METHODS AND COMPOSITIONS FOR DETERMINING GLUCOSE IN BLOOD

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Field of Search 23/230, 230 B; 260/239.3 D; 252/408

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UNITED STATES PATENTS
3,098,717 7/1963 Ferrari, Jr. 23/230

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OTHER PUBLICATIONS
Aloe Scientific Co., Catalog 103, p. 1011, 1041, 1065, 1073

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ABSTRACT

Colorimetric methods and compositions for quantitatively determining the glucose content of blood plasma or serum by heating a deproteinized sample of blood plasma or serum with an alkaline ferricyanide solution, followed by the addition of ferric ions and a 5-(2-pyridyl)-2H-1,4-benzodiazepine or water soluble salts thereof to produce a brilliant purple colored solution which can be quantitated by standard colorimetric means.

11 Claims, 3 Drawing Figures
METHODS AND COMPOSITIONS FOR DETERMINING GLUCOSE IN BLOOD

BACKGROUND OF THE INVENTION

The need for a quantitatively accurate method for the determination of glucose in blood, e.g., plasma and serum, using small amounts of specimen, yet which is simple enough to be effectively utilized in the clinical situation and sufficiently economical for mass screening has long been felt. In addition, it has been considered most desirable that such a method be readily adaptable to an automated sequential or continuous flow system in order that a great many samples may be processed rapidly and with the highest possible accuracy. There is a need for such an automated sequential or continuous flow of system which is capable of highly accurate results before the diagnostic testing of large numbers of persons for the incidence of diabetes among them. A simple accurate test, which is both rapid and reliable, is of great value as an aid in the detection and treatment of diabetes and as an adjunct to routine screening operations in clinics and for periodic screening of patients in hospitals, nursing homes and similar institutions.

Many techniques have been developed for quantitatively determining the glucose content of blood, plasma or serum. One such technique utilizes the enzyme glucose oxidase which catalyzes the oxidation of glucose to gluconic acid. In the more common test, this enzyme is combined with a substance having a peroxidative activity which induces the oxidation of an indicator such as o-tolidine in the presence of hydrogen peroxide formed by the glucose oxidase. This method, though specific, has proved to be too complex, expensive and time consuming for general use.

Other further metric techniques which are adaptable to automated procedures have been found to be not sufficiently sensitive for today’s standards or undesirable in that they require comparatively large volume of specimen.

The diagnostic compositions and methods of the present invention provide a reliable, convenient test for the quantitatively determining of glucose in the blood as well as affording a method whereby the quantitative determination may be carried out in a continuous sequential or flow system. Further, the diagnostic compositions and methods of the present invention overcome many of the disadvantages of the prior art methods of determining glucose in blood by not requiring a high degree of laboratory skill and technology using a small specimen volume, yet being highly accurate in the clinical situation.

BRIEF SUMMARY OF THE INVENTION

In accordance with the invention, a 5-(2-pyridyl)-2H-1,4-benzodiazepine or water soluble salt thereof preferably in combination with a buffer, is added with an aqueous solution of ferric chloride to proteinized plasma or serum which has been treated with an aqueous alkaline ferricyanide solution, whereby a purple solution is obtained which can be quantitated as to its glucose content by standard colorimetric means.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the invention a compound selected from the group consisting of compounds of the formula

\[
3 \text{,653,841}
\]

\[
\text{B is selected from}
\]

\[
\text{and -CH}_2-; \ R_4 \text{ is selected from the group consisting of halogen, hydrogen, trifluoromethyl, nitro and amino; } \ R_4 \text{ is selected from the group consisting of}
\]

\[
\text{hydrogen, lower alkyl and}
\]

\[
\text{and}
\]

\[
\text{and } \ R_3 \text{ and } \ R_4 \text{ where taken together with their attached nitrogen atom form a radical selected from the group consisting of piperalinyl, lower alkyl substituted piperazinyl, pyrrolidinyl, lower alkyl substituted pyrrolidinyl, piperidinyl and lower alkyl substituted piperidinyl; } \ R_4 \text{ is lower alkyl; and } \ R_4 \text{ is selected from the group consisting of lower alkyl and hydrogen and water soluble salts thereof, preferably in combination with a buffer, is added with an aqueous solution of ferric chloride to proteinized blood plasma or serum which has been treated with an aqueous alkaline ferricyanide solution, whereby a purple solution is obtained which can be quantitated by standard colorimetric means.}
\]

Examples of benzodiazepine compounds of formula 1 above which are particularly suitable as the color-forming reagent in the process of this invention include the following:

7-bromo-1,3-dihydro-1-[4-(4-methyl-1-piperazinyl)butyl]-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one;
7-amino-1,3-dihydro-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one;
1-methyl-1-[3-(7-bromo-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one;
1-methyl-1-[3-(7-bromo-5-(2-pyridyl)-1,3-dihydro-2-oxo-2H-1,4-benzodiazepine-1-yl)propyl]urea whose preparation is disclosed in U.S. Pat. No. 3,464,978 issued Sept. 2, 1969;
7-bromo-1,3-dihydro-5-(2-pyridyl)-2H-1,4-benzodiazepine;
7-amino-1,3-dihydro-1-methyl-5-(2-pyridyl)-1H-1,4-benzodiazepine;
7-bromo-1,3-dihydro-3-dimethylaminopropyl-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one-4-oxide;
7-bromo-1,3-dihydro-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one-4-oxide;
7-bromo-1,3-dihydro-1-(β-hydroxypropyl)-2-(2-pyridyl)-2H-1,4-benzodiazepin-2-one; and
3,653,841

7-bromo-5-(2-pyridyl)-1,3-dihydro-1-(3-(N-cyanomethylamino)propyl)2H-1,4-benzodiazepin-2-one whose preparation is described in U.S. Pat. No. 3,644,978.

The term "lower alkyl" as used throughout this specification includes both straight and branched chain alkyl groups having from one to seven carbon atoms such as methyl, ethyl, propyl, isopropyl and the like. The term "lower alkanoyloxy" refers to both straight chain and branched chain aliphatic carboxylic acid moieties such as acetoxy, propanoyloxy, butyroyloxy and the like. The term "halogen" includes bromine, chlorine, fluorine and iodine. Also included within the purview of the present invention are the water soluble acid addition salts of the compounds of formula I above. Any conventional water soluble acid addition salts of the compounds of formula I above may be utilized in the process of this invention to quantitatively determine the iron content of aqueous solutions. Among the acid addition salts which can be utilized in accordance with this invention, includes salts of compounds of the formula I with organic or inorganic acids such as hydrochloric acid, hydrobromic acid, nitric acid, sulfuric acid, acetic acid, formic acid, succinic acid, maleic acid, p-toluene sulfonic acid and the like.

The color differentiation with varying concentrations of ferrous ions produced by the compound of formula I above is such that the concentration of ferrous ions produced by the instant diagnostic reagent composition in situ can easily be determined by standard colorimetric instruments. Furthermore, the compounds of formula I are not sensitive to extraneous sources and therefore are not affected by trace contaminants. The method of this invention provides a simple colorimetric means for quantitatively determining the glucose content of blood plasma and serum.

In accordance with the present invention the glucose content of blood plasma or serum can be determined by first heating a deproteinized sample with an aqueous solution containing ferricyanide ions to form an aqueous solution containing gluconic acid and ferrocyanide ions, cooling the solution and adding an aqueous solution containing ferric ions and a compound of formula I above wherein ferricyanide and ferrous ions are formed and the ferrous ions thus produced react with the compound of formula I, preferably in the presence of a buffer, to produce a brilliant deep purple color and colorimetrically quantitating the amount of glucose present in the sample. This procedure provides a simple and rapid method for quantitatively determining the glucose content of a blood sample which is ideally suited for routine diagnostic use.

In accordance with the present invention, the specimen to be tested is initially treated with a conventional neutral deproteinating agent such as, for example, an aqueous solution of either sodium or barium hydroxide and zinc sulfate, or an acidic deproteinating agent such as, for example, tungstic acid or trichloroacetic acid. Of these, tungstic acid or an aqueous solution of barium hydroxide and zinc sulfate are preferred. The specimen is well mixed with the deproteinating agent and centrifuged at high speed to obtain a clear supernate. A 0.1 ml aliquot of the supernate is then heated to from 90°C to about 100°C, preferably about 95°C with 2.0 ml of an aqueous alkaline solution containing ferricyanide ions. The mixture is rapidly cooled after about 5 minutes heating and treated with 2.0 ml of an aqueous solution containing ferric ions such as, for example, ferric chloride, and 2.0 ml of an aqueous solution of a compound of the formula I. The solutions are mixed and the absorbance of the violet blue color which develops over about 10 minutes is measured at 580 nm against both a standard glucose solution similarly treated and a reagent blank.

The solution containing ferricyanide ions can be made from any water soluble ferricyanide salt which does not otherwise interfere with the reaction such as, for example, potassium ferricyanide or ferric ammonium citrate. Potassium ferricyanide is preferred in the practice of the present invention. This reagent may be made in quantity if so desired and used as needed. The appropriate amount of potassium ferricyanide is dissolved in an aqueous alkaline medium such as, for example, a 2 percent sodium carbonate solution. The quantity of ferricyanide salt utilized in preparing the reagent is variable. However, a sufficient quantity must be utilized to react with all the ferric ions in the specimen to furnish a positive indication of elevated glucose blood levels when the diagnostic method of the present invention is being utilized as a diagnostic or a mass screening tool.

Generally, it is preferred that for each ml. of blood plasma or serum tested, the solution contain from about 1.8 X 10⁻⁸ to about 7.0 X 10⁻⁸ moles of ferricyanide salt, preferably from about 3.5 X 10⁻⁸ to about 5.0 X 10⁻⁸ moles per ml of plasma or serum utilized.

The quantity of ferric ions added to the sample — ferricyanide ion mixture is again variable. However, it is preferred to utilize a quantity of ferric ion slightly in excess of the molar quantity of ferricyanide ions added to the sample. The utilization of such an excess insures that there will be sufficient ferric ions present to react with the ferrocyanide ions generated by the initial reaction between the ferricyanide ion and the glucose in the sample. The ferric ions may be supplied as any water soluble ferric salt which does not interfere with the diagnostic determination such as, for example, ferric chloride, ferric nitrate, ferric sulfate and the like. Of these, ferric chloride is preferred.

The quantity of the compound of formula I which is added to the aqueous mixture is variable. In all instances, however, there must be a sufficient quantity of the compound of formula I present to react with all of the ferrous ions generated by the reaction between the ferric ions and the ferrocyanide ions. This quantity is most conveniently determined by equating the quantity of the compound of formula I with that of the ferric ions to insure the stoichiometry of the chelation reaction.

It is preferred to maintain the test medium at a pH of about 4.0 to about 5.0, preferably about 4.5. This can most easily be accomplished by adding suitable buffers to the ferric ion reagent and the reagent containing the compound of formula I. Buffering these reagents also makes them stable in aqueous solution when they are made up in quantity for large scale laboratory testing.

In general, any recognized buffer pair suitable for the maintenance of such a pH range as described above can be utilized. Preferably, there can be utilized as a buffer pair a water soluble salt of acetic acid and sodium acetate. Of the water soluble salts of acetic acid sodium acetate is preferred. However, ammonium acetate, potassium acetate or other water soluble salt of acetic acid can be used, if desired. Although the quantities of the buffer pair comprising a water soluble acetic acid salt and acetic acid are variable, the present invention contemplates the use of a sufficient quantity of the acetic acid component, e.g. acetic acid, to provide a final test sample having a pH in the range of from about 4.5 to about 5.5. By final test sample is meant a solution containing the ferricyanide ions, the ferric ions and the benzodiazepine color reagent. In general, there is contemplated the preparation of a solution of both the ferric ions and the benzodiazepine color former which contains per liter about 1.0 mole of a water soluble salt of acetic acid to about 1.0 to about 2.0 moles of acetic acid.

From the foregoing description it is evident that the compositions of the present invention may be utilized or handled as prepared aqueous stock solutions, aqueous concentrates or in a dry powder form. In either the concentrate or the powder form, sufficient buffering agents are added to stabilize the compositions whereby the working dilutions are made and maintain the pH of the reaction mixture at between 4.5 and 5.5 preferably about 4.8.

In utilizing the compositions of the present invention, the addition of the compound of formula I to the test system immediately produces the desired purple coloration. The color deepens as the reaction proceeds to completion. The reaction mixture ceases to undergo any color changes discernible to the naked eye after it has been allowed to stand for a short
time at room temperature. Accordingly, in order to insure uniform coloring, the aqueous solution should be allowed to stand until its color appears to have become constant. In general, it has been found that the full development of the purple color will occur over a period of from about 5 to 15 minutes after the addition of the compound of formula I. In most cases 10 minutes is a sufficient period of time to allow for full color development.

The quantitation of the glucose in the colored sample can be carried out by any conventional colorimetric method utilizing standard spectrophotometers such as a Beckman Spectrophotometer, Coleman Spectrophotometer and the like.

The principle of the diagnostic method according to the present invention is based on a series of coupled reactions. Initially, glucose present in the sample undergoing analysis reduces the ferricyanide ion in the added first reagent to ferrocyanide ions, in turn form ferricyanide ions and ferrous ions with the addition of the second reagent which comprises a source of ferric ions such as, for example, ferric chloride, a buffer and a compound of the formula I. The ferrous ions thus generated react with the compound of the formula I to produce a brilliant deep purple color. The purple color is thereafter colorimetrically measured and the glucose content of the sample quantitatively determined.

The quantitative determination of the glucose content in a specimen is carried out as follows: the optical density of the purple color developed in the sample by the method of the present invention is measured against a reagent blank at 580 nm utilizing a standard spectrophotometer such as, for example, a Coleman Spectrophotometer, employing a cuvette with a 19 mm. light path. The quantity of glucose in the specimen is determined in the conventional manner from the absorbance of the specimen with reference to the absorbance of the color produced by a glucose standard similarly treated. The glucose content of the specimen is calculated in accordance with the following formula:

\[
\text{Glucose content of specimen (mg./100 ml.)} = \frac{\text{Absorbance of specimen}}{\text{Absorbance of standard}} \times \text{Glucose content of standard (mg./100 ml.)}
\]

As indicated heretofore, the present invention provides an extremely important diagnostic tool. In addition, the method of the present invention affords a rapid and accurate determination of the glucose content of body fluids such as plasma or serum with results that are characterized by a high degree of reproducibility.

In another aspect of the present invention, the analytical compositions as described are utilized in a method of analyzing the glucose content of body fluids automatically by discrete sequential sampling or by continuous flow apparatus. The latter method consists essentially of mixing specimens in continuous flow with normal saline, dialyzing the mixture to produce an aqueous protein-free solution containing the glucose, mixing the aqueous solution with an aqueous alkaline solution containing ferricyanide ions, passing the resulting mixture through a heating bath to raise the temperature thereof to about 95° C., mixing the heated aqueous solution with an aqueous solution of a ferric salt and a compound of the formula I at a constant pH of from about 4.5 to about 5.5 and passing the resulting solution through an apparatus which quantitatively determines the glucose content thereof photometrically.

FIG. 1 is a schematic flow diagram illustrating a continuous flow automated system for analyzing glucose in biological fluids utilizing the diagnostic composition of the present invention.

FIG. 2 is a recording of the photometric response obtained when utilizing the automated system of FIG. 1.

FIG. 3 is a plot in terms of absorbance of the photometric response illustrated in FIG. 2.

In FIG. 1, a continuous flow automated testing system is, shown schematically wherein a specimen sample to be tested, i.e. serum or plasma, is drawn up in sequence from separate sample cups in the sample plate which rotates at a constant speed to provide the system with 20-60 specimen samples with a 2:1 wash ratio per hour. A sample, so drawn, is mixed in flow with normal saline and passed through a glass mixing coil of conventional design. After the mixture has passed through the mixing coil, it is next pumped through a dialyzor module that is provided with a cellophane membrane or the like through which the glucose passes in aqueous solution by dialysis. The dialyzor module is maintained at a constant temperature of 37° C. The residual, non-diffusible portion of the sample is discarded. As the aqueous glucose solution passes through the dialyzor module membrane it is admixed with an aqueous alkaline solution containing ferricyanide ions, preferably in the form of potassium ferricyanide, the glucose and the ferricyanide ions are passed in solution through a heating bath which raises the temperature of the mixture to 95° C. As this passage takes place the glucose and ferricyanide ions are reacting to form gluconic acid and ferrocyanide ions. The heated aqueous stream is then mixed in continuous flow with an aqueous solution containing ferric ions, preferably in the form of ferric chloride, and a reagent stream comprising the 5-(2-pyridyl)-2H-I,4-benzodiazepine color reagent of formula I. The color reagent, preferably 7-hydroxy-1,3-dihydro-1-(3-dimethylaminopropyl)-5-(2-pyridyl)-2H-I,4-benzodiazepine-2-one is maintained at a pH of about 4.5 to 5.5, preferably at about 5.0. The mixture is then passed through a second mixing coil. As the mixture is in transit through this coil, the ferric ions and ferrocyanide ions react to form ferricyanide ions and ferrous ions which in turn react with the benzodiazepine color reagent to form a brilliant purple coloration. Photometric measurements are then performed at 580 nm in a 15 mm. flow-cell colorimeter, i.e., the absorbance of the solution to be tested is measured at 580 nm in a flow-cell colorimeter using a 580 nm filter. The results of the colorimetric readings are recorded on a conventional recording mechanism.

The continuous flow system illustrated in FIG. 1 aspirates at a rate of 20 to 60 specimens/hour. The rate of flow in ml./min. of the materials entering the system according to a preferred technique is illustrated in FIG. 1. The materials entering the system are pumped into it by any suitable pumping means adjusted to maintain the rate of flow illustrated in FIG. 1. The mechanism for the system of the present invention can be conveniently provided by a manifold assembly prepared in accordance with the system illustrated in FIG. 1 adaptable to the Technicon Autoanalyzer.

In FIG. 2 the absorbance of solutions containing graduated amounts of glucose, e.g. 50 mg./100 ml., 100 mg./100 ml. etc. are plotted as a graph against concentration.

In FIG. 3 the photometric response of solutions containing different concentrations of glucose is demonstrated. The drawing illustrates four separate experiments, each of which represents passage through the automated system of FIG. 1 of a sequence of at least three solutions having glucose concentrations in the order of low to high to low, such as, for example, 50 mg. per 100 ml. to 400 mg. per 100 ml. to 50 mg. per 100 ml. These experiments were conducted to illustrate the sensitivity of the automated system. The difference in the response curve for similar concentration sequences represents a variance in the speed with which they were passed through the system.

The reagents utilized in connection with the automated procedure of glucose determination comprise aqueous solutions of a ferricyanide reagent, a ferric ion containing reagent and the buffered color forming reagent. The ferricyanide reagent comprises sufficient ferricyanide to react with all the glucose in the sample, for example, 0.115 g. potassium ferricyanide dissolved in 1 liter of 0.05 percent sodium hydroxide and 0.9 percent sodium chloride. The ferric ion containing solutions comprises sufficient ferric ions to react with all the ferrocyanide ions formed in the initial reaction, for example, 0.27 g. ferric chloride dissolved in 1 liter of distilled water and
buffered to a pH of about 4.5 with a sodium acetate/acetic acid buffer couple. The color-forming reagent comprises sufficiently color-forming compound to react with the ferrous in the ions formed by the reaction of the ferric ions and the ferrocyanide ions, for example, 2.0 g. of a compound of formula I, 82.0 g. of ammonium acetate and approximately 60.0 g. of glacial acetic acid in a liter of distilled water. The pH of the solution is maintained between about 4.4 and 4.6.

In the practice of the invention according to the automated procedure, iron-free distilled water is pumped through the system for 10 minutes. The system is then switched to reagent and the pumping is continued until a steady base line is obtained on the recorder chart. The base line is set to 0.01A (95 percent transmission).

The standards in the sample tray are aspirated at a rate of 20 to 60 (2:1 wash ratio) samples per hour. The specimens to be analyzed are then sampled, with a standard glucose specimen which is aspirated intermittently to insure qualitative control.

The glucose content of each sample is determined by reference to a calibration curve prepared by plotting the corrected absorbances of the glucose standards against concentrations in mg./100 ml. Table I sets forth a comparison of results obtained when 10 randomly selected plasma specimens were analyzed utilizing the automated and manual glucose procedures of the present invention.

**TABLE I**

<table>
<thead>
<tr>
<th>Specimen No.</th>
<th>Manual</th>
<th>Automated</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>105</td>
<td>101</td>
<td>-4</td>
</tr>
<tr>
<td>2</td>
<td>74</td>
<td>75</td>
<td>-1</td>
</tr>
<tr>
<td>3</td>
<td>89</td>
<td>91</td>
<td>-2</td>
</tr>
<tr>
<td>4</td>
<td>80</td>
<td>87</td>
<td>-7</td>
</tr>
<tr>
<td>5</td>
<td>78</td>
<td>76</td>
<td>-2</td>
</tr>
<tr>
<td>6</td>
<td>108</td>
<td>104</td>
<td>-4</td>
</tr>
<tr>
<td>7</td>
<td>126</td>
<td>118</td>
<td>-8</td>
</tr>
<tr>
<td>8</td>
<td>151</td>
<td>160</td>
<td>-9</td>
</tr>
<tr>
<td>9</td>
<td>94</td>
<td>94</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>77</td>
<td>75</td>
<td>-2</td>
</tr>
</tbody>
</table>

In Table II, the recovery of glucose added to pooled serum aliquots is given. An average recovery of 99.3 percent (range 94.2–103.5 percent) was realized.

**TABLE II—RECOVERY OF GLUCOSE ADDED TO SERUM**

<table>
<thead>
<tr>
<th>Specimen pool</th>
<th>Glucose, mg./100 ml</th>
<th>Glucose added, mg./100 ml</th>
<th>Total glucose, mg./100 ml</th>
<th>Glucose found, mg./100 ml</th>
<th>Recovery percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>29</td>
<td>100</td>
<td>129</td>
<td>129</td>
<td>94.2</td>
</tr>
<tr>
<td>2</td>
<td>39</td>
<td>200</td>
<td>239</td>
<td>234</td>
<td>97.9</td>
</tr>
<tr>
<td>3</td>
<td>102</td>
<td>19</td>
<td>112</td>
<td>112</td>
<td>100.6</td>
</tr>
<tr>
<td>4</td>
<td>105</td>
<td>202</td>
<td>250</td>
<td>250</td>
<td>100.5</td>
</tr>
<tr>
<td>5</td>
<td>102</td>
<td>200</td>
<td>250</td>
<td>265</td>
<td>102.0</td>
</tr>
<tr>
<td>6</td>
<td>27</td>
<td>87</td>
<td>87</td>
<td>88</td>
<td>101.1</td>
</tr>
<tr>
<td>7</td>
<td>37</td>
<td>157</td>
<td>194</td>
<td>191</td>
<td>101.7</td>
</tr>
<tr>
<td>8</td>
<td>37</td>
<td>200</td>
<td>237</td>
<td>240</td>
<td>101.3</td>
</tr>
<tr>
<td>9</td>
<td>44</td>
<td>69</td>
<td>113</td>
<td>112</td>
<td>99.1</td>
</tr>
<tr>
<td>10</td>
<td>69</td>
<td>120</td>
<td>189</td>
<td>181</td>
<td>98.6</td>
</tr>
</tbody>
</table>

For a fuller understanding of the nature and objects of the present invention, reference may be had to the following examples which are given merely as further illustrations of the invention and are not to be construed in a limiting sense.

**EXAMPLE 1**

To a stirred solution off 22.0 g. of 7-bromo-1,3-dihydro-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one in 55.0 ml. of dry N,N-dimethylformamide was treated with 11.0 ml. of a methanolic solution of sodium methoxide (0.0835 mole of NaOCH₃) and stirred for 30 minutes. After "30 minutes" 15.0 ml. of a toluene solution containing 0.0174 mole of γ-dimethylaminopropyl chloride was thereafter added, and the mixture stirred at 75°C. for 5.5 hours. Solvents were removed under reduced pressure and the residual oil was dissolved in 100 ml. of dichloromethane. The resultant solution was washed with water, dried and evaporated. The oil was next dissolved in 100 ml. of ethyl acetate and filtered over 100 g. of activated neutral alumina (Grade I). Using ethyl acetate as the eluent, 7-bromo-1,3-dihydro-1-(3-dimethylaminopropyl)-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one was recovered from the column.

**EXAMPLE 2**

The 7-bromo-1,3-dihydro-1-(3-dimethylaminopropyl)-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one formed in Example 1 was dissolved in sufficient methanol to provide a 10 percent solution. This solution was then saturated with hydrogen chloride. A sufficient amount of ether was added to cause turbidity. The resultant mixture was allowed to cool for several hours. 7-Bromo-1,3-dihydro-1-(3-dimethylaminopropyl)-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one was then dihydrochloride precipitated out on standing and was separated by filtration. The salt was recrystallized from a methanol-ether mixture as pale yellow prisms, M.P. 181°-183° dec.

**EXAMPLE 3**

This example demonstrates the applicability of the test method to either blood serum or plasma.

In the method, aliquots of plasma or serum were added in 0.1 ml. quantities to 2.4 ml. of a tungstic acid solution prepared by mixing one volume 10 percent sodium tungstate and 8 volumes N/12 sulfuric acid. Each mixture was well mixed and then centrifuged at 2,500 r.p.m. for 15 minutes. A 0.1 ml. aliquot of the clear supernatant liquid was treated with 2.0 ml. of a ferricyanide solution which had been prepared by dissolving 0.115 g. of potassium ferricyanide in one liter of a 2 percent aqueous solution of sodium carbonate. The deproteinized fluid ferricyanide mixture was heated for 5 minutes in a boiling water bath, rapidly cooled to about 25°C. and treated with 2.0 ml. of a ferric ion reagent prepared by dissolving 0.27 g. ferric chloride hexahydrate in one liter of an acetate buffer comprising 272.0 g. sodium acetate and 294.0 g. glacial acetic acid, and 2.0 ml. of a solution prepared by dissolving 2.0 g. of the compound produced in Example 2 in one liter of 1M acetate buffer. The solutions were thoroughly mixed and the absorbance of the violet blue color that develops was measured after about 10 minutes against a reagent blank at 580 nm in a Coleman Spectrophotometer using a cuvette with a 19 mm. light path.

The glucose content of the specimens was obtained by reference to a calibration curve prepared by plotting the absorbances (A) given by standard glucose solutions treated in the same manner against concentration or by the Beer-Lambert formula. Utilizing 4.0 mcg. glucose/0.1 ml. as a standard, the concentration of the specimen was calculated according to the formula:

\[
\text{Absorbance of Specimen/Absorbance of Standard} \times 100 = \text{Glucose/100 ml.}
\]

For comparative purposes, glucose analyses were also conducted on a like number of samples utilizing a modified Folin-Wu procedure as described by B. D. Tonks in American Journal of Clinical Pathology, 22:1009, (1952).

The results obtained utilizing the aforesaid two techniques are set forth in the following table:

<table>
<thead>
<tr>
<th>Specimen number</th>
<th>Benzoazepine test (mg. glucose/100 ml.)</th>
<th>Folin-Wu (mg. glucose/100 ml.)</th>
<th>Difference</th>
<th>Difference, percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>210</td>
<td>304</td>
<td>+6</td>
<td>+2.9</td>
</tr>
<tr>
<td>2</td>
<td>263</td>
<td>292</td>
<td>-9</td>
<td>-3.1</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>97</td>
<td>-3</td>
<td>-3.1</td>
</tr>
<tr>
<td>4</td>
<td>210</td>
<td>205</td>
<td>+5</td>
<td>+2.4</td>
</tr>
</tbody>
</table>
The comparative data obtained utilizing plasma specimens was completely analogous to that with serum. Utilizing similar reagents and quantities as were employed in Example 3, tests were conducted utilizing the compound prepared in Example 2 and comparing the results with the glucose content deproteinized plasma prepared as described in B. Klein in Clinical Chemistry, 5: 62, (1959) and designated Somogyi filtrates. The results obtained are set forth in the following table:

<table>
<thead>
<tr>
<th>Specimen number</th>
<th>Benzo-diazepine test (mg. glucose/100 ml.)</th>
<th>Somogyi filtrate (mg. glucose/100 ml.)</th>
<th>Difference (mg. glucose/100 ml.)</th>
<th>Difference (percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>81</td>
<td>84</td>
<td>-3</td>
<td>-3.6</td>
</tr>
<tr>
<td>2</td>
<td>278</td>
<td>294</td>
<td>-20</td>
<td>-6.8</td>
</tr>
<tr>
<td>3</td>
<td>180</td>
<td>184</td>
<td>-4</td>
<td>+2.2</td>
</tr>
<tr>
<td>4</td>
<td>95</td>
<td>103</td>
<td>-8</td>
<td>+8.4</td>
</tr>
</tbody>
</table>

EXAMPLE 5

In an analogous manner to that employed in Examples 3 and 4, serum specimens from 20 randomly selected blood samples obtained from healthy individuals and hospitalized patients were tested for glucose content. For comparative purposes, the glucose content of the same blood sample was also determined utilizing a standard glucose oxidase procedure. The glucose oxidase procedure employed herein is described in detail by R. Richterich and J. P. Colombo in Klin. Woeh., 40, 1208, (1962) and A. Saifer and S. Gerstenfeld in J. Lab. Clin. Med., 51, 448, (1958).

The results obtained utilizing the two techniques are set forth in the following table:

<table>
<thead>
<tr>
<th>Specimen No.</th>
<th>Benzo-diazepine Test</th>
<th>Glucose Oxidase Test</th>
<th>Difference (in mg)</th>
<th>(milligrams glucose/100 ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>82</td>
<td>80</td>
<td>+2</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>98</td>
<td>98</td>
<td>+0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>105</td>
<td>102</td>
<td>+3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>86</td>
<td>83</td>
<td>+3</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>82</td>
<td>86</td>
<td>-4</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>105</td>
<td>112</td>
<td>-7</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>82</td>
<td>76</td>
<td>+6</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>68</td>
<td>70</td>
<td>-2</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>135</td>
<td>129</td>
<td>+6</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>68</td>
<td>70</td>
<td>-2</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>85</td>
<td>80</td>
<td>+5</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>289</td>
<td>288</td>
<td>+1</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>89</td>
<td>84</td>
<td>+5</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>69</td>
<td>70</td>
<td>-1</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>95</td>
<td>98</td>
<td>-3</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>95</td>
<td>95</td>
<td>+0</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>89</td>
<td>84</td>
<td>+5</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>66</td>
<td>67</td>
<td>-1</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>69</td>
<td>78</td>
<td>-6</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>92</td>
<td>98</td>
<td>-6</td>
<td></td>
</tr>
</tbody>
</table>

I claim:

1. A process for quantitating the glucose content of blood serum or plasma comprising:
   a. treating a deproteinized sample of blood serum or plasma with an aqueous alkaline solution containing a source of ferricyanide ions;
   b. heating the mixture from step (a) to a temperature of from about 90°C. to about 100°C.;
   c. adding to the mixture from step (b) a source of ferric iron ions;
   d. reacting the mixture of step (c) with a benzodiazepine compound selected from the group of the compounds of the formula

wherein A is selected from the group consisting of

and

B is selected from the group of

hydrogen, lower alkyl and

n is an integer from 2 to 7; R₁ is selected from the group consisting of hydrogen, hydroxy, lower alkyl, lower alkoxy and lower alkanoyloxy; R₂ is 2-pyridyl; R₃ is selected from the group consisting of lower alkyl, hydrogen, hydrogen, lower alkyl, and

and R₅ and R₆, where taken together with their attached nitrogen atom, from a radical selected from the group consisting of piperazinyl, lower alkyl substituted piperazinyl, pyrrolodinyl, lower alkyl substituted pyrrolodinyl, piperidinyl, and lower alkyl substituted piperidinyl; R₇ is lower alkyl; and R₈ is selected from the group consisting of lower alkyl and hydrogen, and water soluble acid addition salts thereof; and e. colorimetrically quantitating the glucose present by means of said color.

2. The process in accordance with claim 1 wherein said source of ferric ions consists essentially of an aqueous solution of ferric chloride buffered to a pH of between from about 4.0 to about 5.0.

3. The process in accordance with claim 1 wherein said benzodiazepine compound is added as an aqueous solution buffered to a pH of from about 4.0 to about 5.0.

4. The process in accordance with claim 1 wherein said benzodiazepine compound is selected from the group consisting of 7-bromo-1,3-dihydro-1-(3-dimethylaminopropyl)-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one and water soluble acid addition salts thereof.
5. A process for quantitating the glucose content of blood serum or plasma comprising:
   a. treating a deproteinized sample of blood serum or plasma with an aqueous alkaline solution containing a source of ferricyanide ions;
   b. heating the mixture from step (a) to a temperature of from about 90°C to about 100°C;
   c. adding to the mixture of step (b) an aqueous solution containing ferric chloride and a buffer;
   d. adding to the mixture of step (c) an aqueous solution containing a buffer and a benzodiazepine compound selected from the group consisting of compounds of the formula

   \[
   \begin{align*}
   \text{A} & \quad \text{N} \quad \text{B} \\
   \text{R}_1 & \quad \text{R}_2 & \quad \text{R}_3 \\
   \text{C} & \quad \text{H} & \quad \text{R}_4
   \end{align*}
   \]

   wherein A is selected from the group consisting of

   \[
   \begin{align*}
   \text{C} & \quad \text{N} \\
   \text{R}_4
   \end{align*}
   \]

   and

   \[
   \begin{align*}
   \text{C} & \quad \text{N} \\
   \text{R}_4
   \end{align*}
   \]

   B is selected from the group consisting of

   \[
   \begin{align*}
   \text{O} & \quad \text{C} \\
   \text{R}_4
   \end{align*}
   \]

   and \(-\text{CH}_2\)-; \(R_4\) is selected from the group consisting of halogen, hydrogen, trifluoromethyl, nitro, and amino; \(R_4\) is selected from the group consisting of

   \[
   \begin{align*}
   \text{H} & \quad \text{R}_1 - \text{C} - \text{R}_3 \\
   \text{OH}
   \end{align*}
   \]

   hydrogen, lower alkyl and

   \[
   \begin{align*}
   \text{C}_3\text{H}_2\text{N} & \quad \text{R}_4 \\
   \text{R}_3
   \end{align*}
   \]

   \(n\) is an integer from 2 to 7; \(R_3\) is selected from the group consisting of hydrogen, hydroxy, lower alkyl, lower alkoxy and lower alkanoyloxy; \(R_4\) is 2-pyridyl; \(R_5\) is selected from the group consisting of lower alkyl, hydrogen,

   \[
   \begin{align*}
   \text{C} & \quad \text{NH}_2 \\
   \text{O}
   \end{align*}
   \]

   and

   \[
   \begin{align*}
   \text{C} & \quad \text{N} \\
   \text{O}
   \end{align*}
   \]

   and \(R_5\) and \(R_6\), where taken together with their attached nitrogen atom, form a radical selected from the group consisting of piperazinyl, lower alkyl substituted piperazinyl, pyrrolidinyl, lower alkyl substituted pyrrolidinyl, piperidinyl, and lower alkyl substituted piperidinyl; \(R_6\) is lower alkyl; and \(R_6\) is selected from the group consisting of lower alkyl and hydrogen and water soluble acid addition salts thereof and e. colorimetrically quantitating the glucose present by means of said color.

6. The process in accordance with claim 5 wherein said source of ferricyanide ions is potassium ferricyanide.

7. The process in accordance with claim 5 wherein said buffer present in the aqueous solution containing ferric chloride and the aqueous solution containing the benzodiazepine compound is a buffer pair comprising a water soluble salt of acetic acid and acetic acid.

8. A method for the quantitative analysis of the glucose content of blood plasma or serum consisting essentially of providing in continuous flow, the sequential steps comprising:
   a. combining in continuous flow a measured specimen of plasma or serum with an isotonic solution of sodium chloride;
   b. passing said mixture through a separating zone, thereby separating by dialysis in said zone from said mixture a clear aqueous solution;
   c. mixing said clear aqueous solution with a reagent comprising an aqueous solution of a water soluble ferricyanide salt;
   d. passing said aqueous mixture through heating means thereby raising the temperature thereof to from about 95°C to about 100°C;
   e. mixing said heated aqueous mixture by concurrent flow with a reagent comprising a buffered aqueous solution of a color-forming compound selected from the group consisting of compounds of the formula

   \[
   \begin{align*}
   \text{A} & \quad \text{N} \quad \text{B} \\
   \text{R}_1 & \quad \text{R}_2 & \quad \text{R}_3 \\
   \text{C} & \quad \text{H} & \quad \text{R}_4
   \end{align*}
   \]

   wherein A is selected from the group consisting of

   \[
   \begin{align*}
   \text{C} & \quad \text{N} \\
   \text{R}_4
   \end{align*}
   \]

   and

   \[
   \begin{align*}
   \text{C} & \quad \text{N} \\
   \text{R}_4
   \end{align*}
   \]

   B is selected from the group consisting of

   \[
   \begin{align*}
   \text{O} & \quad \text{C} \\
   \text{R}_4
   \end{align*}
   \]

   and \(-\text{CH}_2\)-; \(R_6\) is selected from the group consisting of halogen, hydrogen, trifluoromethyl, nitro, and amino; \(R_4\) is selected from the group consisting of

   \[
   \begin{align*}
   \text{H} & \quad \text{R}_1 - \text{C} - \text{R}_3 \\
   \text{OH}
   \end{align*}
   \]

   hydrogen, lower alkyl and

   \[
   \begin{align*}
   \text{C}_3\text{H}_2\text{N} & \quad \text{R}_4 \\
   \text{R}_3
   \end{align*}
   \]

   \(n\) is an integer from 2 to 7; \(R_3\) is selected from the group consisting of hydrogen, hydroxy, lower alkyl, lower alkoxy and lower alkanoyloxy; \(R_4\) is 2-pyridyl; \(R_5\) is selected from the group consisting of lower alkyl, hydrogen,

   \[
   \begin{align*}
   \text{C} & \quad \text{NH}_2 \\
   \text{O}
   \end{align*}
   \]

   and

   \[
   \begin{align*}
   \text{C} & \quad \text{N} \\
   \text{O}
   \end{align*}
   \]

   and \(R_5\) and \(R_6\), where taken together with their attached nitrogen atom, form a radical selected from the group consisting of piperazinyl, lower alkyl substituted piperazinyl, pyrrolidinyl, lower alkyl substituted pyrrolidinyl, piperidinyl, and lower alkyl substituted piperidinyl; \(R_6\) is lower alkyl; and \(R_6\) is selected from the group consisting of lower alkyl and hydrogen and water soluble acid addition salts thereof and e. colorimetrically quantitating the glucose present by means of said color.
; and R₃ and R₄, where taken together with their attached nitrogen atom, form a radical selected from the group consisting of piperazinyl, lower alkyl substituted piperazinyl, pyrrolidinyl, lower alkyl substituted pyrrolidinyl, piperidinyl, and lower alkyl substituted piperidinyl; R₅ is lower alkyl; and R₆ is selected from the group consisting of lower alkyl and hydrogen thereof and water soluble acid addition salts thereof thereby forming a colored solution; and
f. flowing said colored solution to an analyzing zone and
photometrically determining quantitatively the amount of glucose present during the flow of said colored solution through said analyzing zone.

9. The method in accordance with claim 8 wherein said first reagent and said second reagent are buffered to a pH of from about 4.0 to about 5.0 with a buffer pair comprising a water soluble salt of acetic acid and acetic acid.

10. The method in accordance with claim 8 wherein said colorforming compound is selected from the group consisting of 7-bromo-1,3-dihydro-1-(3-dimethylaminopropyl)-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one and water soluble acid addition salts thereof.

11. The method in accordance with claim 8 wherein said water soluble ferricyanide salt is potassium ferricyanide and said ferric iron salt is ferric chloride.

* * * *