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[54] **METHOD AND REAGENT FOR DETERMINING
 TOTAL CHOLESTEROL IN BLOOD SERUM**
 10 Claims, 4 Drawing Figs.

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260/397.25
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259, 230, 312 A; 252/408, 363.5, 364; 260/397.25

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ABSTRACT: In a method for the determination of total cholesterol in blood serum, plasma or other cell free body fluid the steps of adding a reagent comprising a mixture of ferric acetate $[\text{Fe}(\text{C}_2\text{H}_3\text{O}_2)_3]$ and uranium acetate $[\text{UO}_2(\text{C}_2\text{H}_3\text{O}_2)_2]$ to the fluid whereby the total cholesterol content thereof is solubilized and those chromogens which interfere with the determination are precipitated and separating the liquid phase containing solubilized cholesterol from the precipitate. The cholesterol content is then determined quantitatively, preferably colorimetrically, employing as a color-developing reagent a mixture of ferrous sulfate and sulfuric acid. The invention also includes the ferric acetate-uranium acetate reagent and the ferrous sulfate-sulfuric acid color reagent.

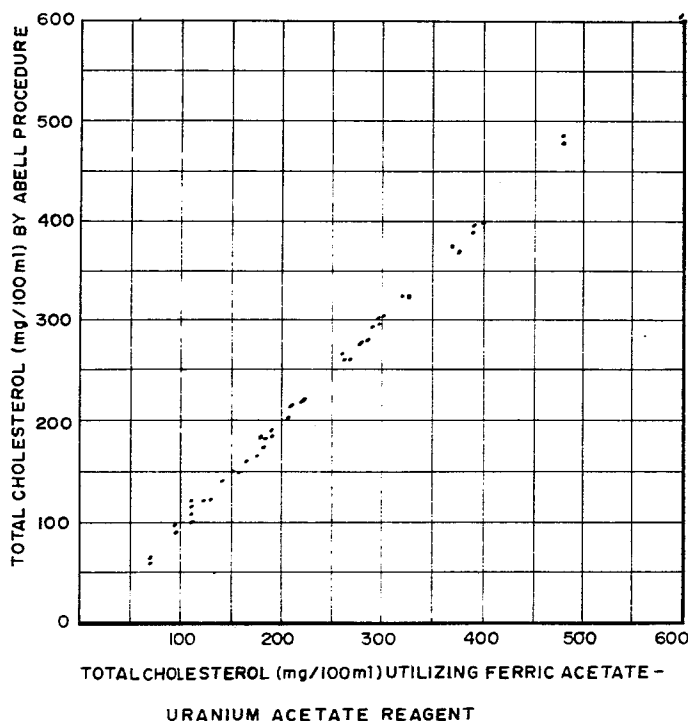


Fig. 2

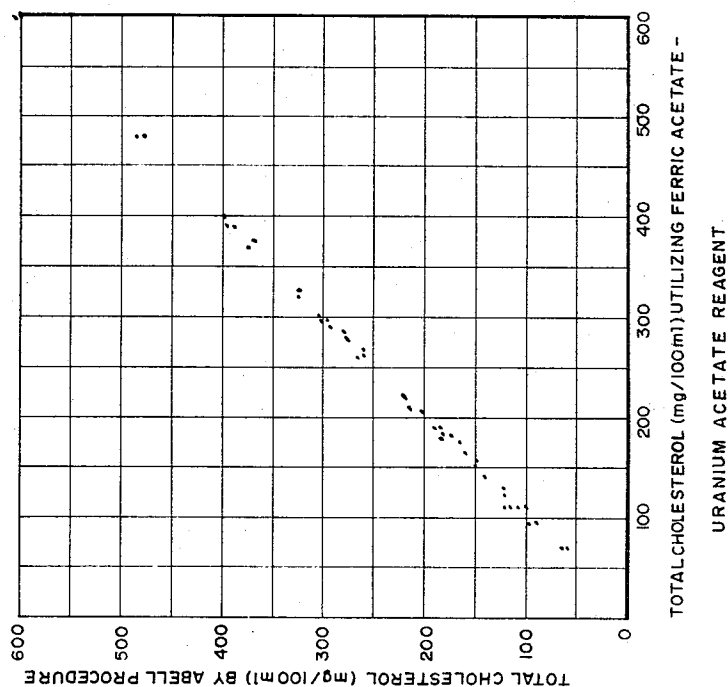
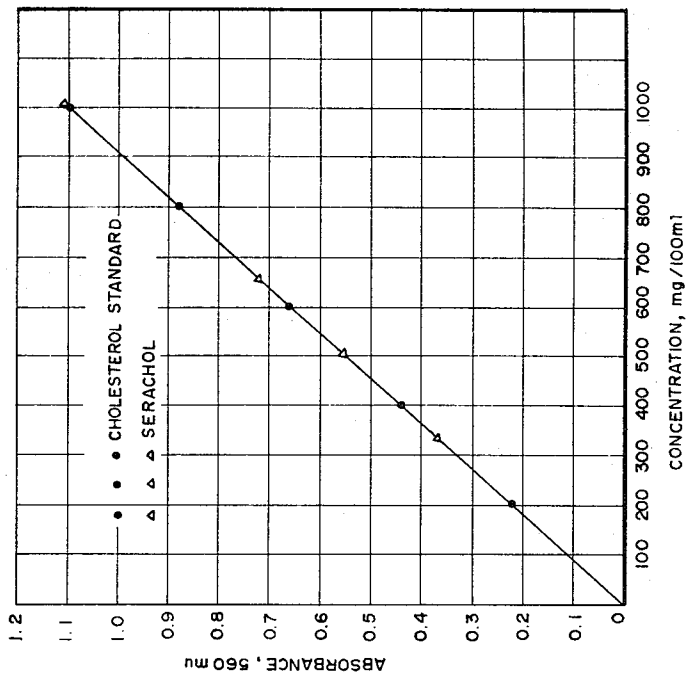


Fig. 1



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Fig. 4

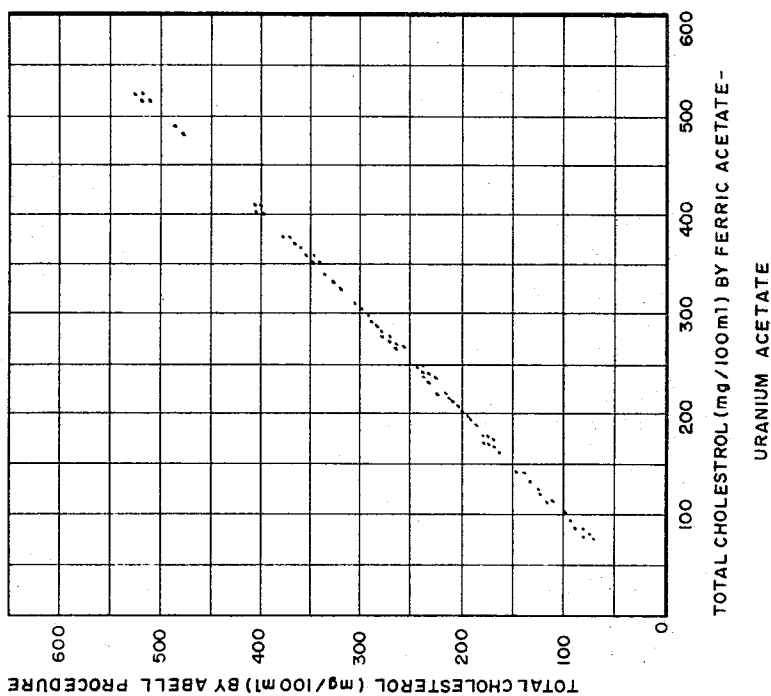
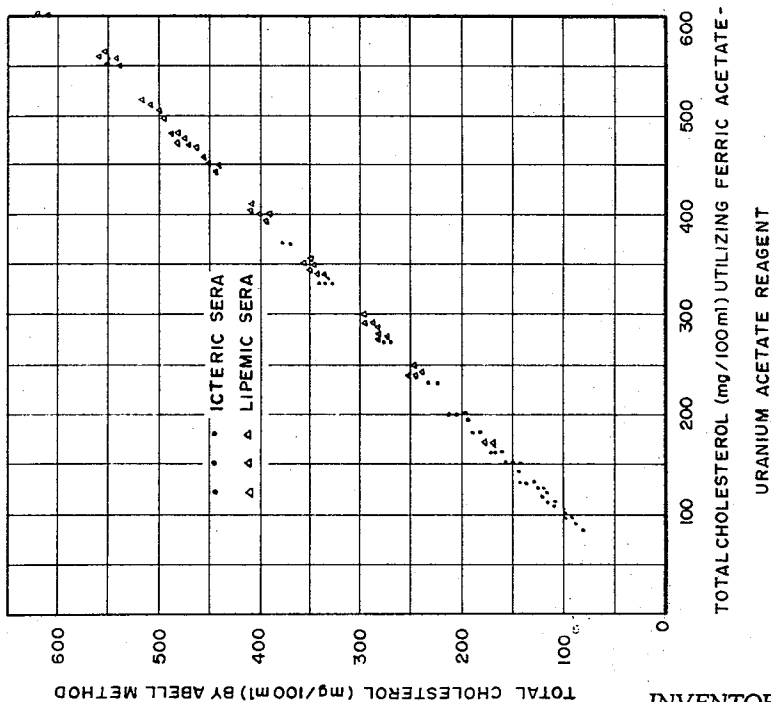


Fig. 3



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METHOD AND REAGENT FOR DETERMINING TOTAL CHOLESTEROL IN BLOOD SERUM

BACKGROUND OF THE INVENTION

The present invention relates to a reagent and method for quantitatively determining the amount of total cholesterol in body fluids, preferably colorimetrically.

Cholesterol exists in substantially all plant and animal cells either in the free form or as an ester. It often exists in admixture with one or more of its derivatives such as dehydrocholesterol, 7-dehydrocholesterol, etc. Cholesterol is an essential ingredient in human blood and is present in constant amounts therein in normal, healthy human beings. The cholesterol level in human blood is usually measured as "total cholesterol" which includes the sum of the free cholesterol and its derivatives. In human blood, these derivatives predominantly comprise esters thereof with the fatty acids contained in human blood.

Recent medical research has unearthed evidence which directly equates the total cholesterol content of blood with certain maladies. For example, the total cholesterol count has been found to be unusually high in the blood of human beings suffering from diabetes, certain diseases of the liver, familial hypercholesterolemia, alcoholism, syphilis, nephritis and other maladies. Most importantly, a high blood cholesterol content has been found to have a direct bearing on the incidence of atherosclerosis and other vascular difficulties.

There have been recently introduced several methods and reagents for quantitatively determining the cholesterol content of human blood. The most successful of these methods involve the colorimetric determination of cholesterol following the addition to serum samples of a reagent adapted to react with cholesterol and its derivatives and form a color. The intensity of this color may be measured in a suitable apparatus such as a spectrophotometer, etc. The amount of cholesterol may then be calculated by comparing this measured intensity with that of a known standard.

Inasmuch as cholesterol and its derivatives are highly insoluble, it is necessary to add a reagent or solvent to the blood serum to solubilize the cholesterol prior to reaction with the color-developing reagent. In the past it has been suggested to employ various materials to solubilize cholesterol. All of the previously employed solubilizing agents suffer from serious disadvantages, however. Although several of the proposed methods effectively solubilize cholesterol, they also solubilize other materials in the serum which interfere with the colorimetric determination, e.g., proteins, bilirubin, lipids, etc. Inasmuch as these materials are color-developing during the conventionally employed colorimetric determinations they will obviously seriously interfere with the obtention of a true reading of the intensity of color or optical density attributable to cholesterol. Furthermore, since the content of these materials in human blood varies from person to person, it is virtually impossible to apply a correction factor in the colorimetric analysis.

The standard Abell method, while it does not suffer from the above-described disadvantage, is extremely complicated and time consuming in order to avoid the solubilization of interfering chromogens.

The prior art is also faced with a serious problem in that to date the reagents employed to develop color by reaction with cholesterol and its derivatives have been found to be unsatisfactory. The most commonly used reagents are the Liebermann-Burchard reagent and a $\text{FeCl}_3\text{-H}_2\text{SO}_4$ mixture. The former comprises a mixture of acetic anhydride and sulfuric acid which develops a green color. The colored reaction product is, however, unstable and requires measurement after the lapse of precise time intervals.

The $\text{FeCl}_3\text{-H}_2\text{SO}_4$ reagent forms a more stable colored reaction product but suffers from the disadvantage that HCl is evolved during the color-forming reaction thereby causing variations in the reaction temperature. The reagent also contributes a yellow color both in the sample under analysis and

in the "blank" or standard with which it is colorimetrically compared thereby interfering with measurement of the optical density.

SUMMARY OF THE INVENTION

By the present invention there is provided a method for the quantitative determination of cholesterol in body fluids which is relatively simple and easy to carry out utilizing only microquantities of the body fluid, yet is extremely accurate and gives results which are highly reproducible.

By the present invention there is also provided a reagent for the colorimetric determination of cholesterol in body fluids which selectively solubilizes cholesterol and does not solubilize those chromogens which interfere with the quantitative determination.

The reagent of the present invention comprises a mixture of ferric acetate and a heavy metal acetate, preferably, uranium acetate $[\text{Fe}(\text{C}_2\text{H}_3\text{O}_2)_3 \text{ and } \text{UO}_2(\text{C}_2\text{H}_3\text{O}_2)_2]$. The reagent is employed in the method of the invention as a lower aliphatic carboxylic acid, preferably, acetic acid solution. This reagent possesses the unique property of being able to solubilize the total cholesterol content of blood serum while simultaneously precipitating those chromogens which interfere with the quantitative determination such as proteins, lipids, bilirubin, etc.

The method according to the present invention comprises intimately admixing the above-defined reagent with a sample of body fluid, separating at least a portion of the liquid phase containing the solubilized cholesterol from the precipitated chromogens and quantitatively analyzing the separated liquid phase for its cholesterol content. Since the interfering chromogens have been substantially entirely eliminated from the liquid phase there is virtually no interference with the analysis. The cholesterol is preferably determined colorimetrically.

The reagent employed to develop the color in the separated liquid phase is preferably a mixture comprising sulfuric acid and ferrous sulfate. When added to the above-described separated liquid phase, an intense purple color is developed. Moreover, no side products having colors which interfere with the colorimetric analysis are produced.

As will be apparent to those skilled in the art, the above-described method is extremely simple and capable of being carried out without the necessity for extensive training of the operator.

DETAILED DESCRIPTION OF THE INVENTION

The method and reagent of the invention are adapted for the solubilization and determination of the total cholesterol in human body fluids, i.e., the sum total of the free cholesterol and cholesterol derivatives such as dehydrocholesterol, 7-dehydrocholesterol, cholesterol esters, etc. It is an advantage of the method and reagent of the invention that the quantitative determination of total cholesterol is not affected to any significant extent by the presence in the body fluid of other steroids, even in amounts exceeding their physiological concentrations.

The invention is applicable for the determination of total cholesterol in any cell-free body fluid. Suitable such fluids include blood serum, blood plasma, cell-free tissue homogenizates, etc.

As described above, the reagent of the present invention comprises a mixture of ferric acetate and uranium acetate $[\text{Fe}(\text{C}_2\text{H}_3\text{O}_2)_3 \text{ and } \text{UO}_2(\text{C}_2\text{H}_3\text{O}_2)_2]$. The reagent is a mixed salt and is preferably employed in the method of the invention as a lower aliphatic carboxylic acid, preferably acetic acid solution. The reagent is conveniently prepared by dissolving ferric hydroxide in acetic acid and then adding uranium acetate. The ratio of ferric acetate to uranium acetate in the mixed salt may vary from about 4:1 to about 4.5:1 by weight. Generally, the carboxylic acid solution may contain from about 0.01 to about 0.1 percent, preferably from about 0.05 to about 0.06 percent, most preferably, about 0.055 percent, by weight, of the mixed salt.

The ferric acetate-uranium acetate reagent of the invention may be utilized to solubilize cholesterol and precipitate the interfering chromogens in the fluid sample analyzed by any quantitative method which depends upon the determination of solubilized cholesterol. Preferably, the reagent is utilized to solubilize the cholesterol for colorimetric analysis wherein the solubilized cholesterol is reacted with a color-forming reagent and the resulting color is measured, i.e., with a spectrophotometer. The reagent is preferably employed in a method, however, which depends upon the reaction of the solubilized cholesterol and a color-developing reagent comprising a mixture of sulfuric acid and ferrous sulfate. As noted above, the utilization of an $\text{H}_2\text{SO}_4\text{-FeSO}_4$ reagent does not result in the production of undesirable color-forming side products or in variations in the reaction temperature. FeSO_4 is difficultly soluble in concentrated H_2SO_4 and is therefore preferably first dissolved in glacial acetic acid prior to admixture with the sulfuric acid.

The method of the invention comprises adding a sufficient amount of the ferric acetate-uranium acetate reagent to the blood serum to be analyzed to solubilize the total cholesterol content thereof and precipitate all of the interfering chromogens contained therein, i.e., proteins, lipids, bilirubin.

Generally, an amount of the reagent is added to the body fluid sample, either in solid or solution form, sufficient to provide a sample to reagent ratio, by weight, of from about 1:50 to about 1:300, preferably from about 1:100 to about 1:250, is sufficient to solubilize the cholesterol and precipitate the interfering chromogens. A sample to reagent ratio of about 1:200 is most preferable.

The ferric acetate-uranium acetate reagent and body fluid sample are intimately admixed and the mixture allowed to stand for a sufficient period of time to ensure complete solubilization of all of the cholesterol contained in the sample and to ensure complete precipitation of all of the interfering chromogens contained therein. The amount of time required is obviously dependent upon a variety of factors, including the size of the sample, amount of reagent employed, the cholesterol and other chromogen content of the sample, etc. Generally, however, where a sample size of from about 0.001 to about 0.1 ml. is to be analyzed and sufficient reagent is employed to provide a sample to reagent ratio by weight of from about 1:50 to about 1:300, a period of time for precipitation of interfering chromogens and proteins of from about 5 to about 15 minutes, preferably from about 5 to about 7 minutes, is usually sufficient.

Following complete solubilization of the cholesterol and precipitation of the interfering chromogens at least a portion of the liquid phase of the body fluid sample is separated from the precipitate. This is most conveniently accomplished by centrifuging the sample to segregate the liquid phase and the precipitated solids. A portion of the supernatant liquid phase is then drawn off and transferred to a suitable container for colorimetric or other quantitative analysis.

The amount of liquid phase drawn off is not critical and will depend upon the particular method contemplated for analysis. Where color for a colorimetric analysis is developed employing the preferred $\text{FeSO}_4\text{-H}_2\text{SO}_4$ reagent of the present invention, a portion of the liquid phase amounting to from about 0.01 to about 5 ml. is generally sufficient for subsequent colorimetric analysis.

As noted above, any conventional colorimetric method may be employed to analyze the body fluid sample. To avoid variations in reaction temperature and the production of side products during the color-forming reaction which interfere with the colorimetric determination, however, it is preferred to employ a colorimetric method which depends upon the color formed by reaction of the solubilized cholesterol with a $\text{FeSO}_4\text{-H}_2\text{SO}_4$ reagent. It has been found that the $\text{FeSO}_4\text{-H}_2\text{SO}_4$ reagent produces a color, the measurement of which colorimetrically ensures a correct analysis.

The reagent preferably comprises a substantially anhydrous mixture of sulfuric acid and ferrous sulfate. The ratio of ferrous sulfate to sulfuric acid, by weight, may range from about

1:10,000 to about 1:30,000, preferably from about 1:15,000 to about 1:20,000. Most preferably a mixture having a ferrous sulfate to sulfuric acid ratio of about 1:18,000 is employed. Optionally, the reagent may contain a small amount of acetic acid which is employed to aid in the solubilization of the FeSO_4 .

In order to develop a sufficiently intense color to enable a precise colorimetric determination of the total cholesterol in the body fluid sample it is generally necessary to add sufficient reagent to the liquid phase containing solubilized cholesterol to provide a reagent: liquid phase ratio, by weight, of from about 1:2 to about 4:1, preferably from about 1:1 to about 3:1. Most preferably, an amount of reagent is employed which provides a reagent: liquid phase ratio of about 1.23:1.

Upon addition of the $\text{FeSO}_4\text{-H}_2\text{SO}_4$ reagent to the liquid-phase aliquot containing solubilized cholesterol an intense purple color is developed. The sample is allowed to stand for a period of time sufficient to permit maximum color development, generally, from about 5 to about 60 minutes. The sample is then analyzed colorimetrically; most conveniently by measuring the optical density of the sample in a spectrophotometer against a blank containing an amount of ferric acetate-uranium acetate solution equal to the size of the liquid-phase portion analyzed and an amount of $\text{FeSO}_4\text{-H}_2\text{SO}_4$ reagent identical to that employed to develop the color therein. It is to be understood, however, that any colorimetric method may be employed to analyze the sample.

The invention will be illustrated by the following nonlimiting example.

EXAMPLE 1

a. Preparation of ferric acetate-uranium acetate reagent

0.5 gm. of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ is converted to $\text{Fe}(\text{OH})_3$ by admixing concentrated ammonia therewith. The $\text{Fe}(\text{OH})_3$ is filtered and washed with water and dried. The $\text{Fe}(\text{OH})_3$ is then dissolved in glacial acetic acid and the volume of the solution made up to 1 liter with further glacial acetic acid. 100 mg. of uranium acetate [$\text{UO}_2(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 2\text{H}_2\text{O}$] is then dissolved in the solution and the resulting solution allowed to stand overnight.

b. Preparation of $\text{FeSO}_4\text{-H}_2\text{SO}_4$ reagent

To 0.1 gm. of anhydrous ferrous sulfate in a flask is added 100 ml. of glacial acetic acid while swirling and then 100 ml. of concentrated sulfuric acid is added. After dissolution of the ferrous sulfate and cooling to room temperature the volume of the solution is made up to 1 liter with concentrated sulfuric acid.

c. Determination of cholesterol

10 ml. of the ferric acetate-uranium acetate reagent prepared according to the above-described process are added to a 16×125 mm. tube provided with a screw cap. 50 μl . of a blood serum sample is added to the tube and thoroughly admixed with the reagent. The tube is allowed to stand undisturbed for 5 minutes. A precipitate is observed to have been formed in the mixture. The mixture is centrifuged for 5 minutes.

A 3-ml. aliquot of the supernatant liquid phase is drawn off with a pipette and transferred to another 16×125-mm. tube, unscratched, provided with a screw cap, which is adapted for use as a cuvette.

2 ml. of the $\text{FeSO}_4\text{-H}_2\text{SO}_4$ reagent prepared according to the above-described process is added to the aliquot. The tube is tightly capped and the contents thoroughly admixed. The tube is allowed to stand for 20 minutes to permit maximum color development. The purple color of reaction is measured in a spectrophotometer at 560 $m\mu$ against a blank consisting of 3 ml. of ferric acetate-uranium acetate solution and 2 ml. of $\text{FeSO}_4\text{-H}_2\text{SO}_4$ reagent.

EXAMPLE 2

In order to demonstrate the effectiveness of the method and reagent of the present invention for quantitatively determining

total cholesterol in human blood serum, the procedure of example 1 is followed in analyzing six different icteric blood sera. The same six sera are also analyzed according to the procedure set forth in example 1 but employing as the solubilizing reagent, ferric acetate, prepared according to example 1a with the exception that no uranium acetate is added to the solution. The total cholesterol is measured in mg./100 ml. by comparing the measured optical densities with those of standard cholesterol solutions which were analyzed according to the same procedure. The same six sera are also analyzed according to the well-known Abell procedure which is commonly accepted as the most accurate method, although difficult, tedious and time consuming, for quantitatively determining cholesterol. The Abell procedure is described in J. Biol. Chem., Vol. 195, page 357 (1952) and in Standard Methods in Clinical Chemistry, Vol. 2, page 26 (1958).

The results are set forth in table 1.

TABLE 1

Serum No.	Mg Cholesterol/100 ml		
	Abell Procedure	$\text{Fe}(\text{C}_2\text{H}_3\text{O}_2)_3 - \text{UO}_2(\text{C}_2\text{H}_3\text{O}_2)_2$	$\text{Fe}(\text{C}_2\text{H}_3\text{O}_2)_3$
1.....	122	122	133
2.....	165	166	172
3.....	235	233	243
4.....	167	168	172
5.....	375	375	379
6.....	127	129	135

As is apparent from table 1, the results obtained according to the method of the present invention compare favorably with the highly accurate results obtained according to the Abell procedure. When employing the ferric acetate reagent, however, which solubilizes both the total cholesterol and interfering chromogens, the results obtained are higher in each instance, thus demonstrating the effectiveness of the reagent of the invention in eliminating the interfering chromogens.

EXAMPLE 3

In order to demonstrate the effectiveness of the $\text{FeSO}_4\text{-H}_2\text{SO}_4$ color-developing reagent, the procedure of example 1 is followed in analyzing six different icteric sera. In one analysis of the six sera, the $\text{FeSO}_4\text{-H}_2\text{SO}_4$ reagent is employed. In the second analysis, H_2SO_4 alone is employed to develop the reaction color. In both of the foregoing analyses, the ferric acetate-uranium acetate reagent of the invention is utilized. In the third analysis the Abell procedure is followed. The results are set forth in table 2.

TABLE 2

Serum No.	mg. Cholesterol/100 ml.		
	Abell Procedure	$\text{H}_2\text{SO}_4\text{-FeSO}_4$	Conc. H_2SO_4
1.	375	375	400
2.	307	305	326
3.	168	170	176
4.	127	128	132
5.	172	173	180
6.	126	129	135

As is apparent from table 2, the results obtained when following the method of the invention and employing the $\text{FeSO}_4\text{-H}_2\text{SO}_4$ color-developing reagent compare favorably with those obtained following the Abell procedure. When employing only H_2SO_4 , however, the results obtained are consistently higher.

EXAMPLE 4

The procedure of example 1 is repeated employing samples from a serum pool containing 82 mg./100 ml. cholesterol to which were added varying amounts of cholesterol. The results are set forth in table 3.

TABLE 3

Cholesterol Added (mg./100 ml.)	Total Cholesterol Content (mg./100 ml.)	Cholesterol Recovery (mg./100 ml.)	Percent Recovered
82	82	82	100.0
162	162	162	100.0
182	182	182	100.0
281	281	281	99.5
381	381	381	99.6
476	476	476	98.8

EXAMPLE 5

The procedure of example 1 is followed utilizing ferric acetate reagents having different concentrations in acetic acid. A high cholesterol content serum was employed (400 mg./100 ml.). The results are set forth in table 4. As is apparent, a concentration of ferric acetate of about 5.6 mEq/l. is sufficient for cholesterol solubilization and subsequent color development. By employing ferric acetate as the solubilizing component of the reagent rather than the conventionally employed ferric chloride, the objectionable evolution of HCl occasioned by the use of the latter is avoided.

TABLE 4

Ferric Acetate Reagent Concentration (meg./l.)	Absorbance
1.9	0.170
2.8	0.193
5.6	0.197
11	0.197
22	0.197
33	0.197

EXAMPLE 6

The procedure of example 1 is followed with the exception that the amount of $\text{FeSO}_4\text{-H}_2\text{SO}_4$ reagent is varied over six separate runs. The results are set forth in table 5.

TABLE 5

Volume of $\text{H}_2\text{SO}_4\text{-FeSO}_4$ ml.	Absorbance
0	0.145
1.5	0.165
1.75	0.185
2.0	0.195
2.25	0.180
2.5	0.150

These results indicate that the optimum ratio of ferric acetate-uranium acetate reagent to ferrous sulfate-sulfuric acid reagent is about 3:2.

7

EXAMPLE 7

The procedure of example 1 is followed in analyzing two serum samples. In one analysis the color-developing reagent employed is $\text{FeSO}_4\text{-H}_2\text{SO}_4$. In the second analysis, an equivalent volume of concentrated H_2SO_4 is employed. The optical densities are measured at different time intervals. The results are set forth in table 6.

TABLE 6

Time Intervals Mins.	Absorbance $\text{FeSO}_4\text{-H}_2\text{SO}_4$ Reagent	Absorbance H_2SO_4 Reagent
5	0.215	0.190
10	0.223	0.194
15	0.225	0.194
20	0.225	0.192
30	0.225	0.190
40	0.225	0.188
50	0.225	0.185
60	0.225	0.183

As is apparent, the color developed employing the $\text{FeSO}_4\text{-H}_2\text{SO}_4$ reagent is more intense and is more stable than that produced when employing H_2SO_4 alone. When using the latter, the color produced is less intense than when using the former and it fades on standing.

8

EXAMPLE 8

The procedure of example 1 is followed in analyzing a standard cholesterol sample (200 mg./100 ml.) and a serum sample. The optical densities are measured at various wavelengths. The optical densities of the blank are measured against water. The results are set forth in table 7.

TABLE 7

Wavelength $\text{m}\mu$	Absorbance Blank	Absorbance Standard	Absorbance Serum
400	0.218	0.116	0.096
450	0.012	0.130	0.115
500	0.003	0.162	0.148
550	0.002	0.215	0.190
560	0.002	0.223	0.195
580	0.002	0.212	0.176
600	0.002	0.175	0.130
650	0.002	0.097	0.070
700	0.004	0.055	0.035

As is apparent, the absorbance of the blank is ideally low, demonstrating the lack of contribution of interfering color-producing side reaction products by the method of the invention. Moreover, as is apparent from the table the standard and serum samples evidence identical peaks at about 560 $\text{m}\mu$.

9

EXAMPLE 9

The procedure of example 1 is followed in setting up a calibration curve. Various cholesterol standards and Serachol solutions (commercial cholesterol standard solutions manufactured by Warner-Chilcott) of various concentrations are analyzed and the absorbancies plotted against the known concentrations. The thus-obtained calibration curve is set forth in FIG. 1.

As is apparent, the calibration curve is absolutely linear, even up to 1,000 mg./100 ml., thus evidencing the unusually extensive analytical range of the method and reagent of the invention.

8

EXAMPLE 10

The procedure of example 1 is followed in analyzing various randomly selected serum samples. The same samples are also analyzed according to the Abell procedure. The results are plotted against each other in terms of mg. cholesterol/100 ml. The resulting scattergram is depicted in FIG. 2.

As is apparent the linearity of the scattergram indicates that the results obtained according to the method of the invention are highly accurate over a wide cholesterol concentration range.

11

EXAMPLE 11

The procedure of example 1 is followed in analyzing various samples of both icteric sera and lipemic sera.

The same samples are analyzed according to the Abell procedure. The results (mg. cholesterol/100 ml.) are plotted in the scattergram set forth in FIG. 3.

Again, the results demonstrate the effectiveness of the reagent and method of the invention in eliminating the interference of chromogens such as bilirubin, proteins, lipids, etc. with the quantitative determination of cholesterol by a colorimetric method.

12

EXAMPLE 12

The procedure of example 1 is followed in analyzing serum samples which have been preliminarily extracted according to the method of Abell, i.e., 0.5 ml. of Abell's extract is evaporated to dryness, reconstituted with 3 ml. of the ferric acetate-uranium acetate reagent solution and admixed with 2 ml. of the $\text{FeSO}_4\text{-H}_2\text{SO}_4$ reagent.

The procedure of Abell is followed in analyzing the same samples. The results are depicted in the scattergram set forth in FIG. 4. As is apparent therefrom, the method and reagent of the invention may be employed equally well in analyzing serum samples by whatever method utilized to extract the cholesterol therefrom.

It is readily apparent from the foregoing examples that the method and reagent of the invention enable precise quantitative determinations of cholesterol with relative ease and simplicity of operation.

We claim:

1. A method for the colorimetric determination of total cholesterol in a cell free body fluid sample comprising:

a. intimately admixing said sample and an acetic acid solution of a mixed salt comprising ferric acetate and uranium acetate and allowing the mixture to stand for a period of time sufficient for the cholesterol in said sample to be solubilized and the chromogens contained in said sample which interfere with said colorimetric determination to be precipitated,

b. separating at least a portion of the liquid phase containing solubilized cholesterol from said precipitate, and

c. analyzing said liquid phase colorimetrically by measuring the optical density thereof after the addition thereto of a color-developing reagent.

2. A method according to claim 1 wherein said color-developing reagent is a mixture of ferrous sulfate and sulfuric acid.

3. A method according to claim 1 wherein the amount of total cholesterol in said sample is determined by comparing the optical density measured in step c with that of a standard.

4. A method according to claim 1 wherein the weight ratio of ferric acetate to uranium acetate in said mixed salt is from about 4:1 to about 4.5:1.

5. A method according to claim 4 wherein said acetic acid solution contains from about 0.05 to about 0.06 percent by weight of said mixed salt.

6. A method according to claim 4 wherein the weight ratio of said sample to said reagent is from about 1:50 to about 1:300.

7. A method according to claim 2 wherein the ratio of ferrous sulfate to sulfuric acid by weight in said color-developing reagent is from about 1:10,000 to about 1:30,000.

8. A method according to claim 2 wherein the ratio of said color-developing reagent to said liquid phase containing said solubilized cholesterol, by weight, is from about 1:2 to about 4:1.

9. A method according to claim 2 wherein said color-developing reagent is substantially anhydrous.

10. A method for the quantitative colorimetric determination of total cholesterol in a cell-free body fluid sample comprising:

- a. intimately admixing said sample with about 200 parts by weight based on the weight of said sample of an acetic acid solution containing about 0.055 percent, by weight, of a mixed salt comprising ferric acetate and uranium acetate, the weight ratio of ferric acetate to uranium acetate being from about 4:1 to about 4.5:1,
- b. allowing the mixture to stand for from about 5 to about 10 min. whereby the total cholesterol in said sample is

solubilized and the chromogens contained in said sample which interfere with said colorimetric determination are precipitated,

- c. centrifuging said mixture to segregate said precipitate from a liquid phase containing solubilized total cholesterol,
- d. separating 3 ml. of said liquid phase from said segregated mixture,
- e. adding to said separated liquid phase 2 ml. of a reagent comprising a mixture of sulfuric acid and ferrous sulfate wherein the ratio of ferrous sulfate to sulfuric acid, by weight, is about 1:18,000 whereby a purple color of reaction is developed,
- f. analyzing said liquid phase colorimetrically by measuring the optical density thereof, and
- g. determining the total cholesterol content of said sample by comparing said measured optical density with that of a standard.

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