2 carbon atom bearing the –OH group. If inversion of the 5-membered phospholane ring is slow there would be two diostereomers present. This suggestion is supported by the proton coupled ^{31}P NMR spectrum, which shows three peaks (a triplet-like structure) with a $^{3}J_{P\text{-}H}$ coupling of 9.25 Hz, suggesting two overlapped doublets. Phospholane derivatization of lyso lipids have not been reported before and this may be an area for further studies. Derivatized 1,2-dipalmitoyl glycerol produced a resonance at 148.26, which was comparable with the chemical shift value reported in literature for diglycerides.[23] 3-Palmitoyl-*sn*-glycerol produced two peaks at 146.4 ppm and 147.58 ppm, corresponding to derivatized secondary and primary hydroxyl groups, respectively.

Although sphingomyelin carried only one hydroxyl functional group, it produced multiple resonances after derivatization which was consistent in several trials. This may be again attribute to slow inversion of phospholane ring with respect to two adjacent carbon centers and spacial arrangement in solution. The possibility of sphingomyelin caring any impurities were ruled out by ^{1}H and ^{31}P NMR spectra of authentic compounds.

Human serum

The ^{31}P NMR spectrum of human serum was recorded prior to derivatization to identify the naturally occurring phosphorous signals. The aqueous fraction shows mainly inorganic phosphate, which appears at −0.596 ppm, along with small amounts of phosphocholines, sugar phosphates and phosphoethanolamine. The lipophilic fraction mainly consists of phosphocholines/lysophosphocholines located at 0.5 ppm. The ^{31}P NMR spectrum did not

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show any additional resonances in an extended region of −40 ppm to 220 ppm in the lipid extract samples. After derivatization with CTMDP, newly formed P-O groups derived from hydroxyl groups appeared in the 144.5 – 150 ppm region, while P-O groups derived from carboxylic acids appeared at 134.5 – 135.5 ppm.

Human serum was derivatized according to two protocols. In the first procedure, lipids were extracted from serum as described above, while in the second procedure serum aliquots were lyophilized to dryness and reconstituted in the pyridine/deuterated chloroform stock solution. There were two distinct differences seen in the ^{31}P NMR spectra of samples from these two protocols. The concentration of cholesterol was found to be higher in extracted samples than in reconstituted samples. This can be attributed to the high solubility of cholesterol in 100% chloroform compared to its solubility in stock solution (4:1(v/v) pyridine: chloroform). On the other hand, free fatty acids were less abundant in the extracted samples than in the lyophilized samples. This may be attributed to a lower extraction efficiency of some short chain fatty acids in chloroform or loss of material during the extraction procedure. Of the two protocols, lyophilization followed by derivatization is recommended for clinical applications. The optimal quantity of serum for detection by ^{31}P NMR was assessed and samples sizes from 10 ml to 0.5 ml were studied. It was found that even 0.5 ml of serum could produce useable spectra for the study. However, 1.0 ml serum samples were used for a majority of the experiments. Because these are extracted samples, there is a potential for further analyte pre-concentration and detection using, for example microcoil NMR.[29–31]

Figure 3(b) shows the spectral region between 134 and 150 ppm. Ten hydroxyl-containing metabolites were clearly detected, while three metabolites and some non-resolved metabolites were seen in the carboxylic acid region. In comparison, non-derivatized human serum (Figure 3(a)) does not show any ^{31}P NMR resonances in this region. Based on the data in Table 2 as well as other literature sources, seven metabolites could be identified in this region. Derivatized cholesterol appears at 144.93 ppm and this signal represents all unesterified cholesterol species in serum. In contrast, individual identification and quantification of esterified and non-esterified cholesterol using 1D ^{1}H NMR is ambiguous due to the strong spectral overlap of proton signals. In this study distinct identification and quantification of the total non-esterified fraction of cholesterol is possible. Mono and diglycerides can also be distinguished by this methodology, whereas derivatized 1,2 diglycerides in serum appear at 148.26 ppm, while for mono glycerides, derivatized primary and secondary hydroxyl groups appear at 147.6 and 146.54 ppm, respectively. Human serum carries a considerably high content of triglycerides; however, due to lack of hydroxyl groups, triglycerides cannot be detected by this methodology. Regardless of the length of the carbon chain, polar head group or unsaturation, all lyso lipids produced two resonances at 146.68 ppm and 146.74 ppm in serum. Serum free fatty carboxylic acids appeared at 134.78 ppm. In addition, two other distinct phosphorous resonances and several unidentified resonances can be seen in the derivatized carboxylic acid region. Compounds such as derivatized syringic acids can appear in this region.[23] Further studies are needed to identify these unknown resonances in this region.

Reproducibility studies were performed by derivatizing the same split human serum sample in triplicate. This methodology showed high reproducibility with standard deviation of ≤8% for all peaks using the 3 replicates. Figure 4 shows the 144 – 150 ppm region of the ^{31}P NMR spectra for five derivatized serum samples from healthy individuals. All spectra showed good consistency with some intensity variations. We would anticipate significantly larger variations in samples from individuals with lipid pathologies. This set of samples was analyzed with cyclohexanol as an internal standard, which is evident in the spectra at 145.2 ppm. The components marked with an asterisk are impurities arising from cyclohexanol.

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The average concentration of several compounds was calculated using sample triplicates, and these results are tabulated in Table 3.

Conclusions

Chemical derivatization of human serum with 2-chloro-4,4,5,5-tetramethyldioxaphospholane provides an approach complimentary to that of conventional NMR methods for the identification and quantitation of a number of lipophilic compounds. The method provides sufficient sensitivity and spectral resolution, and derivatized species have unique and well-resolved resonances located in the 135–150 ppm region of the ^{31}P NMR spectrum. The number of lipid components identified is comparable to those identified by ^{1}H NMR. The addition of sample pre-concentration and microcoil NMR detection would provide an avenue for sensitivity enhancement and further metabolite identification. In addition, 2D ^{1}H-^{31}P NMR would be useful for resolving lipid components which overlap in the 1D spectra. This methodology offers the possibility of identification of a number of additional lipophilic compounds that contain hydroxyl and carboxylic acid functionalities in different chemical environments. This approach is consistent with the requirements for a fast screening method for lipid pathologies in human serum making possible the efficient use of ^{31}P NMR spectroscopy alongside ^{1}H NMR spectroscopy in metabolomics studies.

Acknowledgments

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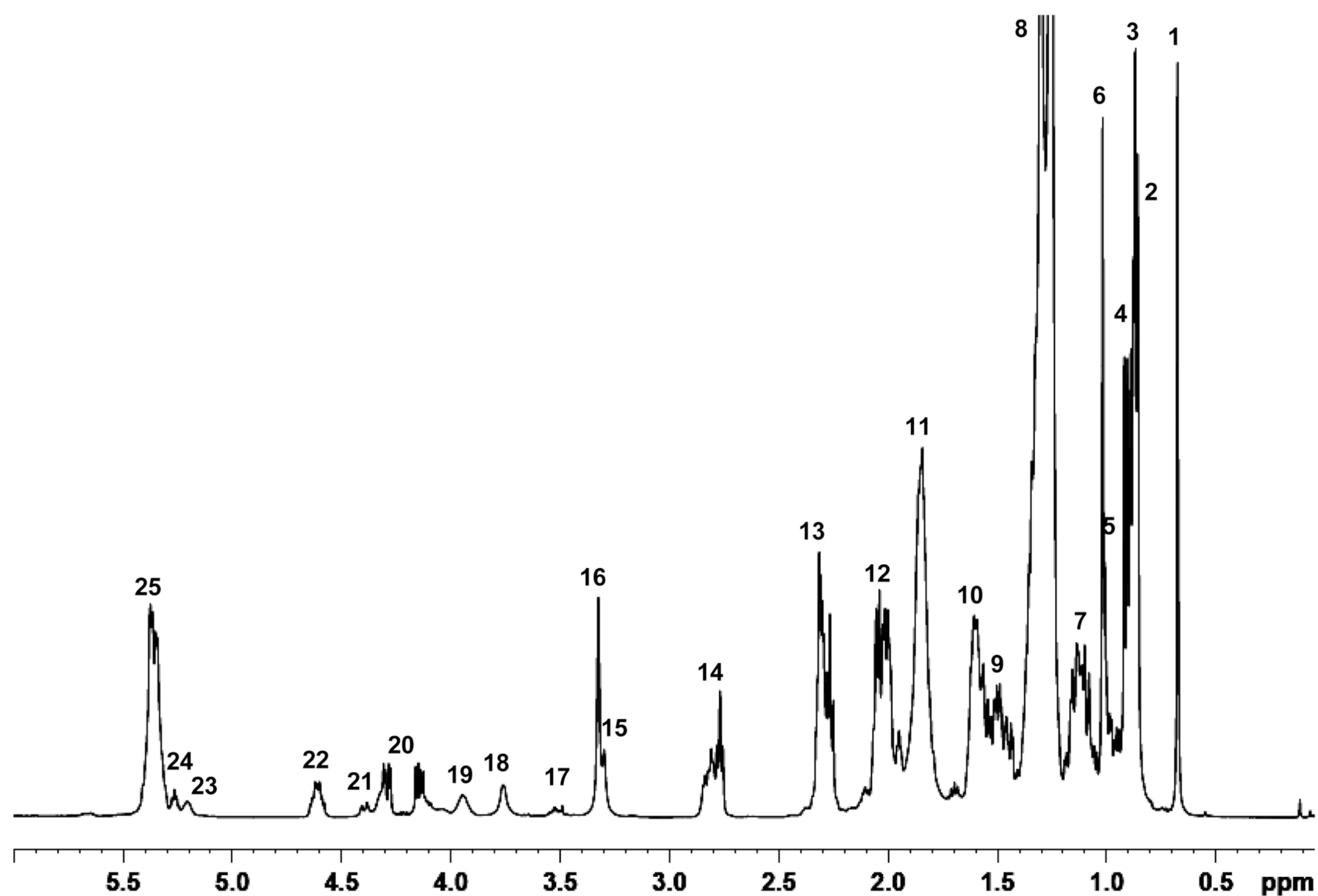

Figure 1.

^{1}H NMR spectrum of a serum extract from a healthy individual (commercial sample). Assignments for the numbered peaks are listed in Table 1.

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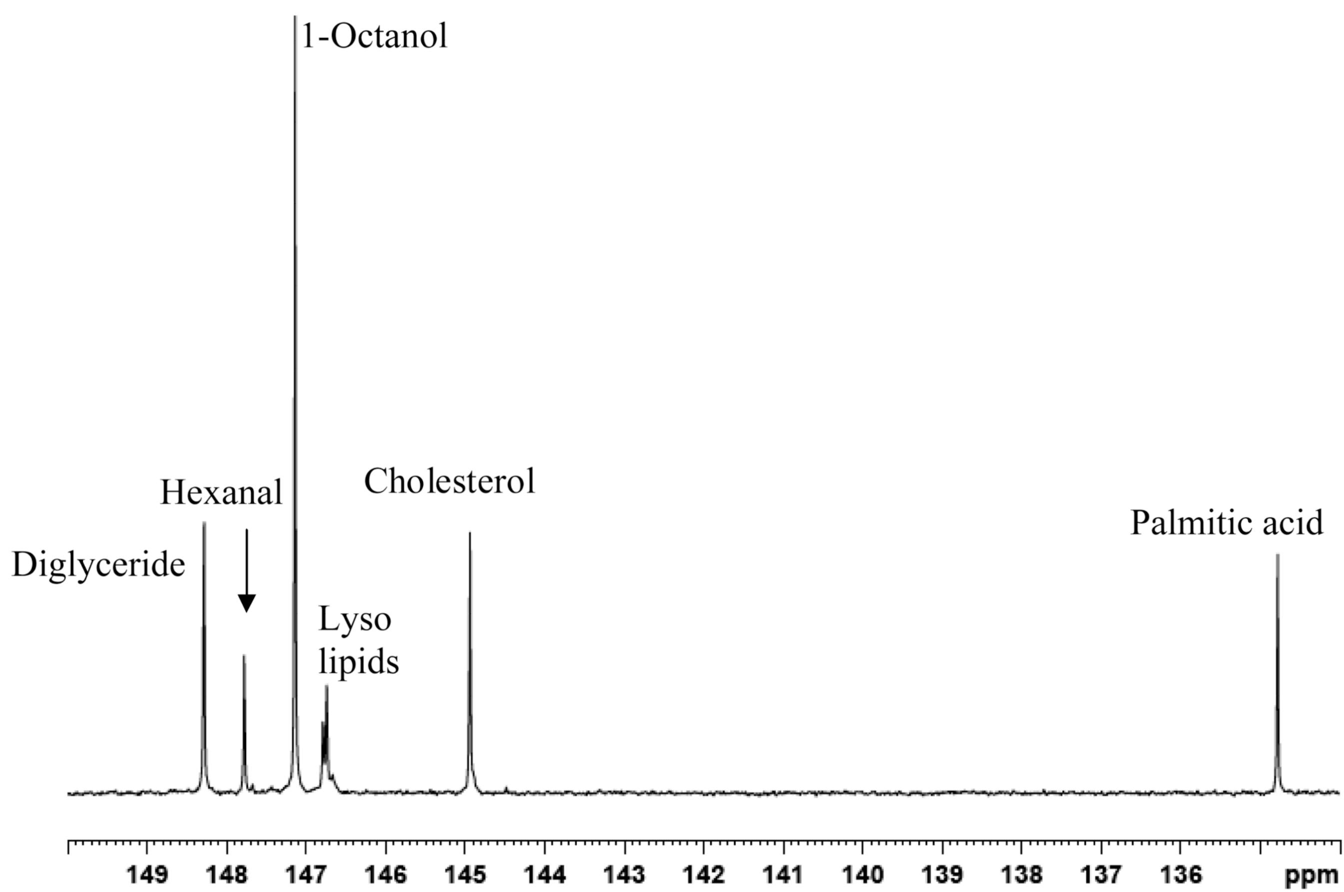

Figure 2.
A portion of the ^{31}P NMR spectrum of a mixture of six derivatized representative compounds.

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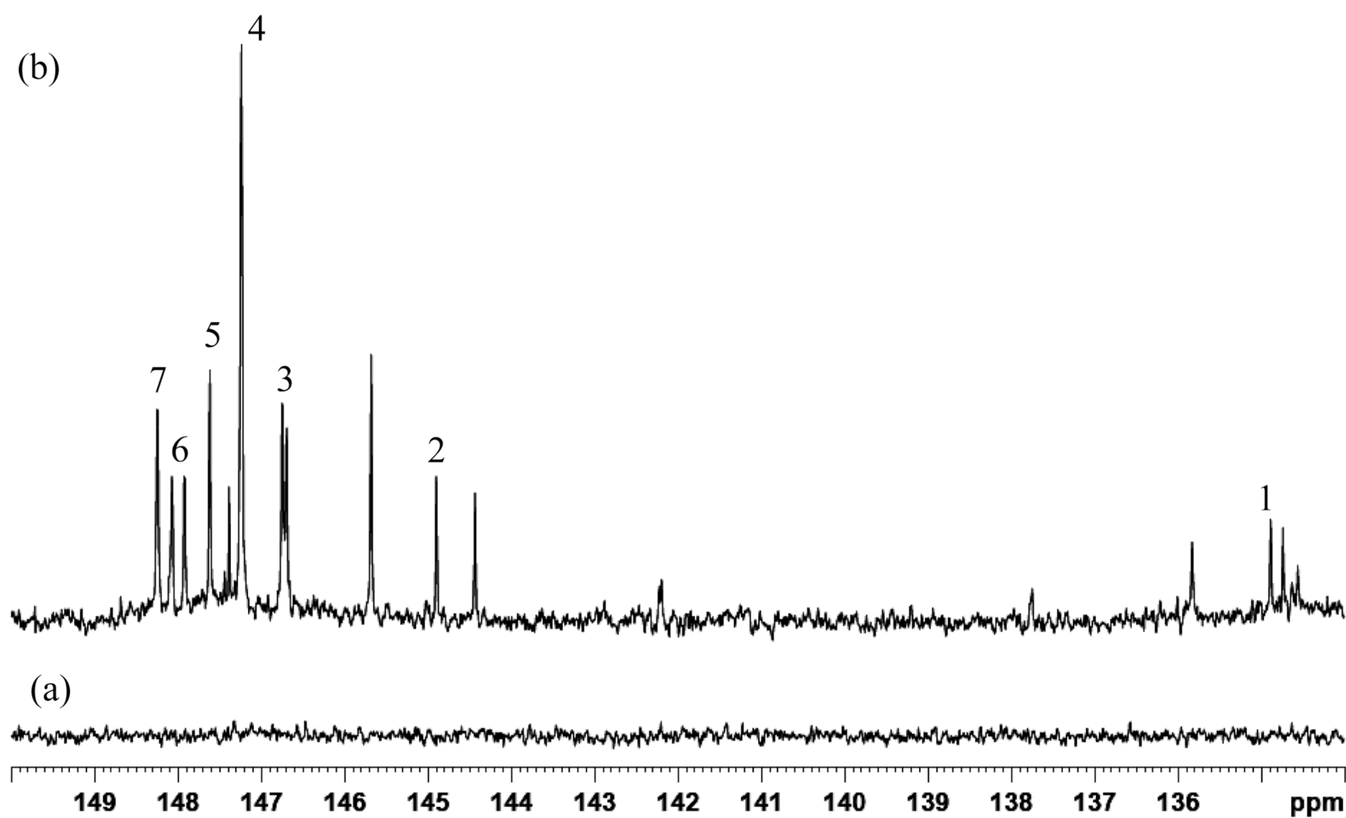

Figure 3.
A portion of the ^{31}P NMR spectrum of derivatized serum from a healthy individual. Derivatized compounds include: free fatty acids **1**; cholesterol **2**; lyso lipids **3**; possible fatty aldehydes **4**; phosphotidyl mono glycerides (primary) **5**; free n-alkanol **6**; 1,2-diacylglycerol **7**.

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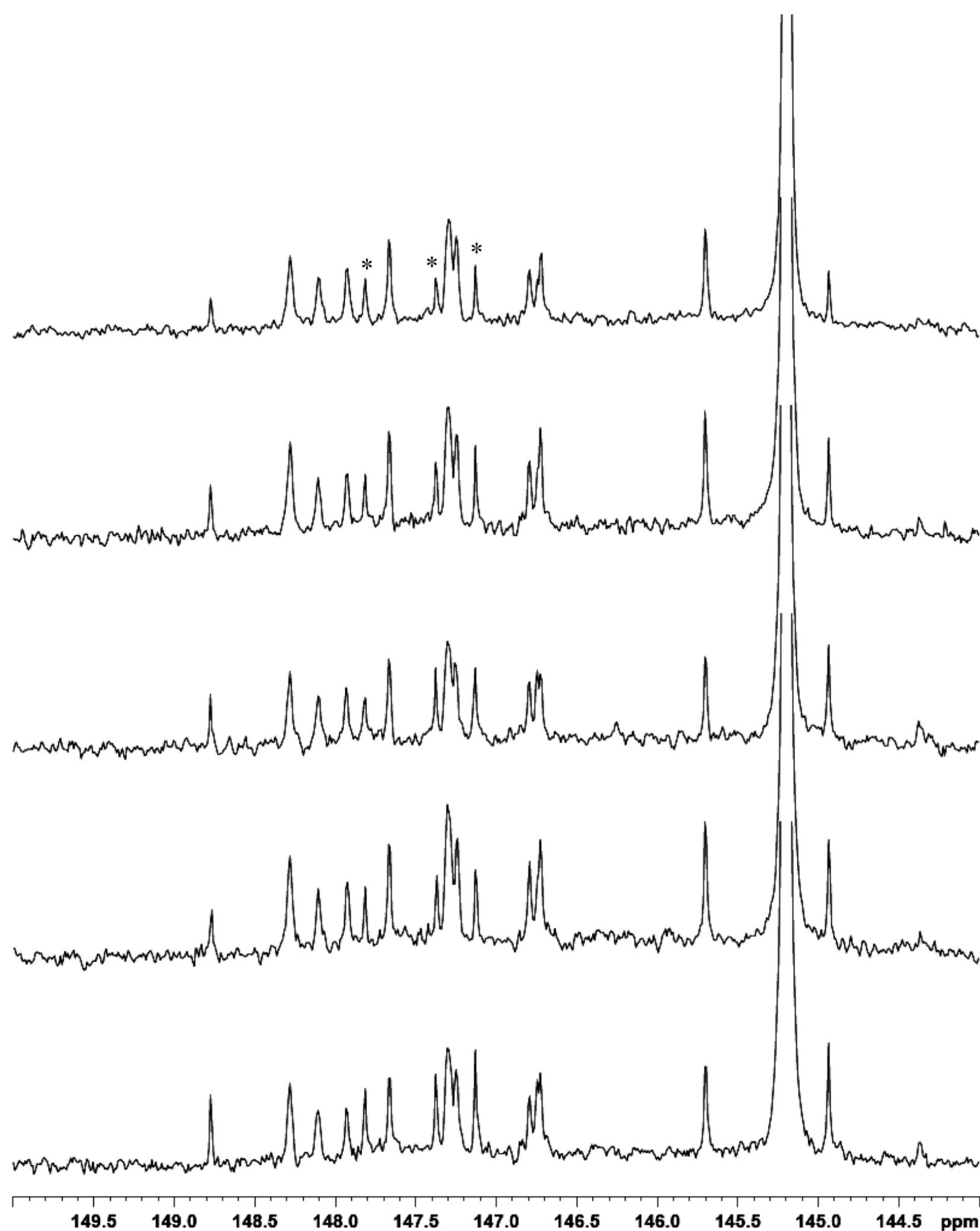

Figure 4.

A portion of the ^{31}P NMR spectrum of derivatized serum from 5 healthy individuals with added cyclohexanol (2µl; 145.2 ppm) as an internal standard in the stock solution. Impurities arising from cyclohexanol are marked with an asterisk.

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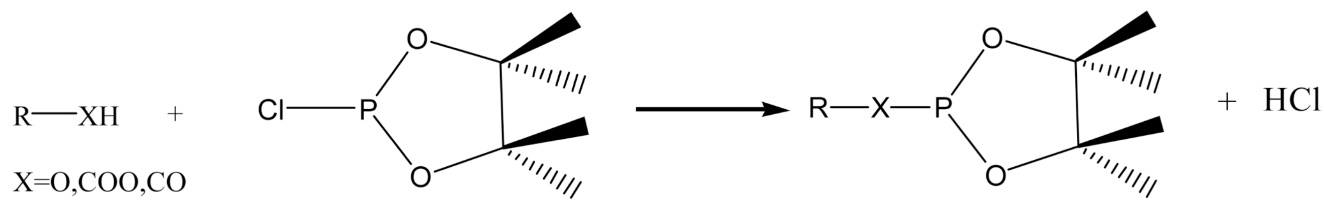

Scheme 1.

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Table 1

Resonance assignments for signals identified in the lipid extract of serum

Peak	Assignment
1	Total cholesterol C-18 CH_3
2	Total cholesterol C-26 and C-27 CH_3
3	Fatty acid terminal CH_3
4	Total cholesterol C-21 CH_3
5	Free cholesterol C-19 CH_3
6	Esterified cholesterol C-19 CH_3
7	Free and esterified cholesterol protons
8	Fatty acyl chain CH_2
9	Free and esterified cholesterol protons
10	Fatty acyl chain $C\underline{H}_2CH_2CO$
11	Free and esterified cholesterol protons
12	Fatty acyl allyl -CH_2-
13	Fatty acyl $C\underline{H}_2CO$
14	Fatty acyl diallylic proton
15	Sphingomyelin
16	Choline
17	Free cholesterol C-3 H
18	Phosphatidylcholine N-CH_2
19	Glycerophospholipid backbone
20	Glycerophospholipid backbone
21	Phosphatidylcholine PO-CH_2
22	Esterified cholesterol $C\underline{H}OCOR$
23	Glycerophospholipid backbone
24	Glycerol backbone
25	Fatty acyl CH=CH

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Table 2

^{31}P resonance assignments for representative compounds after CTMDP derivatization

Compound	**^{31}P NMR chemical shift**
5-cholesten-3β-ol-7-one	144.93
Cholesterol	144.93
Monoglyceride	147.6 (primary)
L-α-Phosphatidyl-DL-glycerol	146. 80
1,2 dipalmitoyl glycerol	148.26
1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate	146.74, 146.68
1-1auroyl-2-hydroxy-*sn*-glycero-3-phosphocholine	146.74, 146.68
1-behenoyl-2-hydroxy-*sn*-glycero-3-phosphocholine	146.74, 146.68
Sphingomyelin	Multiple resonances
Inositol	146.78
1-hexanol	147.18
1-octanol	147.18
Hexanal	147.70
Octanoic acid	134.78
Palmitic acid	134.78
Oleic acid	134.78

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Table 3

Concentrations of lipid species in healthy human serum measured after derivatization.

Compound	Concentration (μM)
Free carboxylic acids	53.1
Free cholesterol	77
Lyso lipids	252
Fatty alcohols	354.5
1,2-diglycerides	147