Cholesterol Methodology for Human Studies

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ABSTRACT

A classification and review of the methodology involved in the determination of serum cholesterol for human (or animal) studies are presented. The purpose of both is to enable selection of a technique appropriate for the assay intended with a reasonable understanding of its advantages, disadvantages and limitations. The various methods discussed include direct reaction systems, partial isolation systems and complete isolation systems, as well as screening, reference and definitive procedures. The interferences that could occur are considered, especially those caused by hemoglobin, the turbidity in lipidemia, and bilirubin, as well as interferences caused by optical aberrations and chemical reactants. The various instrumental methods used to determine cholesterol or a substitute determinand such as hydrogen peroxide are discussed, including spectrophotometry, electrochemistry and densitometry of electrophoretically separated proteins.

INTRODUCTION

The beginnings of modern cholesterol methodology date from 1885-1909 (1-3), when color-forming and precipitation reactions for cholesterol were developed, of which modifications are still used for the determination of serum cholesterol. The keys to the technology were the Liebermann-Burchard (LB) color reaction (1,2) and the digitonin precipitation reaction (3) for cholesterol, encompassing a combination of principles and ideas that enabled procedures to be evolved for the determination of free, esterified and total cholesterol. With relatively little alteration, but with some analytical honing, these are still in use. A novel development of the early fifties involved attempts to determine cholesterol in serum directly without a prior separatory purification step (4,5). Although there was some resistance to such apparently bold technology—a resistance which waxed and waned—the idea of direct determination not only persisted, but apparently extended to other serum determinands (the constituent determined, also analyte). It was demanded by most of the simplified robots, the large devices with automation characteristics precluding, for the most part, the preparation of extracts and filtrates or other more complicated separatory steps.

While the LB reaction was enjoying peak use, the cholesterol oxidase reaction was discovered (6,7) and some preliminary procedures were suggested (8,9), although none of them were suitable for routine use. An important development was the appearance of a relatively simple and less cumbersome technique employing cholesterol oxidase (cholesterol: oxygen oxido-reductase, EC 1.1.3.7) in a process in which the hydrogen peroxide generated was used in a peroxidase (EC 1.11.1.7)-coupled reaction to generate a colored product (10). The next critical addition involved the inclusion of cholesterol esterase (EC 2.1.1.13) (11) in the procedure which eliminated the need for the alkaline saponification of cholesteryl esters, which did not react with the oxidase. Introduction of 4-aminoantipyrene and phenol from the Trinder reaction for glucose (12) into the peroxidase-coupled reaction for determining the hydrogen peroxide resulted in a complete procedure. From this point, the optimization of matrix characteristics, along with the substitution of other reagents for 4-aminoantipyrene and phenol, contributed to the overall improvement of a single-pipetting, one-step analytical technique (13,14). A modified procedure employed catalase to convert the hydrogen peroxide and methanol to formaldehyde. Then, by coupling the formaldehyde with acetylacetone and ammonia, the Hantzsch reaction (15) was used for the determination of the peroxide. Thus far, this modification has not been used as commonly as the Trinder reaction.

This discussion will focus on current technology in a manner that will assist the reader in selecting from a wide array of available methods. An all-inclusive classification will be suggested, built on well received published reviews (16-20). However, it will not attempt arrangement of methods on the basis of the final color reactions, thereby avoiding the problems engendered by classifying them as functions of terminal equilibrium reactions (16). Instead, it will group methods according to the manner of sample treatment that ultimately leads to the quantitative reaction selected, because, after all, the final step of a methodological scheme is not the method. Obviously, direct determinations may be complex, even though the procedure only calls for the addition of serum to the reagents. This

single-pipetting step may initiate a series of sequential chemical reactions, terminating in an equilibrium reaction of a color formation, which is used to make the final measurement.

METHODS

Direct Reaction

In this class of methods, no separation or partial separation of the analytical phases occurs. Obviously, when all constituents of the sample are present and it is simply mixed with reagents, maximal perturbance by potential interferences may be expected. These effects could depend on the instrumental methods used to determine the equilibrium product, such as photometry or electrochemical analysis. Generally, spectrophotometry is used because that is how most determinands are assayed in the clinical laboratory. Electrochemical analysis is or can also be used for the determination of cholesterol, but this is still relatively rare.

Blank determinations are probably necessary. However, often the only blank included is the reagent blank, which may be inadequate if sources of irrelevant absorption are present in the sample. The most common natural interferences encountered in serum are bilirubin, hemoglobin and the turbidity in hyperlipidemia. Sometimes, only one interfering substance is present, but encountering 2 or more is a distinct possibility. The use of a serum blank in which one component of the reagent may be left out will sometimes be feasible, provided the substance remains spectrally identical both in the serum blank solution and in the final reaction system, for then an absolute correction can be made. But if the substance to be blanked is a static reactor in the blank and a dynamic reactor in the specimen, serum blanking may be difficult (21). Sometimes the interfering material has a complex reactive nature and can form more than one kind of reaction product, as can be true for bilirubin. It has been shown that it may then generate different irrelevant absorptions in the sample blank and in the sample (22). In that case, the correction for a variable dynamic interference may be erroneous (23).

The main advantage of the direct approach is, of course, that the procedure is simple because the sample is merely added to the reagents; this simplicity also facilitates automation or mechanization, especially with robots which can handle only 1- or 2- part reagents at best. The major disadvantage of the direct procedure is that, since no phase separation such as extraction or dialysis occurs, maximal interference effects not only are possible, but prevalent. In the case of absolute or constant errors from these interferences, little correction may be required. In the case of proportional (relative) errors, the principle of standard additions may be used (24). If the interfering material is an interactor enhancing or inhibiting the reaction, then the standard addition technique, by means of taking advantage of this interacting process, will allow correct values to be closely approximated, even though incorrect absorbance signals are obtained.

Attempts to overcome the problem of interference in direct procedures by correcting at some wavelength other than the measuring wavelength have been made using bichromatic spectrophotometry (25). Several of the automatic instruments now use dual wavelength measurements for most of their determinations in biological fluids (e.g., Hycel M., Abbott ABA-100, 200 and VP, and TECHNICON STAC), whereas other instruments use dual wavelength measurements only where it is believed to be essential (e.g., Dupont ACA). However, it is obviously difficult to assure that one side-wavelength absorbance used for any interference that one might encounter in a biological specimen will subtractively correct the peak-wavelength absorbance for that interference. In addition, the choice of the 2 wavelengths is severely limited by the abridged character of the filter photometers in the automatic instruments. In order to make such a correction accurately, dual wavelength monochromators capable of narrow waveband selection are preferable; the nature of the interference should be known and no other interference can be tolerated at either wavelength, unless it gives identical readings at both wavelengths. Such severe restrictions would make the dual wavelength approach difficult when the limited wavelength monochromators of present instruments are used, unless there is little or no interference present.

In direct methods where enzyme reagents are used for a final colorimetric reaction, free and total cholesterol determinations are possible, depending on whether cholesterol esterase is present in the reagents.

Partial Purification — Use of Organic Solvents

In this class of methods a partial sample cleanup is effected primarily by a separatory process, involving extraction from the proteins with water-soluble solvents (26) or liquid-liquid extractions of the cholesterol from the proteins into organic solvents (27). In the liquid-liquid extractions, this transfer is aided by having a water-soluble component in the extraction mixture, e.g., chloroform-methanol. Free and esterified cholesterol are determined in the

extract using digitonin, which precipitates free cholesterol and separates it from its esters.

The main requirement for blank determinations after partial purification is the reagent blank. Many interfering materials are removed during the extraction process. Digitonin precipitation also aids in separating the cholesterol from most but not all interfering substances. The main drawback with digitonin precipitation is that other 3-β hydroxy sterols are precipitated by the saponin along with cholesterol, although their molar absorptivities in either the LB or the ferric chloride reactions may be considerably lower than that of cholesterol.

An advantage of partial purification is obviously that the equilibrium reaction mixture is purer, as many, though not all, of the interferences are eliminated (28). However, the potential for automation decreases as the complexity of a procedure increases.

In the early matrices used for the LB reaction, esterified cholesterol produced considerably more color than did free cholesterol, which was a significant disadvantage of partial purification. Therefore, procedures for total cholesterol, involving alkaline saponification followed by extraction from the alkali, were developed which virtually eliminated LB-reactive compounds other than cholesterol from the equilibrium reaction (28). This method became the reference by which other proposed procedures are judged even today (18).

A novel approach to partial purification involves electrophoresis of serum in cellulose acetate, filter paper or a transparent gel such as agarose (29-32). Several of these techniques have been proposed as a means of separating the carriers of cholesterol, the lipoproteins. They are visualized by overlaying them with enzyme reagents, which results in a color complex involving the hydrogen peroxide-peroxidase oxidative coupling action of 4-aminoantipyrene and phenol. This procedure has several theoretical advantages over chemical fractionation of serum by selective precipitation of several of the lipoproteins. It is also simpler to carry out than ultracentrifugation followed by the determination of cholesterol in the separated fractions.

Complete Isolation of Cholesterol

The first nearly successful attempt to isolate cholesterol completely involved extraction, then saponification, to convert esters to free cholesterol followed by saponin precipitation with digitonin (3) or tomatine (33) and washing of the insoluble digitonide or tomatide. The dried precipitate was then dissolved and determined by the LB equilibrium reaction (34). This process also obviated the problem that esterified cholesterol gives a color that is ca. 17% more intense than that of free cholesterol, (35) as an apparent matrix phenomenon (18). The procedure is designated "nearly successful" because the extraction and precipitation steps cannot ensure the absolute purification of the determinand.

A number of chromatographic systems involving thin layers, filter paper and columns were elaborated which give more complete isolation than saponin precipitation (18). Gas chromatography with various detectors (36,37), including the mass spectrometer (38), has been applied to the determination of cholesterol in serum.

Screening, Reference and Definitive Procedures

Screening large populations for their serum cholesterol concentrations has been simplified by the invention of automation. This mechanized approach to rapid performance of a large number of determinations has made it possible to examine, without great expenditure of labor, all incoming patients in hospitals as well as outpatients.

The advent of rather accurate cholesterol procedures based on enzyme reagents has not only improved the screening capabilities, but also increased the reliability of the analysis with a concomitant reduction of the doubtful range above and below the normal range. The mechanical simplicity of direct enzymatic reagent techniques has enabled automation to be applied to a large number of clinical samples. The accuracy approaches that of the reference procedures and the normal or reference range can now be more reliably defined.

Thus far, no attempt to use enzyme reagents in the final step of a reference procedure has been published. The procedures accepted as reference procedures still terminate in the LB reaction, i.e., the Abell et al. (28) and Schoenheimer-Sperry (34) procedures. However, since the reference procedure on which these 2 techniques are based is the gravimetric Windaus procedure (3), it should also be considered a reference procedure even though it is unlikely that it would be performed now.

There is no agreement on definitive procedures (18), but likely candidates include gas chromatography with a mass spectrometer as detector or some version of high performance liquid chromatograph. Undoubtedly, the results obtained with well-designed procedures terminating in enzyme reactions will closely approximate the definitive values and easily match the current reference values.

DISCUSSION

Interferences

The naturally occurring interfering materials commonly encountered in serum specimens are bilirubin, hemoglobin and the turbidity in severe lipemia. A variety or sample blanks are described for direct procedures in which an attempt is made to correct for a static or dynamic interference encountered. Two kinds of blanks commonly used are prepared in the following way. The blank reagent is made up with a missing reactive component. This omission can either allow a static interference to represent the total background of irrelevant absorbance or it can permit a dynamic interference to react while the determinand, cholesterol, is unreactive. Dual wavelength blanks are used to make a correction at some wavelength at which the interference is assumed to be identical to that at the peak wavelength of the cholesterol reaction or to apply the proportion of interference at some wavelength to correct the peak wavelength (25).

Since most modern clinical methods are mechanized and limited in the complexity of sample handling by the automated systems, there has been a tendency to use direct handling and thus interference has been maximized. Of the 3 interferences mentioned, the simplest to correct is that resulting from hemoglobin, because its color effect is easily eliminated using a serum blank. Such a treatment, incidentally, is possible only with a genuinely unreactive interfering substance, i.e., a static interference.

Bilirubin presents a much more formidable problem for the analyst (39). It reacts independent of the cholesterol reaction in strong acid to form stable biliverdin. It also interacts in the final step of the peroxidase-coupled enzyme reagent systems and competes with the 4-aminoantipyrene-phenol as a substrate of that reaction. Fortunately, in this instance, the residual bilirubin and perhaps its oxidized product almost completely substitute for the intended oxidation product derived from 4-aminoantipyrene and phenol. The result is a useful compensating error. But, if one now subtracts a serum blank, as some suggest, the final result will be too low (40). However, in the case of colorless drugs which could act similar to bilirubin in the peroxidase-coupled reaction, the final results would be too low with or without a serum blank, a fact already demonstrated for the virtually identical glucose oxidase reaction (41). In this instance, no compensatory color would be present to counteract the loss of color from the peroxidase substrate. It is easy to correct for excess color resulting from the generation of biliverdin from bilirubin if the bilirubin concentration is known. This is ca. 5 mg/mg bilirubin for the LB reaction and ca. 0.7 mg/mg for the ferric chloride reaction. One reason for the difference in magnitude of error for these 2 acidic systems is that the LB reaction for cholesterol produces a peak at the wavelength where biliverdin has a spectral peak, whereas biliverdin shows a minimum in its absorption spectrum at the absorption maximum of the ferric chloride reaction. Another reason is inherent in the molar absorptivities of the 2 acidic reactions for cholesterol. The ferric chloride reaction for cholesterol is much more sensitive than the LB reaction.

The problem in severe lipemia is more complicated than suspected at first glance. In direct reactions of the strongly acidic systems at high dilutions, the turbidity may clear up as a result of the dilution and the nature of the medium. However, at the nearly neutral pH of the enzyme reagent systems severely lipemic specimens may cause turbidity in the final solution, which may require correction, e.g., by a serum blank. If one attempts to clarify the serum by ultracentrifugation in the presently popular micro-ultracentrifuges sold particularly for that purpose, some other factors may have to be considered. One factor involves the lipids in the chylomicrons that are centrifuged off. They contain a small quantity of cholesterol, but perhaps it is small enough to be tolerable as a negative error. However, the removal of a very high concentration of chylomicrons could decrease the volume of serum enough to concentrate the remaining cholesterol by as much as 20-30% (42). This phenomenon has been a problem in other assay systems (43).

Enhancement and inhibition of the ferric chloride reaction for cholesterol by bromide and thiouracil, respectively, have been described when they are present in serum. These interacting species produce proportional errors. Unlike independent side reactions, which cause absolute errors, proportional errors are correctable by the method of standard additions (24).

Electrochemical methods for the determination of cholesterol are relatively new (44-46). They are based on the measurement by the oxygen electrode of the rate of oxygen used up during the cholesterol oxidase reaction or on the measurement by a peroxide electrode of the rate of formation of the peroxide generated in the same reaction. Any side reaction involving either oxygen or peroxide could, in theory, affect the reaction. So far, the oxygen electrode technique has been free of such criticism, although one report suggests that it is not as precise as it could be (47).

Standardization

A previous review of cholesterol methodology provides a rather complete discussion of the problems of standardization of the several procedures commonly used (18). There are differences in reactivity between free and esterified cholesterol in LB reactions which vary as a function of the differences in matrices. There is also the dilemma of what to use as a standard for direct reactions in automated instrumental determinations. If one uses the values for a standard serum the concentration of which has been determined by a reference procedure, there may be considerable background absorbance in the samples to be analyzed, which may be quite different from that of the standard.

The temperature of reaction can disrupt LB procedures if the standard is prepared in a solvent such as glacial acetic acid, which generates little heat upon mixing with LB reagent, whereas serum with its high water content generates relatively much more heat for a temperature-dependent reaction (23).

In enzyme reagent systems, 3 alternatives are currently available. In one, nonesterified cholesterol is dissolved in isopropanol, and this water-soluble solvent generates a molar absorptivity similar to the one obtained with the cholesterol of the sample (48). In order to avoid the use of organic solvents entirely, aqueous standards have been prepared using a detergent to solubilize nonesterified cholesterol (49,50). This standard can be used for the strong acid systems and for the enzyme reagent systems. Morpholine cholesterol hemisuccinate is a practical cholesterol standard in aqueous solution, as shown for the ferric chloride and LB reactions (51). When cholesterol is determined in a multiphasic analyzer, a serum secondary standard is necessary, because each sample is subjected to a variety of tests simultaneously. For this, previously assayed pooled human serum is required and the background problems described earlier in this discussion must be considered (18).

Equilibrium Reactions

A variety of equilibrium reactions and some kinetic reactions have been described in the course of evolution of the determination of cholesterol (18). These have mostly involved reagents which react with cholesterol to generate a measureable product, usually a pigment. Sometimes, the reagent is determined along with the cholesterol e.g., by weighing an insoluble complex. The gravimetric method was actually the first successful approach to the determination of cholesterol (3). Digitonin quantitatively reacts with the free hydroxyl group of the A ring to form an insoluble complex which, when washed free of impurities, provides an accurate weighing form of cholesterol. Some years later, reactions with the carbohydrate moiety of the pentasaccharide precipitating agent provided an indirect determination of cholesterol by assaying for the concentration of saponin involved in the precipitation (52,53). The LB reaction, which was invented some years earlier, provides a simpler photometric solution to the problem. The reagent, a mixture of acetic anhydride, acetic acid and sulfuric acid, produces green products with both free and esterified cholesterol or with the cholesterol digitonide. The LB reaction has undergone many modifications through the years and is still widely used either in the form of the sodium sulfate-stabilized reagent of Huang et al. (54) or the *p*-toluene sulfonic acid technique of Pearson et al. (5).

In 1953, another color reagent was proposed for generating pigment by a direct reaction with cholesterol. It is a mixture of ferric choride in an acetic acid-sulfuric acid milieu, which produces a stable purple compound with considerably higher molar absorptivity than the one produced by the LB reaction. It has also generated several modifications, including ferrous sulfate (55), ferric perchlorate (56) and a mixture of ferric acetate-uranium acetate and ferrous sulfate (57). The direct reaction with serum was replaced by a partial isolation procedure (58), but then reverted to the direct reaction (56). All of these are still used, some manually and some in automated procedures. Interferences, including reagent contamination, e.g., nitrite in sulfuric acid (59) and drug interference, e.g., bromide and thiouracil, have been encountered (60,61). The presence of other steroids in tissue determinations has been described, leading to claims for the advantages of one procedure (57) over others (52-64). However, in a direct comparison of the equilibrium reactions themselves, where constant concentrations of interfering material were used, these claims have been determined to be unfounded (60).

Additional spectrophotometric reactions for the peroxidase-coupled step are certain to be proposed in the future, and if they are more sensitive, they should be useful for the determination of low cholesterol concentrations, e.g., in serum fractions with HDL cholesterol or in cerebrospinal fluid (65). They could be based on fluorescence (66), chemiluminescence (67), or color reactions (68,69). An increase in sensitivity would also be useful for kinetic

studies, where only a portion of the signal of the equilibrium reaction is used (70).

Reaction Mechanisms

The reaction mechanisms of the enzyme reagents are well understood for both the Trinder and Hantzsch reactions (18). Here, oxygen is used and cholstenone is formed along with hydrogen peroxide. The procedures based on reagent oxygen or either of the 2 reaction products, hydrogen peroxide or cholestenone, clearly show that the mechanism is correct. The mechanisms of the LB and iron reaction are more obscure because the products are difficult to identify or isolate. In a rigorous proof of mechanism (71), it was shown that both reactions begin with the dehydration to a common carbonium ion, but then follow separate oxidative pathways. The LB reaction proceeds to a measurable but unstable green pentaenylic ion, which can be oxidized to a yellow cholestahexaene sulfonic acid, a product which has also been a measuring form. The iron reaction is described as proceeding oxidatively through a dienylic carbonium ion to a trienylic ion and finally to a stable purple tetraenylic ion, the compound which is measured.

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