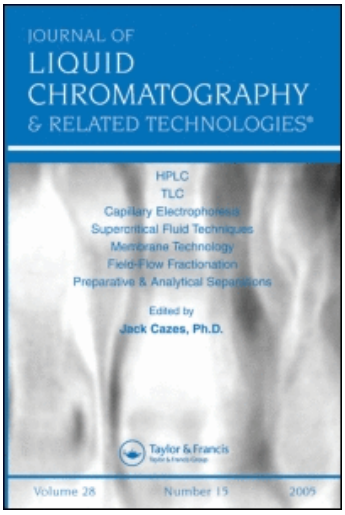

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A Comparison of High Performance Gel Permeation Chromatography and Nuclear Magnetic Resonance Spectroscopy in the Analysis of Plasma from Normal Subjects and Cancer Patients

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A COMPARISON OF HIGH PERFORMANCE GEL PERMEATION CHROMATOGRAPHY AND NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY IN THE ANALYSIS OF PLASMA FROM NORMAL SUBJECTS AND CANCER PATIENTS*

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ABSTRACT

High Performance Gel Permeation Chromatography (GPC) was evaluated as an alternative to the more expensive Nuclear Magnetic Resonance (NMR) spectroscopy technique presented by Fossel and co-workers (1) for cancer detection using human plasma. These two techniques show a biphasic relationship which can be explained on the basis of the relative amounts of the lipoprotein levels present in the plasma and a good correlation (Table 1) with total

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triglyceride concentrations obtained from standard blood tests. The major difference in the GPC elution profiles (254 nm) of plasma from normal individuals and that from cancer patients occurred in the peak eluting at the void volume. This peak has a retention time consistent with very low density lipoprotein (VLDL) and is elevated in most cancer patients and in normal patients with triglyceride levels greater than 200 mg/ml. The use of these techniques as a screening test for cancer in an asymptomatic population needs further evaluation.

INTRODUCTION

For over thirty years cancer researchers have searched for a possible tumor marker in plasma, sera and other biological fluids (2,3). At present no marker for general screening of cancer is in clinical use; certain markers for malignancies of specific organs have a role in clinical management post-treatment (4).

It has been observed (5) that in serum from patients with malignancies, there is a notable decrease in linoleic acid and a marked increase in stearic and palmitic acids. Barclay and Skipski (6) found over a decade ago that the presence of cancer in man influences the metabolism of lipoproteins. Some distinct patterns were (a) an elevation of very low density lipoprotein (VLDL) concurrent with low levels of α-lipoproteins, specifically high density lipoprotein (HDL_2); and (b) unusually high values for HDL_1, together with low values for other HDL's.

In a study of 133 cancer patients Bucovaz (7) found that B-protein levels accurately reflected the patients' response to chemotherapy or surgery. A chromatographic procedure was later used for the purification of this protein from human serum (8).

The water-suppressed proton NMR spectrum of plasma is dominated by the resonances of plasma lipoprotein lipids. Fossel and co-workers (1) have suggested that measurement of the mean line widths of the methyl and methylene resonances correlates with the presence or absence of malignant tumors. They concluded that this correlation is a potentially valuable

approach to the detection of cancer and the monitoring of therapy. Since its publication in November 1986, this test has been actively evaluated in a number of laboratories (9), but few detailed reports have appeared.

High performance gel permeation chromatography is a simple and inexpensive technique which can be used for the separation of human serum proteins and lipoproteins (10-12). We have therefore used it to determine if there is a difference in the plasma protein elution profiles between cancer patients and normal subjects, in the hope that these differences can be used as potential tumor indicators or to follow the progress of a cancer treatment. The results obtained from the above analyses will be compared to those obtained by nuclear magnetic resonance spectroscopy (NMR) using the procedure described by Fossel et al. (1).

EXPERIMENTAL

Materials:

Lipoprotein standards, very low density lipoprotein (VLDL #L-2264), low density lipoprotein (LDL #L-2139) and high density lipoprotein (HDL #L-2014), were purchased from Sigma Chemical Company. The gel filtration molecular weight standards and gel permeation column were obtained from Bio-Rad Laboratories. Potassium phospates, mono and dibasic, were purchased from Fisher Scientific Company.

Equipment:

A Perkin Elmer Series 4 Chromatograph and a Hewlett Packard 1040A HPLC Detection System interfaced with a Hewlett Packard 85 personal computer equipped with a 82901M Flexible Disc Drive were used in all the analyses. Peak areas were electronically calculated using a Hewlett Packard 3357 integration system. Proton NMR spectra were obtained using a GE-NMR NT-300 wide bore spectrometer with a 1280 data system.

Chromatographic Conditions:

Plasma samples (25 µl injection volume) were analyzed on a 300 x 7.5 mm Bio-Sil TSK-400, gel permeation column using a mobile phase of 25 mM potassium phosphate, pH 7.0, at a flow rate of 1.0 ml/min. The effluents were monitored at 254 and 414 nm. Phosphate buffer solutions were made by adding 4.35 grams of dibasic potassium phosphate (K_2HPO_4) to 1 liter of deionized distilled water and titrated to pH 7.0 with 25 mM mono-basic potassium phosphate (KH_2PO_4, 3.40 grams in 1 liter of water). The buffer was filtered and degassed before use and maintained under helium throughout the experiment.

Proton NMR Spectra:

Temperature was maintained at 21-22°C and calibrated with neat ethylene glycol externally. The 5 mm proton probe was detuned by 3 MHz to prevent line-broadening caused by radiation damping. The instrument was then shimmed to obtain a water resonance of 4 Hz or less in width before NMR spectra on intact plasma were obtained nonspinning and unlocked, with the following parameters: 16 acquisitions, 6 sec presaturation at the water line with ca. 4 watts of continuous wave decoupler power in the external mode; 90° (18 µsec) acquisition pulse; spectral width ± 1300 Hertz (Hz); acquisition time 1.57 sec; 8192 points zero filled to 16384; line broadening (LB) 2 Hz. The shimming was done on each sample by an operator who observed the free induction decay. A digital resolution of 0.63 and a LB of 2.0 Hz gives an overall resolution of 1.26 Hz compared to 2.93 Hz used by Fossel.

Analyses of NMR spectra were obtained as follows: Expanded plots of 10 Hz per cm with the CH_2 peak approximately 20 cm high were made of the CH_2/CH_3 region. Baselines and line widths were obtained as in Fossel's procedure (1). The average line width ($\Delta\nu_{1/2}$) was obtained as the average of the widths at half-height of the CH_2 and CH_3 resonances centered near 1.3 and 0.88 parts per million (ppm), respectively.

Preparation of Plasma for GPC and NMR Analysis:

The whole blood (7-10 ml) was collected into lavender top, evacuated glass tubes containing EDTA and immediately placed on ice until processing. The plasma was prepared for analysis by centrifugation of the whole blood at 3,000 x g (5,000 rpm) for 20 minutes at 0-4°C in a Beckman J-21B centrifuge using a JA-34 rotor. The clear yellow supernatant (plasma) was separated from the red blood cells using a pasteur pipet and stored at 4°C. (Discolored (red) plasma was discarded because the presence of lysed cells was shown to interfere with both GPC and NMR tests.) NMR and GPC analyses were performed within 24 hours of collection.

Patient Selection:

Blood samples were collected from normal employee volunteers during routine periodic health evaluations at three locations, Frederick Cancer Research Facility (FCRF), Frederick, MD; National Institutes of Health (NIH), Bethesda, MD; National Cancer Institute (NCI). Blood was obtained from cancer patients under treatment for a variety of different malignancies at FCRF and NCI. Informed consent was obtained from all participants who completed a short data sheet providing information on recent diet, smoking, medications, day in menstrual cycle for women, and general medical history.

RESULTS AND DISCUSSION

Chromatograms obtained from the GPC separations of plasma from a healthy individual and a treated breast cancer patient are shown in Figures 1A and 1B, respectively. These profiles are typical of the results obtained from a selection of normal individuals and patients with a variety of malignant conditions. The peak eluting at a retention time of approximately 6 minutes represents the high molecular weight (>10^6 Daltons) plasma lipoprotein particles and aggregates. The large peak eluting at a retention

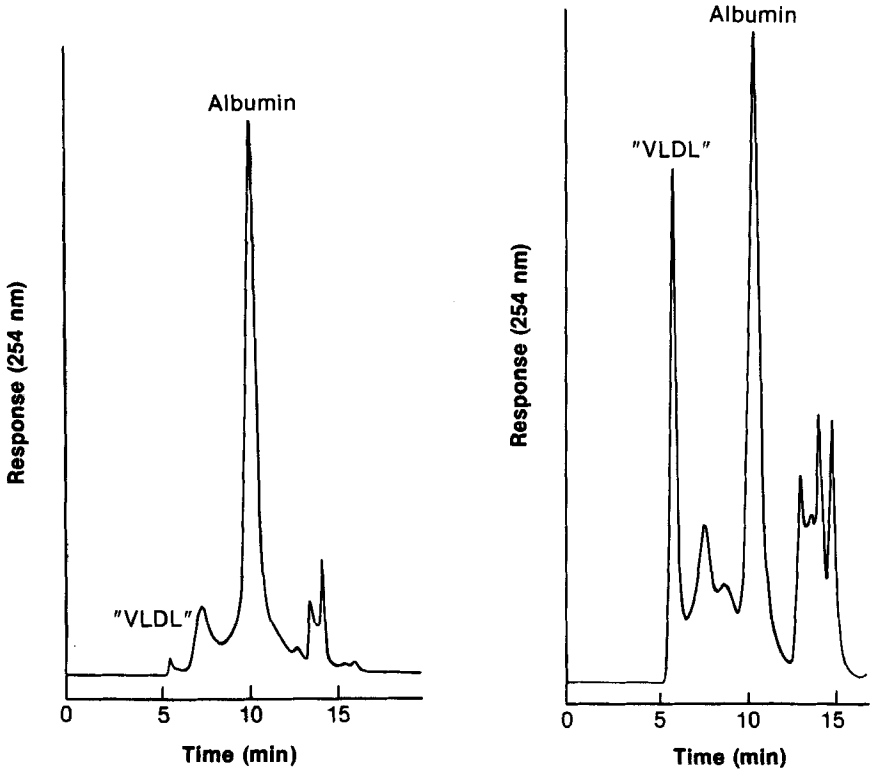

FIGURE 1

A Typical GPC Profile from Plasma of a Normal Individual, (experimental details in text).

B Typical GPC Profile from Cancer Plasma of a Breast Cancer Patient Who Had Been Treated, (experimental details in text).

time of approximately 11 minutes is consistent with albumin, a major constituent of human plasma. The major difference between the two chromatograms in Figures 1A and 1B is the increase in the peak eluting at 6 minutes found in the majority of cancer patients.

This high molecular weight peak which elutes in the void volume (retention time = 6 minutes) was shown to contain VLDL, based on comparisons with the elution profiles of Sigma Standards: VLDL, LDL and HDL (Figure

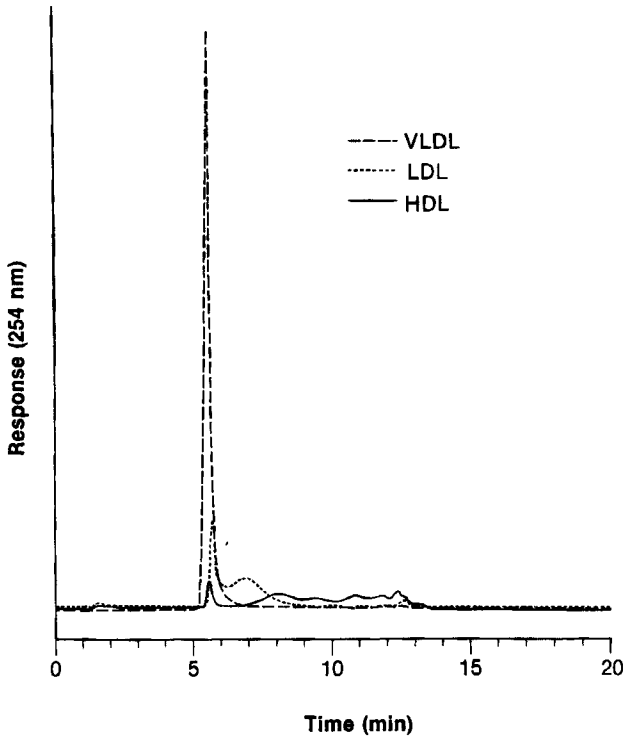

FIGURE 2

GPC Profile of Sigma Standards from Human Plasma, (experimental details in text).

2). Interference with the area of this peak from chylomicrons was minimal because the plasma was obtained from individuals who had fasted for 12 hours. This peak may also contain other high molecular weight material and therefore we shall designate it in the subsequent discussion as "VLDL". The increase in the area of this peak in cancer patients correlates with the increase in triglyceride content of their sera. It is, therefore, reasonable to conclude that the increase in peak area is due to increased VLDL content of the plasma. This is in agreement with data found by Barclay and Skipski (7) as discussed in the introduction.

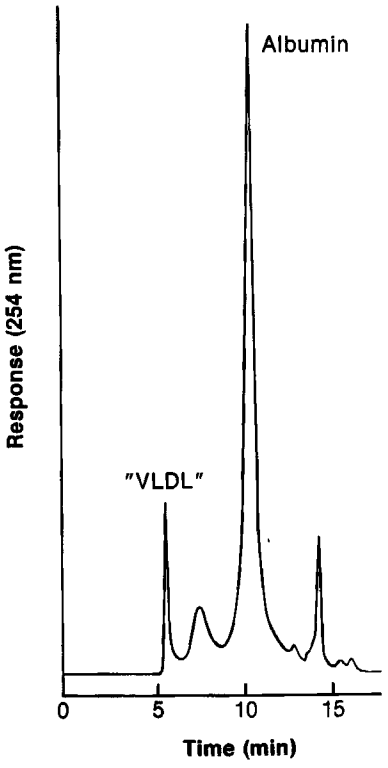

FIGURE 3

Typical GPC Profile from Plasma of a Normal Individual with High Triglycerides, (experimental details in text).

Normal individuals with triglyceride levels greater than 200 mg gave elution profiles similar to those of cancer patients. This can be seen by comparing Figure 3 with Figure 1B. In the normal control group, it was observed that there is a correlation between "VLDL" and triglyceride concentrations. These results are summarized in Figure 4 which shows a plot of "VLDL" areas vs. triglyceride concentrations. This correlation is not surprising since 55% of VLDL is triglyceride (13).

An additional peak with a molecular weight of approximately 400,000 was observed in the plasma of cancer patients when the column effluent was

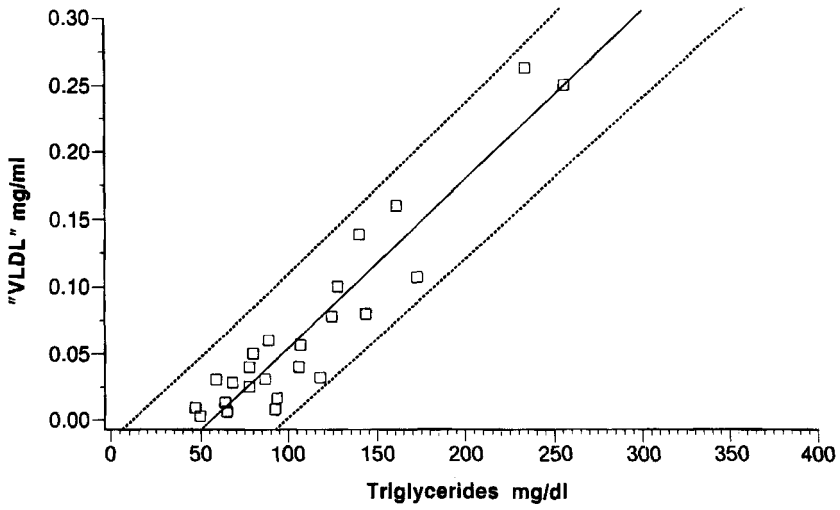

FIGURE 4

Plot of "VLDL" Concentrations (mg/ml of Protein Obtained from Sigma Standard VLDL #L-2264) Versus Total Triglyceride Concentrations Obtained from Clinical Blood Analysis.

monitored at 414 nm. It was absent from the chromatograms of most normal individuals (Figure 5A and 5B). The significance of this peak as a possible tumor marker is under investigation. There appears to be an interference at the same retention time if the blood is lysed on collection.

The basis of the Fossel test (1) is that the NMR spectra for cancer patients exhibit an average line width at half height for CH_2 and CH_3 peaks that is narrower than those from individuals without cancer. In the NMR spectra of a typical triglyceride, tripalmitin (Figure 6A), a narrow average line width at half height is observed for the CH_2 peak because a large number of CH_2 groups occupy similar environments and for the CH_3 peak because the three CH_3 groups are in nearly identical environments. The ratio of peak heights (and areas) for the NMR signals from CH_2 protons to CH_3 protons is also large. The NMR spectrum of cholesterol, the other major

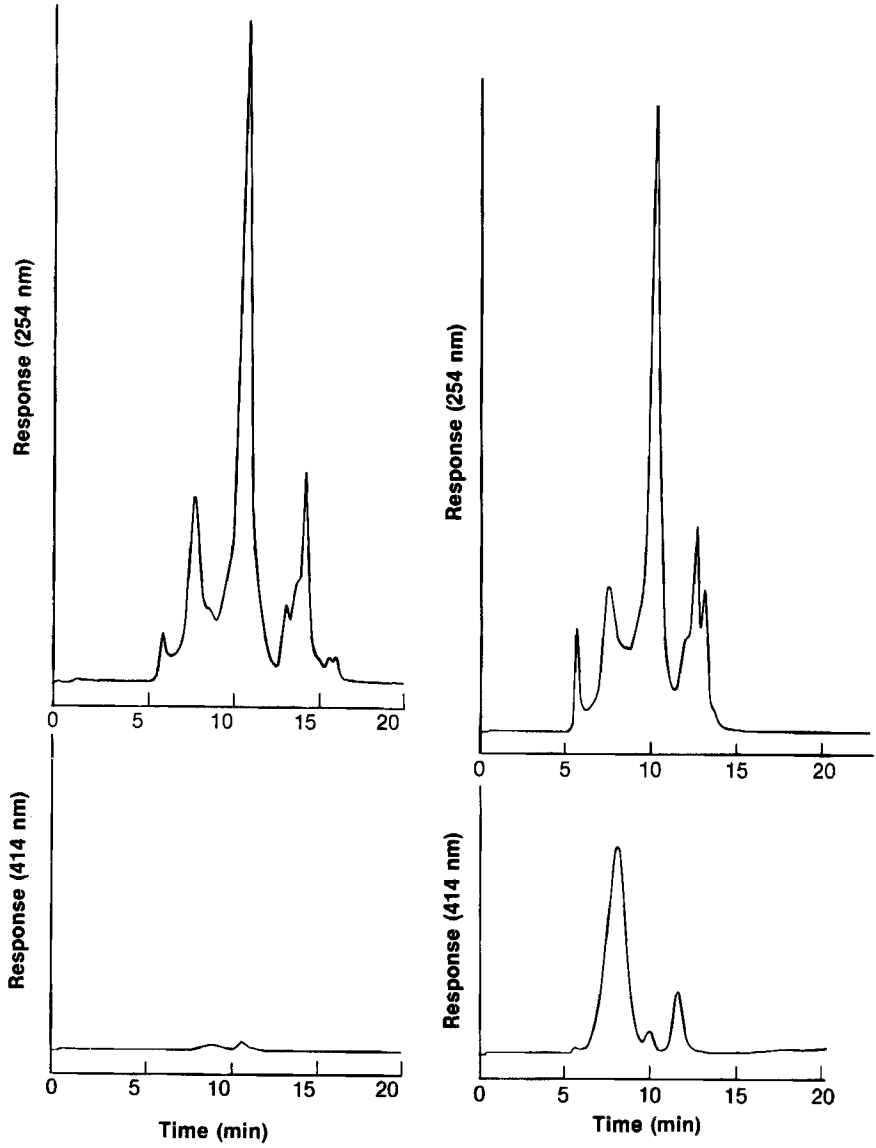

FIGURE 5

A GPC Elution Profile from Plasma of a Normal Individual Monitored at 254 and 414 nm, (experimental details in text).

B GPC Elution Profile from Plasma of a Treated Lung Cancer Patient Monitored at 254 and 414 nm, (experimental details in text).

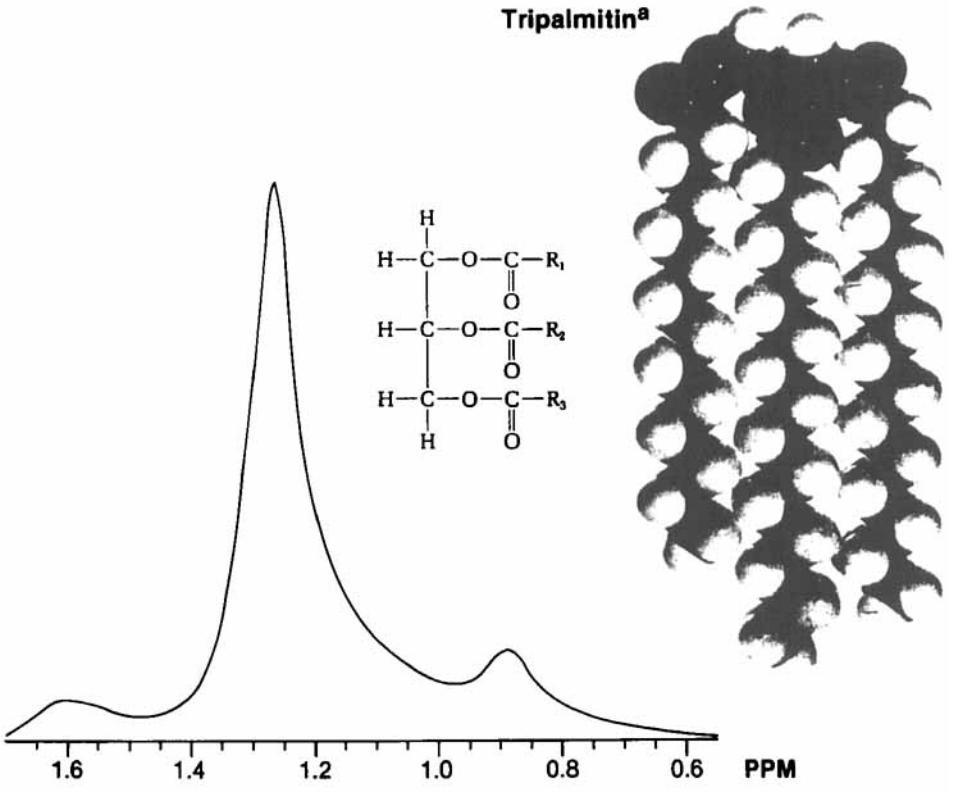

FIGURE 6A

NMR Spectrum (LB = 15 Hz) of the CH_2 and CH_3 Regions of Tripalmitin in $CDCl_3$ a) Structures from Lehninger "Biochemistry", Worth Publishers, Inc. 1970, p. 193.

component in lipoproteins that is NMR sensitive, (Figure 6B) demonstrates the heterogeneous environments for the proton resonances that occur in the 0.88 ppm and 1.3 ppm regions of the spectrum. Long chain esters of cholesterol will have spectra which are a sum of cholesterol plus triglycerides. Therefore, if a lipoprotein particle has a larger percentage of triglycerides, it would have an NMR spectra with a narrow average width at

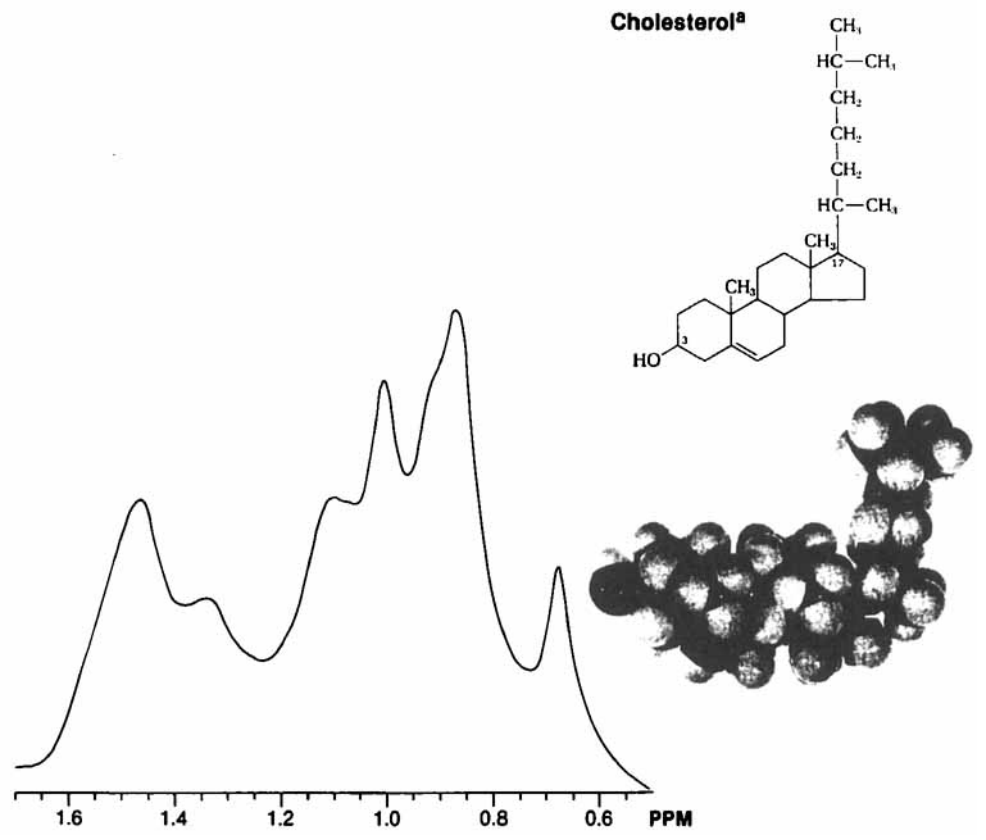

FIGURE 6B

NMR Spectrum (LB = 15 Hz) of the CH_2 and CH_3 Regions of Cholesterol in $CDCl_3$ a) Structures from Lehninger "Biochemistry", Worth Publishers, Inc., 1970, p. 202.

half height for the CH_2 and CH_3 signals. The NMR spectra of the Sigma Standards, VLDL, LDL and HDL are shown in Figure 7 for comparison.

The results obtained from the above GPC study were compared to those obtained by the NMR analysis of the same plasma samples to see if this inexpensive HPLC procedure can be used as an alternative or complement to the NMR procedure proposed by Fossel and co-workers (1). The results of the

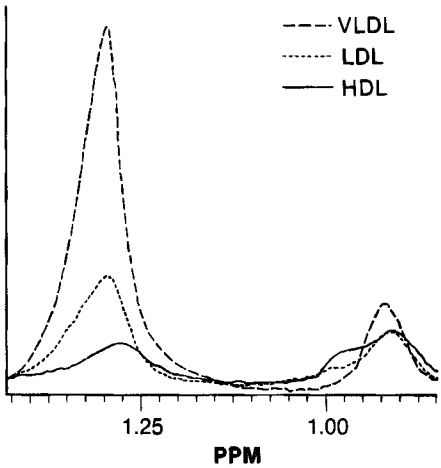

FIGURE 7

NMR Spectra of Sigma Standards from Human Plasma, (experimental details in text).

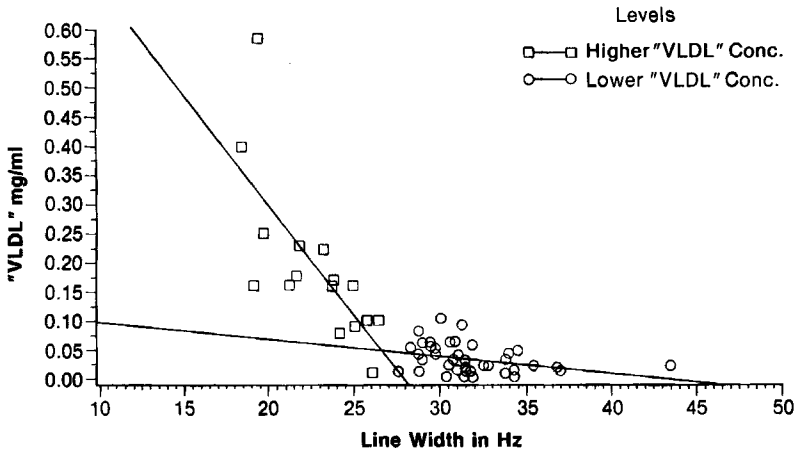

FIGURE 8

Plot of "VLDL" Concentration Obtained by GPC (see Figure 4) Versus NMR Line Width.

TABLE 1

STATISTICAL COMPARISON BETWEEN NMR AND HPLC DATA

VARIABLE	N	MEAN	STD DEV	MEDIAN	MINIMUM	MAXIMUM
Hz	177	28.0022	4.5590	28.8000	18.5000	43.5000
"VLDL" (mg/ml)	177	0.1458	0.2341	0.0630	0.0010	1.7300

CORRELATIONS BETWEEN NMR AND HPLC DATA

	CORRELATION COEFFICIENT	P<n
PEARSON	-0.52763	0.0001
SPEARMAN	-0.74750	0.0001
KENDALL TAU B	-0.55882	0.0001
HOEFFDING DEPENDENCE COEFFICIENTS	0.24495	-

comparison between GPC and NMR are illustrated in Figure 8 which shows a plot of "VLDL" concentration obtained from the GPC vs NMR line widths. It can be seen from the plot that a biphasic relationship exists between NMR line widths and "VLDL" concentration. Figure 9A and 9B show the NMR and GPC analysis of a healthy individual and a cancer patient, respectively. It is proposed that labs without 300-500 MHz NMR capability could use this GPC procedure as an alternative to the NMR test (1).

If either test is to be successful as a screening test for an asymptomatic population, at least a correction will have to be found for the effects of triglyceride levels. The use of these tests to follow cancer therapy is being evaluated. Our laboratories have evaluated the precision and accuracy of these tests; the results of these evaluations will be presented at a later date (14).

A theoretical difficulty facing a "general" marker is the unlikelihood of any one biochemical parameter correctly characterizing all neoplastic cell proliferations while excluding all non-neoplastic ones. Practical

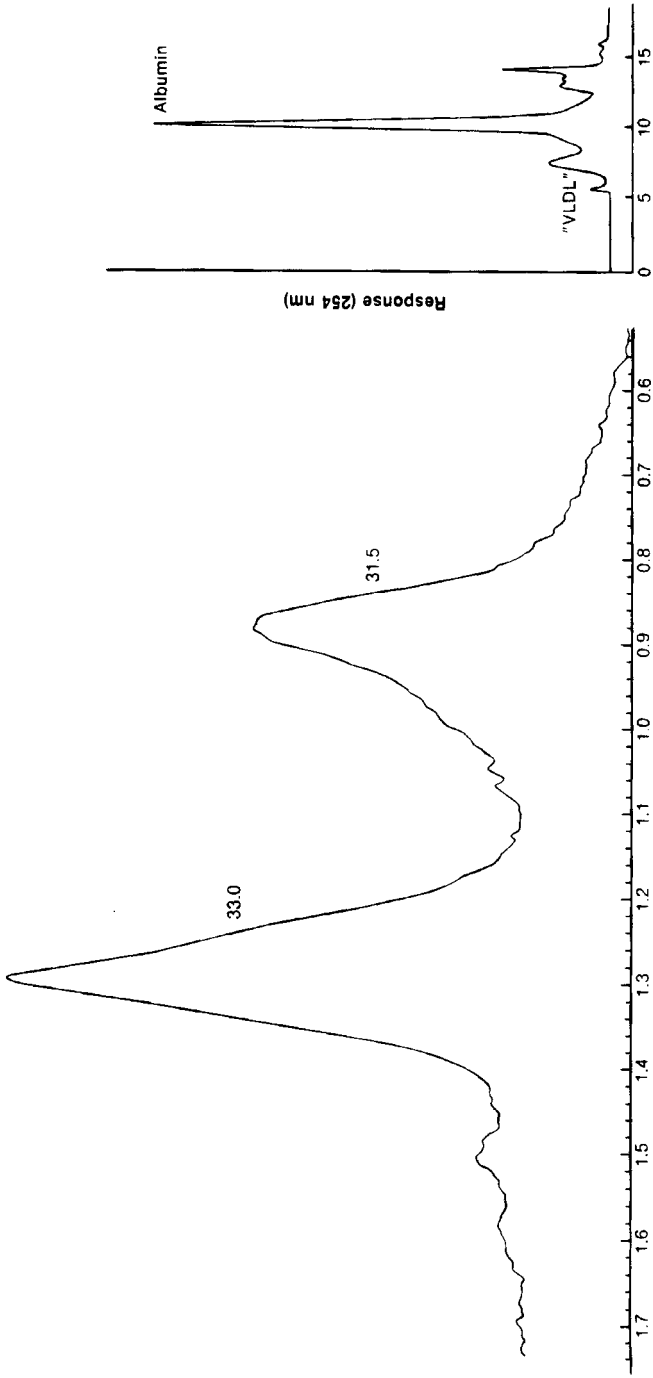

FIGURE 9A NMR and GPC of Plasma from a Normal Individual, (experimental details in text).

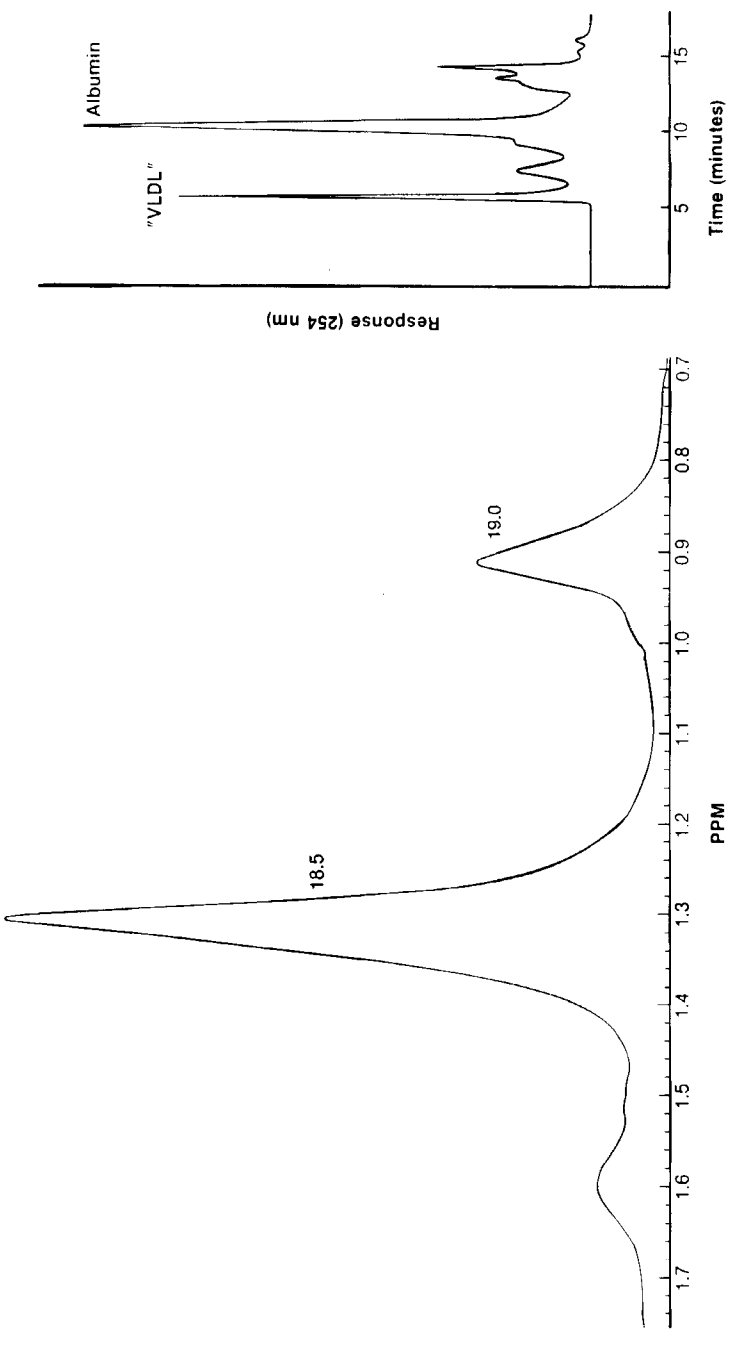

FIGURE 9B NMR and GPC of Plasma from a Cancer Patient, (experimental details in text).

challenges include cost-effectiveness and the difficulty of applying even a highly specific test to the general population because of a high number of false positives. These difficulties should not dissuade investigators since new technologies can be expected to improve assays and create new, highly targeted procedures for detecting genetic pathology on the molecular level. Even if assays do not achieve immediate clinical utility, markers may serve to shed light on mechanisms of neoplastic transformation or promotion, and thus advance our understanding of carcinogenesis.

CONCLUSION

The areas of the GPC peak "VLDL" from plasma, monitored at 254 nm, were shown to have a biphasic relationship with the NMR line widths obtained from the technique described by Fossel (1). Both GPC and NMR tests show good correlations (Table 1) with total triglyceride levels from normal individuals obtained from routine blood analysis.

These tests indicate an increase in VLDL in the plasma of cancer patients compared to that of normal individuals with triglycerides less than 200 mg/ml. This is in agreement with studies by Barclay and Skipski in the 1970's (6,7). It is not clear if other disease states will contribute to such GPC profiles or narrow NMR line widths.

ACKNOWLEDGEMENT

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REFERENCES

1. Fossel, E.T., Carr, J.M. and McDonagh, J., New Eng. J. Med. 315, 1369 (1986).

2. McIntire, K.R., Hospital Practice 19, 55 (1984).

3. Biological Markers in Neoplasia: Basic and Applied Aspects, R.W. Ruddon, editor, Elsevier, NY, 1978.

4. Chu, T.M., Morphological Tumor Markers, Ed. G. Seifert, Springer-Verlag, Berlin, 19-45 (1987).

5. Barclay, M. and Skipski, V.P., Prog. Biochem. Pharmacol. 10, 97 (1975).

6. Barclay, M. and Skipski, V.P., Prog. Biochem. Pharmacol. 10, 76 (1975).

7. Bucovaz, E.T., Private Communication, Sept. 1986. Also Business Week, Sept. 14, 167 (1987).

8. Lynn, W.R., Macleod, R.M., Morrison, J.C., Whybrew, W.D. and Bucovaz, E.T., Biochem. Biophys. Methods 12, 57 (1986).

9. Society of Magnetic Resonance in Medicine, Sixth Annual Meeting and Exhibition, August 1987, New York City, NY, G.N. Chmurny, et al, Abstract 28; O. Sezer, et al, Abstract 29; S.D. Buchthal, et al, Abstract 30; W.B. Mackinnon, et al, Abstract 58; G. Williams, et al, Abstract 59; K. Bjorq et al, Abstract 60; I.M. Armitage, et al, Abstract 61 and S.D. Otvos, et al, Abstract 62, P. Wilding, Clinical Chemistry, in press.

10. Okazaki, M., Ohno, Y. and Hara, I., J. Chromatogr., 221, 257 (1980).

11. Okazaki, M., Sharaishi, K., Ohno, Y., and Hara, I., J. Chromatogr., 223, 285 (1981).

12. Clifton, P.M., Mackinnon, A.M. and Barter, P.J., J. Chromatogr., 414, 25 (1987).

13. "Biochemistry", 2nd Ed. N. V. Bhagavan, J. B. Lippincott Company, Philadelphia, PA, p. 936 (1978).

14. Chmurny, G.N., et al, paper in progress and Smith, I.C.P., et al, paper in progress.