Determination of Cholesterol Using a Novel Magnetohydrodynamic Acoustic-Resonance Near-IR (MARNIR) Spectrometer

ROBERT G. BUICE, JR., and ROBERT A. LODDER*

Division of Medicinal Chemistry and Pharmaceutics, College of Pharmacy, University of Kentucky Medical Center, Lexington, Kentucky 40536-0082

Near-IR spectrometric determination of minor constituents of biological systems is complicated by the fact that near-IR spectra of these materials vary in different chemical and physical environments. In such cases, wavelength selection methods and full-spectral techniques such as partial least-squares and principal component regression (which weight each wavelength in calibration) produce excess error because they must attempt to model both variations in major constituents and variations in the analyte. A magnetohydrodynamic acoustic-resonance near-IR (MARNIR) spectrometer can determine major constituents of biological materials noninvasively and nondestructively, leaving the near-IR spectrum of the analyte to be used quantitatively with less prediction error.

Index Headings: Ultrasonic; Blood analysis; Hydrogen bonding.

INTRODUCTION

Epidemiological studies have shown that reduction of blood cholesterol levels decreases the risk of atherosclerosis, ischemia, and myocardial infarction. It has been shown that a 1% reduction in plasma cholesterol leads to a 2% reduction in cardiac events for individuals at risk for heart disease.[1] A target level for total blood cholesterol of 185-200 mg/dL has been established for healthy individuals. This target level is below the national average serum cholesterol concentration. With many Americans at risk for cardiovascular disease, widespread screening of the population could be instrumental in lowering escalating medical expenditures in the United States. However, the tests currently available for cholesterol have several disadvantages. The tests are invasive and cause discomfort to the patient, which usually decreases willingness to submit to regular cholesterol screening. Many tests are also expensive, and some tests for mass screening have been shown to be inaccurate. A 1985 study showed that 47% of 5000 laboratories volunteering for the study could not get a test result within 5% of the true cholesterol value of a standard sample.[2] This error is significant since the clinical risk brackets are only 10% wide.[3] The development of an accurate noninvasive assay for cholesterol in all its forms would be of great benefit in preventive medicine.

It appears possible to develop noninvasive near-IR cholesterol assays. A near-IR technique has been developed to create false-color images of cholesterol in developing atheromas in rats.[4] Near-IR spectroscopy has been successfully used to determine cholesterol in serum samples acquired from patients.[5] The determination of cholesterol by near-IR spectrometry presents several problems, the most important arising from the fact that the near-IR spectrum of an analyte is dependent on the environment of the analyte. Figure 1 is a normal probability (NP) plot of the ordered residuals from a robust fit line correlating the near-IR spectra of 162 human serum samples to total cholesterol concentration. If cholesterol concentration were the major factor influencing the spectra used to develop the robust model, the residuals should be randomly distributed, giving a straight line in the NP plot.[5] However, three distinct linear segments are visible in the NP plot, indicating three separate distributions in the spectra with different means and standard deviations. The three distributions suggest that three different robust models should be used to determine cholesterol spectrometrically. t-tests were performed to determine the probability of the groups indicated in Fig. 1 having the same level of certain background constituents. The results of these t-tests are shown in Table I. The most prominent feature in Table I is that the level of sodium differs greatly between the residual groups. The levels of albumin, protein, glucose, and triglycerides also differ among the cholesterol residual groups, but none to the extent of sodium. Therefore, varying sodium ion concentration constitutes a major source of error in the near-IR determination of cholesterol.

If the levels of all background constituents are known, a far more accurate near-IR determination of cholesterol could be made. It is possible to determine sodium, protein, triglycerides, albumin, and glucose through invasive and time-consuming procedures, but the goal is a rapid noninvasive analysis. The development of the magnetohydrodynamic acoustic resonance near-infrared (MARNIR) spectrometer is an important step in the direction of noninvasive near-IR imaging and analysis.

The MARNIR spectrometer collects three spectra simultaneously: a near-IR spectrum, an acoustic-resonance (AR) spectrum, and a magnetohydrodynamic (MHD) spectrum. In combination these three spectra give information about specific chemical interactions, bulk properties, and ionic properties of a sample. MARNIR spectrometry allows the simultaneous determination of cholesterol and the background constituents that cause errors in the near-IR calibration curve.

Principal component analysis of typical near-IR spectra shows that only the first 15 to 20 factors contain useful information. Beyond 20 factors, a Wald-Wolfowitz runs test applied to the loading vectors shows that the com-

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*** Author to whom correspondence should be sent.**

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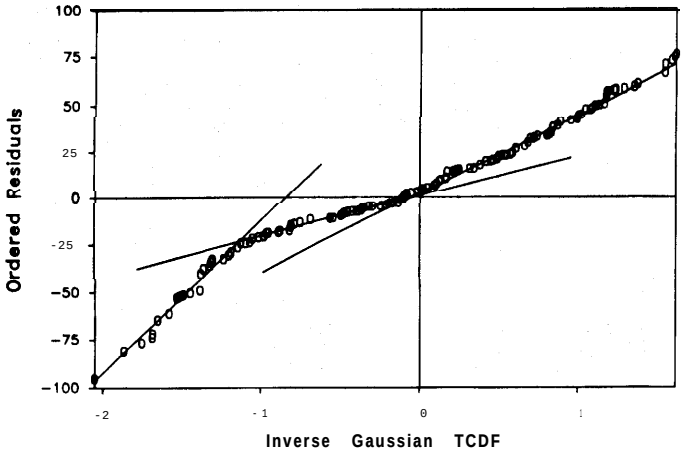

FIG. 1. Normal probability (NP) plot of the ordered residuals from a robust fit line correlating the near-IR spectra of 162 human serum samples to total cholesterol concentration.

ponents usually model merely random noise. The 20 useful factors correspond to the 20 largest sources of spectral variation in the sample, which often arise from the 20 components of the sample present in highest concentration. Most of the important constituents of biological materials (including cholesterol and lipoproteins) are not in the list of the 20 constituents present in tissue in the highest concentration. MARNIR analysis can be used to remove some large sources of near-IR spectral variation from the samples by allowing different calibration models to be created for different levels of a major sample constituent, such as aqueous sodium ion. The levels of the major component are determined from the AR and MHD spectra obtained simultaneously from the sample. Inside each calibration set one or more major constituent concentrations are held constant, freeing some factors to model components of interest and by that process producing a more accurate model.

EXPERIMENTAL

The near-IR monochromator used in the MARNIR spectrometer was a commercially available device. The acoustic-resonance spectrometer and the means for recording a magnetohydrodynamic spectrum, however, had to be constructed in the laboratory. Acoustic-resonance spectrometry (ARS) is primarily sensitive to the bulk properties of a sample (for example, density, viscosity, and major constituents that affect such bulk properties). ARS is based on the measurement of changes in acoustic wave velocity and acoustic wave absorbance in samples.[6] ARS has been used to differentiate among intact tablets of varying types and to predict the dissolution rate of carbamazepine tablets.[7] ARS has also been used to quantify oil and gas mixtures.[8] The magnetohydrodynamic effect is the generation of a current as ions move in a magnetic field. The magnitude of the MHD electrical signal is proportional to the ion concentration.

The MARNIR spectrometer is shown in Fig. 2. A Macintosh IIci personal computer (Apple Computer, Cupertino, CA) connected to an analog-to-digital converter (Acrosystems, Beverly, MA) drives a frequency generator (Extech Instruments, Waltham, MA). The sine wave created by the frequency generator is converted into a sonic

TABLE I. Probability[a] P[data H_o:$\mu_1 = \mu_2$] for cholesterol groups and various background constituents.

	Albumin		Sodium	
	Group 2	Group 3	Group 2	Group 3
Group 1	88	23	11	2
Group 2	—	27	—	1
	Triglycerides		Protein	
	Group 2	Group 3	Group 2	Group 3
Group 1	1	24	82	13
Group 2	—	45	—	23
	Glucose			
	Group 2	Group 3		
Group 1	5	63		
Group 2	—	19		

[a] Probability given as % by a two-tailed t-test.

wave by a piezoelectric film (Atochem Sensors, Norristown, PA). The acoustic wave is propagated down a quartz tube until it interacts with a sample placed at the vertex of the tube. The acoustic energy passes into the sample and back into the rod where it interferes with the original acoustic wave. A second transmitting film driven in phase with the first and located beneath the sample holder also can be used to launch an acoustic wave through the sample and into the tube. Resonance is achieved when a standing wave is established in this mechanical system. The resonance condition of the system is detected as acoustic intensity by a third piezoelectric film, which converts the acoustic energy to electrical energy. The resulting voltage is amplified and sent through rectifier and integrator circuits and returned to the A/D where it is collected on the computer for analysis.

When the input acoustic frequency is scanned from 0 to 60 kHz, an acoustic-resonance spectrum is obtained like the one shown in Fig. 3. The peaks represent resonances of the system, which are either enhanced or attenuated by interactions with the sample placed at the vertex of the quartz tube.

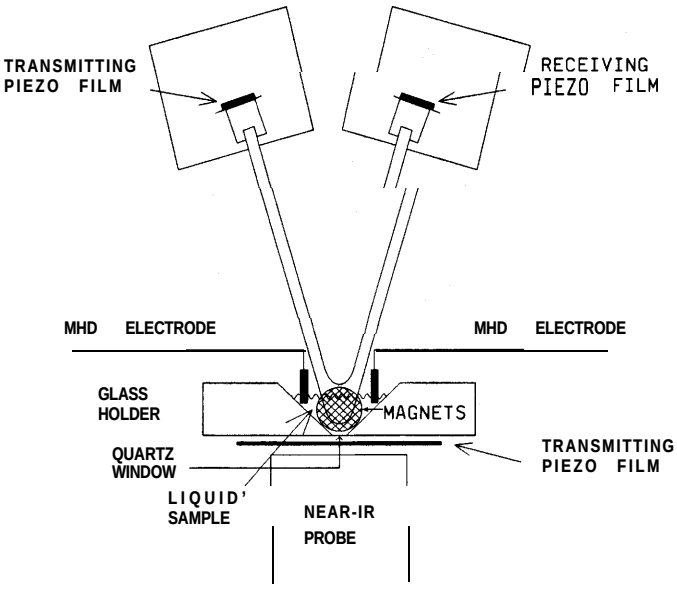

FIG. 2. Diagram of the sample compartment in the magnetohydrodynamic acoustic-resonance near-infrared (MARNIR) spectrometer.

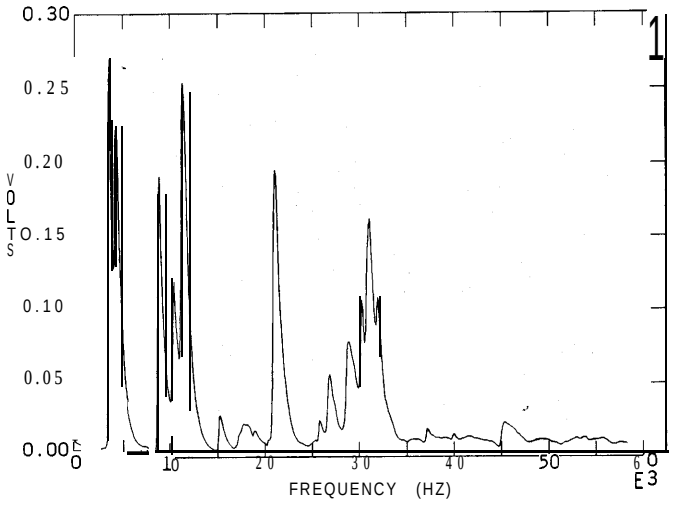

FIG. 3. **A representative acoustic-resonance spectrum of a standard cholesterol sample.**

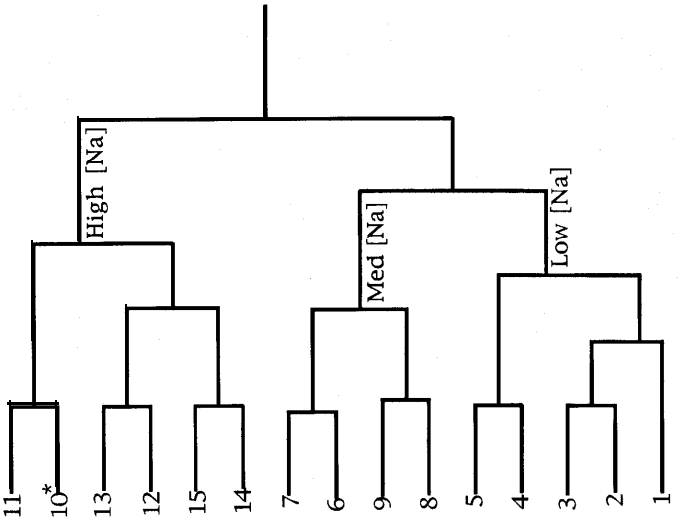

FIG. 4. **A complete-linkage dendrogram showing three groups of five spectra for 15 samples prepared with 5 cholesterol concentrations and 3 sodium ion concentrations. This grouping indicates that it is sodium concentration that dominates the clustering of cholesterol samples, not cholesterol concentration.**

When the input acoustic wave interacts with a liquid sample placed at the vertex of the quartz tube, the acoustic wave causes the ions in the solution to oscillate at the frequency of the acoustic wave. The movement of the ions in a magnetic field causes current flow in the leads of electrodes placed in the sample cell. The MHD effect is used to find the ionic strength of the sample, which is valuable because ions in solution perturb the water bands of the near-IR spectrum through their effect on hydrogen bonding. The inability of simple near-IR spectrometry to model simultaneously changes in large numbers of these effects creates errors in the near-IR determination of cholesterol.

Data collection was performed on a Macintosh IIci using software written in BASIC (Microsoft, Redman, WA). Data analysis was performed on an IBM 3090 6005 vector supercomputer using software written in Speakeasy (Speakeasy Computing, Chicago, IL) and on a Macintosh IIci using software written in FORTRAN (Language Systems, Herndon, VA).

Three sets of cholesterol standards were prepared to test the hypothesis that the MARNIR principle improves near-IR determination of cholesterol. A cholesterol standard of 5.0 mg/mL in 2-propanol (Sigma, St. Louis, MO) was diluted with water and 2-propanol to give O.O-, 0.5, l.O-, 1.5, 2.0-, 3.0-, 4.0-mg/mL solutions of cholesterol with a consistent ratio of 2-propanol to water. (Each of these solutions contained 20% water.) Each cholesterol standard was prepared at three sodium concentrations: 0.00, 0.8752, and 1.75 mg/mL. The cholesterol concentrations approximated physiological levels, while the maximum sodium level was only 23 % of the typical physiological concentration (to prevent the "salting out" of the cholesterol).

RESULTS AND DISCUSSION

The standards were all scanned by MARNIR spectrometry; however, each component spectrum was obtained individually instead of simultaneously because only one small-signal op amp had been completed (one amplifier is required for the ARS signal and one for the MHD signal). The acoustic-resonance spectrum showed no correlation to cholesterol concentration. This poor correlation was not unusual because ARS is sensitive to bulk properties determined by the 2-propanol and water concentrations, which were held constant.

The near-IR spectra of the cholesterol samples were treated with multiplicative scatter correction to eliminate a small baseline shift. The spectra obtained at 351 near-IR wavelengths were then projected as points in 351-dimensional space. A complete-linkage cluster analysis performed with the use of the Euclidean distances between the spectral points produced the dendrogram in Fig. 4. Complete-linkage cluster analysis was selected because it attempts to connect points together in the dendrogram (or tree) that lie in the same hypersphere in hyperspace. In addition, complete-linkage cluster analysis requires no *a* priori information about the samples, so the tree reflects the largest differences and similarities among the samples. Figure 4 shows that the tree has three major branches that correspond to the three sodium concentrations. (The cluster analysis misclassified sample #10 as a high sodium sample though it was a medium sodium concentration. Occasional errors were expected because Table I suggested some overlaps between the sodium groups.) The most important feature of Fig. 4 is that the dendrogram shows three groups of five spectra. This grouping indicates that sodium concentration dominates the clustering of cholesterol samples, not cholesterol concentration. If cholesterol concentration were the dominant force in determining the clustering in the near-IR spectra, then five groups of three spectra would have been observed. The dendrogram shows that the sodium concentration must be known to calculate an accurate near-IR calibration for cholesterol.

The samples were sorted into categories based on the magnitude of the magnetohydrodynamic effect observed at an acoustic input frequency of 6 kHz. The low sodium concentrations produced a mean MHD signal of 0.8 V. The medium sodium concentration produced a voltage

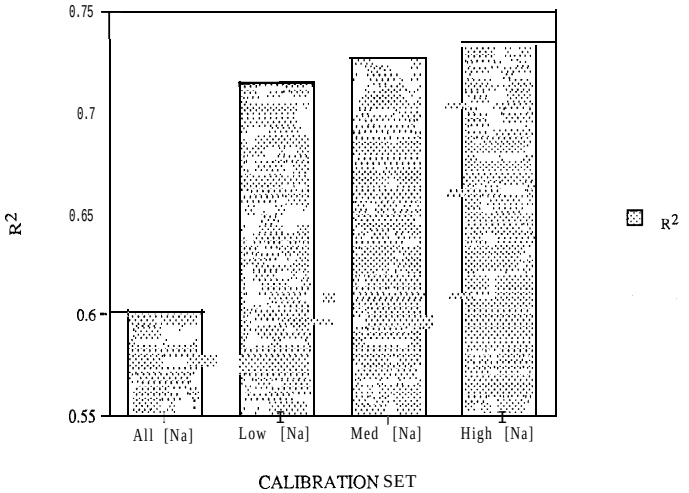

FIG. 5. **The r^2 of the PCR models increases when the sodium concentration is known. The highest correlations are observed at the highest sodium concentrations because the signal-to-noise ratio improves in the MHD signal from the prototype spectrometer. All four PCR models passed cross-validation with an F-test (P = 0.05).**

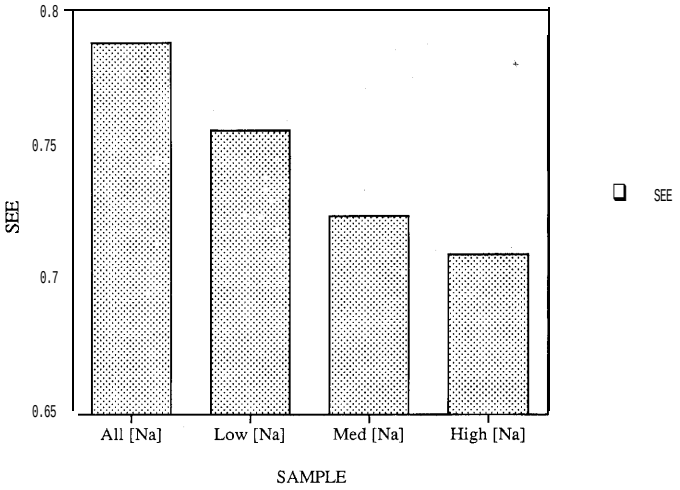

FIG. 6. **The standard error of estimate (SEE) decreases when the sodium concentration is known. The lowest errors are observed at the highest sodium concentrations because the MHD signal was noisy in the prototype spectrometer. All four PCR models passed cross-validation with an F-test (P = 0.05).**

of 0.4 V, and the high sodium concentration a voltage of 0.6 V. The low sodium concentrations were actually 0 mg/mL sodium, so the electrode in the sample cell acted as a bare antenna that picked up the rf emanating from the unshielded piezoelectric transmitter. When sodium ion was present, the electrode circuit was effectively closed and the MHD voltage was proportional to the sodium ion concentration.

To perform leave-one-out cross validation, we added samples with 0.5, l.O-, 2.0-, and 1.5-mg/mL cholesterol concentrations to the calibration set. Principal component regression (PCR) using the three components with the highest t-statistics was performed on all 27 sample near-IR spectra. The results are shown in the first column (all [Na]) in Figs. **5 and 6.**

PCR was also used to form three models based on the groups determined by the magnitude of the magnetohydrodynamic effect. Figure 5 shows the increasing r^2 of the models when the sodium concentration is known. Figure 6 shows the decreasing standard error of estimate (SEE) when the sodium concentration is known. The lowest errors appear at the highest sodium concentrations because the MHD signal was noisy in the prototype spectrometer. All four PCR models passed cross-validation with an F-test (P = 0.05), demonstrating that the improved performance is not merely a random effect of the sample number.

The standard samples were prepared with the use of cholesterol in 2-propanol and water, and the concentration of sodium in the sample had to be kept low to prevent precipitation. In humans cholesterol is found in lipoproteins that are water soluble, and the sodium concentration is approximately 7.7 mg/mL,[9] which should greatly increase the magnitude of the MHD effect and improve the results shown in Figs. 5 and 6.

CONCLUSION

The results show that it is possible to develop a near-IR assay for cholesterol, but the levels of background constituents also must be known for an accurate determination. MARNIR spectrometry allows the simultaneous acquisition of three spectra: acoustic-resonance, near-IR, and magnetohydrodynamic, which allow the determination of some background constituents in serum samples. The addition of lock-in amplification to the ARS and MHD op amps and complete shielding of the piezoelectric transducers should greatly improve the results by increasing the signal-to-noise ratio of the ARS and MHD signals. Work is currently underway to determine LDL by MARNIR spectrometry and to sort the samples into albumin groups as well as sodium groups for calibration and prediction. Albumin is expected to have an acoustic absorbance which is different from that of other constituents and should produce an identifiable effect in the acoustic-resonance spectrum.

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