The Measurement of Total Fatty Acid in Serum

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ABSTRACT

A review of the four most popular approaches to the measurement of total serum fatty acid is presented. Hydroxylamine, copper soap, and sulfophospho-vanillin methods are summarized briefly, while acidimetric techniques are considered in somewhat greater detail. The highly sensitive acidimetric method of Massion and Seligson is presented. In this technique, serum is saponified in alcoholic KOH. Mineral acid is added to precipitate the fatty acid moieties which are extracted into petroleum ether. Following solvent evaporation, a buffered solution of methyl red indicator is added. A shift of indicator color related to the concentration of fatty acid in the standards or the serum sample is produced and quantitated spectrophotometrically. The method is highly accurate, lacking interference, semi-micro, rapid, and versatile.

Until about 10 to 15 years ago, precise chemical measurements of the various components of serum lipid were relatively difficult. By contrast, the quantitation of total serum lipid by gravimetric means had been well established for over two decades. As cholesterol and phospholipid methods were improved and became more popular, the necessity for total lipid measurement waned. However, a complete lipid profile of the patient seemed to demand a total lipid value as long as triglyceride concentration was not provided.

With the advent of good triglyceride methodology, the need for the measurement of total serum lipid has been eclipsed. However, it is possible that an easily standardized, precise, rapid procedure which accurately reflects the total lipid content might still prove to be clinically useful. Fatty acid constitutes approximately 60 percent of the total serum lipid. One percent of the fatty acid is bound loosely to albumin, while triglycerides contain 25 percent, phospholipids contribute 20 percent and cholesterol esters are the source of the remaining 14 percent. “Free” cholesterol is the only lipid fraction not associated with a fatty acid group. Because of this wide distribution, the accurate measurement of total serum fatty acid is an excellent method of estimating total serum lipid.

Many approaches to the measurement of total fatty acid have been developed and are outlined by Henry.21 Four will be considered: hydroxylamine methods, copper soap methods, sulfophospho-vanillin methods and acidimetric methods.

Hydroxylamine Methods

Feigl's15 spot test for carboxylic acids and esters is the basis of the hydroxylamine approach. In this method, esters are warmed in an alkaline hydroxylamine media to produce hydroxamic acid, which forms a red to lavender complex with ferric ion in acid solution. Following the development of a quantitative measurement
of fatty acids in industrial oils,\textsuperscript{22} Bauer and Hirsch\textsuperscript{6,7} and Stem and Shapiro\textsuperscript{8,7} applied the method clinically. Though workable, major problems resulted from excessive turbidity in the presence of elevated cholesterol concentrations and instability of the final color owing to a competition for ferric ion between chromogen and water.\textsuperscript{19}

Many modifications have been suggested. These include optimization of the reaction conditions,\textsuperscript{33,35} adding an oxidant to destroy excess hydroxylamine and reduce fading of the final color,\textsuperscript{19} introducing nonaqueous reagents\textsuperscript{2,31} to obviate the anhydrous conditions needed to prevent cholesterol turbidity, eliminating the initial extraction step\textsuperscript{41} to increase ease and reduce the introduction of interfering solvent impurities, and modifying the extraction step\textsuperscript{9,18} so that a cholesterol assay could also be performed.

In spite of many improvements it has been shown\textsuperscript{21} that alcohols, lactones, carboxylic anhydrides, polyesters of polycarboxylic acids, acetic acid, citrates, oxalates, salicylates, and carotene\textsuperscript{20} will all interfere with the final color. In addition, many authors\textsuperscript{3,7,9,19,20,31,36} have demonstrated that different fatty acids will produce different molar absorptivities. This not only introduces a built-in variability of 4 to 10 percent,\textsuperscript{7,9} but also confounds the selection of a suitable standard.

Hydroxylamine methods were undoubtedly worthwhile at one time but have been largely superseded. The disadvantages to this approach include the confusing array of conditions promoted, the large number of interfering substances and the variability of the color reaction.

Copper Soap Methods

The copper soap methods were originated by Ayers\textsuperscript{4} in 1956 who measured the fatty acids in cosmetic and textile emulsions. The first clinical method\textsuperscript{24} applied the reaction to non-esterified fatty acids extracted from serum. The fatty acids were added to a copper nitrate-triethanolamine reagent and the resulting copper soaps transferred into chloroform to produce a color. The detection limit of the method was improved 200 fold with the introduction of diethylthiocarbamate\textsuperscript{18} and a further twelve-fold with diphenylcarbazide.\textsuperscript{26} Even greater sensitivity was achieved with bis-cyclohexanoneoxalylidihydrazone.\textsuperscript{23} Because of its extreme sensitivity, the method has been applied to the measurement of free fatty acids and the fatty acids contained on thin-layer chromatography plates. Because it is a technically difficult procedure which suffers from phospholipid interference, it has been applied only once to the measurement of total fatty acid.\textsuperscript{5}

Sulfo-Phospho-Vanillin Methods

The sulfo-phospho-vanillin methods were developed in Europe,\textsuperscript{12,36} and recently introduced into the American literature.\textsuperscript{16,37,34} The recent laboratory goal to perform all chemical determinations in a single tube has made this method a popular commercial product.

The method is initiated by a hot sulfuric acid digestion of serum and a red color is developed in phosphovanillin reagent.\textsuperscript{17} The theoretical basis of the reaction is poorly understood but has been recently examined by Knight and co-workers\textsuperscript{25} who attribute the color production to the interaction of phosphovanillin reagent with carbon to carbon double bonds. They suggest that concentrated H\textsubscript{2}SO\textsubscript{4} reacts at the unsaturated bond to produce a carbonium ion. The carbonium ion then reacts with the carbonyl group of vanillin to form a colored compound which is stabilized by resonance and absorbs maximally at 525 to 530 nm. The phosphoric acid reacts with vanillin to produce a phosphate ester which enhances the reactivity of the carbonyl group.
Although it is a pleasingly simple and rapid method with stable reagents, few interferences, small sample requirement and reproducible results (relative standard deviation = 3.5 percent), it has one serious fault owing to its dependence on the presence of double bonds. It has been observed that approximately 30 percent of the fatty acids in normal serum are saturated, 50 percent contain one double bond and 10 percent contain two. These amounts vary from 3 to 16 percent in the normal population. Because of this 16 percent variation, there is an automatic 16 percent uncertainty in any result in the normal range. In abnormal metabolic states such as diabetes, liver disease, and hypothyroidism, the variation is even larger.

The problem in standardization is severe. Since pure palmitic and stearic acids produce no color, they are obviously unsuitable. The two double bonds present in linoleic acid will produce a different degree of color from the single double bond of oleic acid and corn oil will not react the same as olive oil. Lyophilized serum standards are often used in a compromise attempt to provide useful standardization. Even if these serum standards are evaluated using gravimetric techniques, they cannot hope to represent the range of unsaturation seen in human serum.

Acidimetric Methods

The most satisfactory methods, in our opinion, are those based upon the hydrolytic liberation of fatty acids followed by titration with alkali.

The first successful method, devised by Stewart and White, involved saponification of the lipids contained in a Bloor extract of serum, followed by exact neutralization of the sodium hydroxide used in hydrolysis with hydrochloric acid. The mixture was boiled to liberate ketones and absorbed atmospheric CO₂. Finally, the fatty acids were titrated using phenolphthalein as an indicator. In addition to being technically demanding, small errors in neutralization could produce huge errors in the final result. Another approach to the problem of exact neutralization was to collect the soaps on a Gooch filter and then to remove the alkali with copious water washes. This method was further improved by Man & Gildea.

A slightly different principle was employed by Milroy in 1928. After extraction, saponification and neutralization, the fatty acids were re-extracted into petroleum ether. Nile blue dye was added and the color produced in the extract was compared visually to a series of graduated standards in a comparator block.

Three decades later, Dole developed a procedure for “free” fatty acids using the acidimetric approach. After extraction of serum, a two phase solution of organic extract and aqueous indicator solution was formed. Nitrogen was bubbled through to provide intimate mixing and to drive off absorbed CO₂. In 1959, Albrink modified this technique to measure total serum fatty acid.

Mosinger eliminated the problems of a two phase titration system in his method of measuring free fatty acid. After extraction and saponification, the fatty acids were removed into a solution of sodium barbital and phenol red in a mixture of heptane and ethanol. The resulting color was quantitated spectrophotometrically.

Employing the best features of the earlier works, Massion and Seligson developed a method for the measurement of total serum fatty acid. Serum was saponified without extraction and mineral acid added to precipitate the fatty acid moieties which were extracted into petroleum ether. Following solvent evaporation, a buffered methyl red indicator reagent was added. By judicious adjustment of the buffer a range of indicator shift could be produced which spanned the concentration of fatty acids.
acid present in the serum aliquot. The resulting color was quantitated spectrophotometrically.

**Methodology**

**REAGENTS**

*Potassium hydroxide, 33 percent* (stock solution). Ten grams of KOH are added to 20 ml of deionized water. The reagent is prepared fresh weekly.

*Potassium hydroxide, 2 percent in ethanol*. Six ml of the stock solution of 33 percent KOH is added to 94 ml of absolute ethanol. The reagent is prepared fresh daily.

*Hydrochloric acid, (1.8 N)*. Seventy-five ml of concentrated hydrochloric acid is added to 425 ml of deionized water.

*Petroleum ether (B.P. 39 to 54°C)*. Redistilled.

*Methyl red, (0.2 percent)*. (Fisher Scientific Co., No. M-219). Two hundred mg of powdered methyl red are dissolved in 100 ml of 95 percent ethanol. Solution is slow, occurring in 2 to 3 days unless accelerated by constant stirring. The final concentration is adjusted photometrically.

*Ethanol, (95 percent)*. Reagent grade.

*Sodium acetate, (1 M)*. Thirteen and six-tenths grams of sodium acetate are dissolved in 100 ml of deionized water.

*Sodium hydroxide, (1 N)*. Methyl red indicator solution (buffered). Variations in dye lots are minimized by photometrically controlled preparation. One liter of 95 percent ethanol is made alkaline by adding 10 ml of 1 N sodium hydroxide. Enough 0.2 percent methyl red solution is added to bring absorbance to between 0.095 and 0.100 (usually 10 to 13 ml) at a wavelength of 500 nm. Two (2.0) ml of 1 M sodium acetate is added. One normal hydrochloric acid is added dropwise with frequent mixing or constant stirring until a faint red-orange tinge persists. Additional acid is added carefully until the solution has an absorbance of 0.200 ± 0.005. If this point is passed, dilute sodium hydroxide is added. Excessive dilution with aqueous solutions should be avoided. The indicator solution is now at a balanced point near the anion end of the titration curve and will respond to minute increments of fatty acids. The color of this reagent may fade slightly in the first few hours but will remain unchanged for at least a month if stored in a brown bottle or in the dark. Eventually the solution will bleach and sensitivity will be lost. The shape of the standard curve obtained by addition of fatty acids can be adjusted by varying the concentration of sodium acetate buffer. More acetate will flatten the curve; less will steepen the slope. Increased amounts of methyl red will raise the blank absorbance and steepen the slope of a standard curve.

*Standard solution of tripalmitin*. Purified tripalmitin (215 mg) is dissolved in 200 ml of redistilled heptane (petroleum ether is usable but is less desirable because of its lower boiling point) to make a solution of 4.00 μEq per ml. The standard curve is set up to read directly in μEq per ml or mEq per liter of serum. Carefully measured portions of standards are measured to be equivalent to 40, 30, 20, 10 and 5 μEq per ml of serum by pipetting 2.00, 1.50, 1.00, 0.50 and 0.25 ml, respectively, of the solution into 15 x 150 mm glass culture tubes with a screw top. The solvent is evaporated by a stream of filtered air, leaving the standard as a dry, white residue. When capped, these are stable for at least two months, so a number of sets can be made at once. These standards are processed with 0.2 ml of water added as a serum substitute.

**Procedure**

Two-tenths ml of serum is pipetted into a screw-top glass culture tube and 2 ml of alcoholic potassium hydroxide are added.
A teflon-lined cap is applied tightly and the tube is heated for one hour in an 80°C water bath. The tube is uncapped, and 2.0 ml of water are added. After recapping, it is heated again for 10 minutes.

The 1.8 N hydrochloric acid (0.60 ml) is added and the solution mixed by inversion. After cooling to room temperature, 4.00 ml of petroleum ether are added and the tubes recapped and shaken vigorously for 2 minutes. Traces of mineral acid in aqueous droplets are removed from suspension in the petroleum ether by centrifugation at 2,000 rpm for five minutes.

Two ml of the petroleum ether supernatant are transferred to another culture tube and the solvent evaporated by a gentle stream of air. This can be hastened by warming in a 50 to 60°C water bath.

Three ml of the methyl red indicator reagent are added and the tubes are capped tightly before shaking vigorously for 1 to 2 minutes. Additional shaking may be necessary for large residues.

The absorbance of the solution is read in a spectrophotometer set at 502 nm wavelength with 95 percent ethanol as a blank. The standard curve is obtained by plotting absorbance against concentration on rectilinear graph paper. The concentrations of fatty acids in samples are read from the curve in μEq per ml or mEq per l. The very few abnormal sera that contain more than 40 mEq per liter can be diluted with saline and rerun, but it is easier to dilute the end mixture of fatty acid and methyl red indicator reagent with additional volumes of reagent until readable on the standard curve. This is possible since no blank value obtained from hydrolysis of reagents and redistilled petroleum ether.

**Calculation**

Since the standards produce a curve that does not follow Beer's Law, concentrations of samples cannot be calculated from a single standard and must be read directly from the curve.

**Sources of Error**

**Mineral Acid Contamination**

Extreme caution must be exercised to avoid the aqueous interface at the time of transfer of the aliquot of fatty acid in petroleum ether.

**Incomplete Solution of Residue Into Methyl Red Reagent**

Shake until all flakes of residue are dissolved.

**Normal Range**

Adults: 8 to 18 mEq per liter.
Children: No information available.

**Discussion**

The advantages to the described procedure are as follows:

1. It is relatively accurate since the extraction of fatty acid is nearly complete, while at the same time, interference from atmospheric CO₂ and endogenous short chain organic acids such as lactic, acetic, and beta-OH butyric acid is avoided. In addition, the color development is due to carboxylic acid groups. Since by definition these are part of the configuration of fatty acids, there can be no variation in color development owing to differences in the carbon chain. Furthermore, the reaction may be standardized by pure fatty acid.

2. It is relatively reproducible. Duplicates agree within 1 percent and the day to day relative standard deviation can be maintained at 4 percent.

3. It is semi-micro, requiring no more than 0.2 ml of serum.

4. It is reasonably fast and up to 50 specimens can be analyzed by a single technician within 3 hours.

5. The system is adaptable to the analyses of fat from any source such as tissue
or stool. It has been employed in the measurement of serum lipase and can be used for measuring non-esterified fatty acids.

Conclusion

There are many methods available for the measurement of total serum fatty acid. The method of Massion and Seligson offers a high degree of accuracy, precision, and versatility. If further clinically oriented studies like that of Postma and Stroes continue to support the worth of the measurement of total serum fatty acids as a fine screen, it may fill a need as a routine determination.

References