

June 6, 1972

B. KLEIN
 COLORIMETRIC METHODS AND COMPOSITIONS FOR
 DETERMINING IRON IN BLOOD

3,667,915

Filed April 28, 1970

2 Sheets-Sheet 1

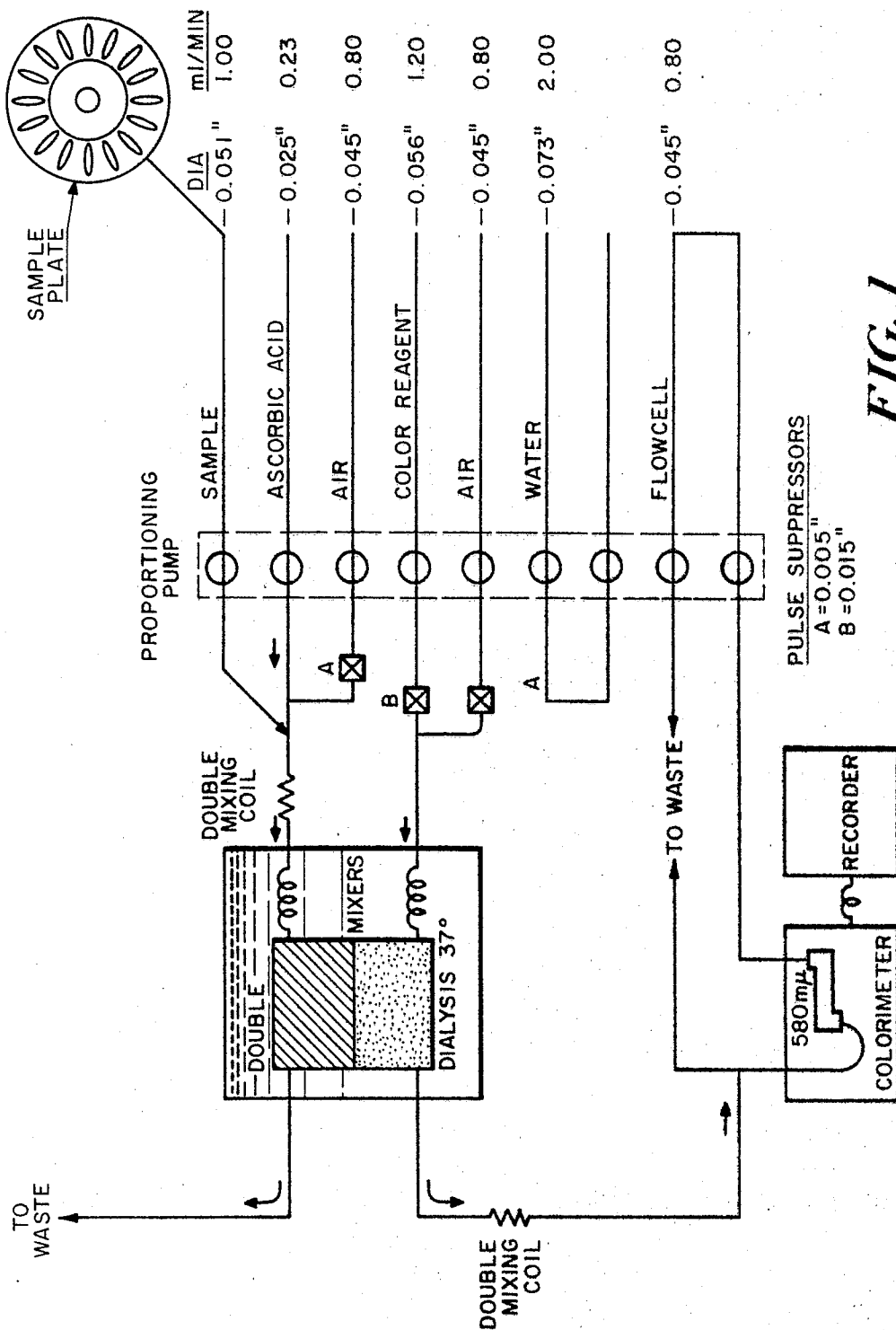


FIG. 1

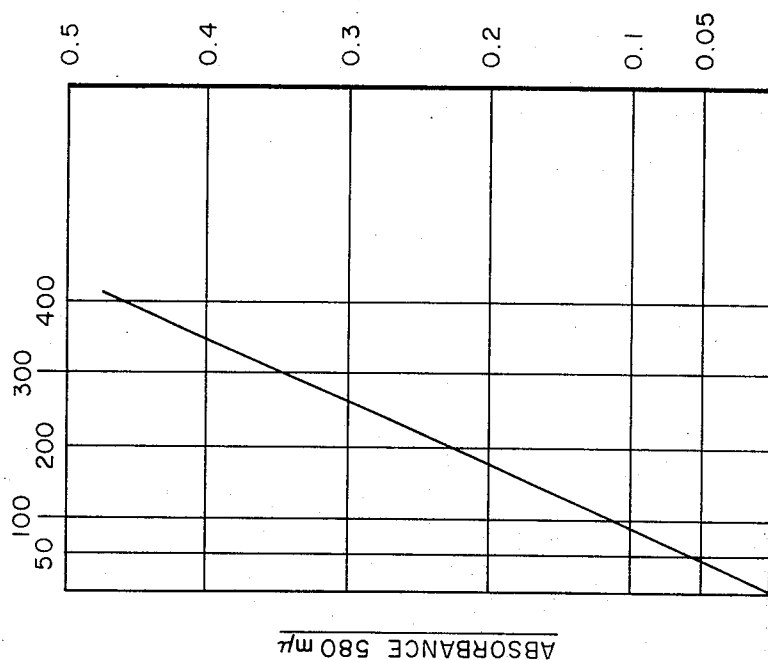
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2 Sheets-Sheet 2



$\mu\text{g Fe}/100\text{ ml}$
FIG. 3

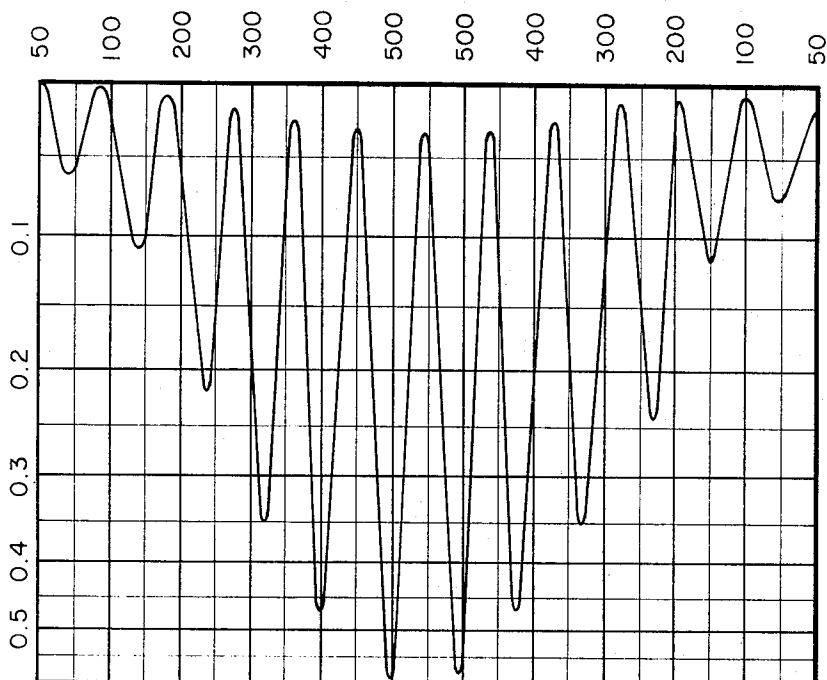


FIG. 2

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3,667,915

COLORIMETRIC METHODS AND COMPOSITIONS FOR DETERMINING IRON IN BLOOD

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Continuation-in-part of application Ser. No. 764,616,
Oct. 2, 1968. This application Apr. 28, 1970, Ser.
No. 32,755

Int. Cl. G01n 21/26, 31/22, 33/18

U.S. Cl. 23—230 B

6 Claims

ABSTRACT OF THE DISCLOSURE

A diagnostic kit is described comprising as a first reagent a reducing agent and, as a second reagent, a buffered 5-(pyridyl)-2H-1,4-benzodiazepine, or a water-soluble acid addition salt thereof. The benzodiazepine is buffered with a buffer pair comprising a water-soluble salt of acetic acid and either ascorbic acid or acetic acid. Also described is a method of utilizing these reagents in aqueous solution in a continuous flow quantitative analysis of the iron in serum.

RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Pat. application Ser. No. 764,616 filed Oct. 2, 1968 now abandoned.

BACKGROUND OF THE INVENTION

Numerous methods and techniques have been devised in the prior art for quantitatively determining the iron content of body fluids, e.g. whole blood and serum. Many of these processes utilize a reaction of the iron in either its ferric or ferrous state with various color-forming reagents such as ammonium thiocyanate, bathophenanthroline, dipyridyl and the like. These known reagents suffer from the disadvantage of forming an unstable color or, being too sensitive to trace iron contaminants thus making methods utilizing them unreliable. In some instances, these known color-forming reagents do not produce a sufficient color differentiation between the blank and the sample prepared from whole blood or serum to be tested. This makes the quantitative determination of the iron content in the sample difficult to carry out by commonly used colorimetric instruments, such as, for example, the Coleman or Spectronic 20 Spectrophotometer. The aforementioned disadvantages also pertain to an adaptation of the prior art color-forming reagents to a continuous flow system, since by its very nature, a continuous flow system must be at the same time, both accurate and insensitive to trace contaminants.

Another disadvantageous feature of prior art procedures of colorimetrically determining the iron content of body fluids is that, usually, separate reagents must be used for each step in the process. The necessity of utilizing separate reagents for each step of a multistep procedure increases the possibility of an error and/or a contaminant being introduced into the determination. This disadvantage is compounded where the color reagent utilized is sensitive to contaminating matter. A further disadvantage inherent in prior art diagnostic systems requiring a separate reagent for each step in the procedure is that a diagnostic kit embodiment is often not feasible due to the number of separate reagent containers and the like which must be utilized. The use of a number of separate reagent containers in a diagnostic kit also necessitates exacting instructions which may make its use unattractive for large scale diagnostic operations or where semi-skilled laboratory personnel must be utilized.

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The present invention provides means for overcoming all of the shortcomings of the prior art procedures and compositions alluded to herein. In accordance with the present invention, a method of quantitatively determining the iron content of serum or whole blood is provided which is accurate, produces results which are reproducible with a high degree of reliability and is adaptable to be packaged as a two-reagent diagnostic kit. The diagnostic kit as contemplated herein eliminates or at least minimizes the possibility of error inherent in the use of diagnostic methods requiring separating handling of reagents in multistep procedures.

BRIEF SUMMARY OF THE INVENTION

The present invention is concerned with a diagnostic kit suitable for the quantitative determination of iron in body fluids as well as a method for making such a determination utilizing the diagnostic reagents contained therein in aqueous solution in a continuous flow system.

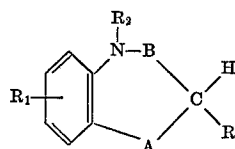
More particularly, the diagnostic kit as contemplated herein comprises a first reagent comprising a reducing agent and a second reagent comprising a 5-(2-pyridyl)-2H-1,4-benzodiazepine or water soluble salt thereof in combination with a buffer.

DETAILED DESCRIPTION OF THE INVENTION

The compositions utilized in the form of a diagnostic kit for a single analysis or in a continuous flow system for large scale diagnostic screening contain, as a first reagent, a reducing agent.

In the practice of the invention, any known chemical reducing agents can be used. These include, for example, ascorbic acid, hydrazine sulfate, thioglycolic acid, sodium thioglycolate, sodium hydrosulfite, sodium metabisulfite, sodium bisulfite, sodium sulfite, hydroxylamine hydrochloride, hydroxylamine sulfate, hydroquinone, stannous chloride, etc. In the preferred embodiment of the invention, either thioglycolic acid or ascorbic acid is utilized as the reducing agent. The use of thioglycolic acid or ascorbic acid is advantageous since it permits the reduction of the protein-free iron from the ferric state to the ferrous state to be accomplished at room temperature. The quantity of reducing agent, preferably thioglycolic acid or ascorbic acid, which is used is not particularly critical. In all instances a sufficient amount is used to insure complete conversion. Advantageously, when quantitating the iron content of whole blood, there can be provided, for example, from about 7.2×10^{-4} moles to about 1.6×10^{-3} moles of reducing agent per ml. of whole blood utilized, preferably from about 7.2×10^{-4} moles to about 1.44×10^{-3} moles of reducing agent per ml. of whole blood utilized. When quantitating the iron content of blood serum, there can be provided, for example, from about 1×10^{-6} moles to about 2×10^{-6} moles of reducing agent per ml. of serum utilized.

The second reagent of the compositions of the present invention comprises a buffered compound selected from the group consisting of compounds of the formula



wherein A is selected from the group consisting of



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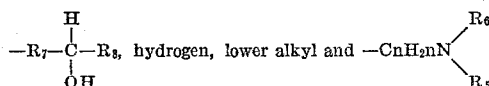
and



B is selected from



and $\text{---CH}_2\text{---}$; R_1 is selected from the group consisting of halogen, hydrogen, trifluoromethyl, nitro and amino; R_2 is selected from the group consisting of



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 and hydrogen; R_6 is selected from the group consisting of lower alkyl, hydrogen,



and $\text{---C}\equiv\text{N}$; 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_7 is lower alkyl; and R_8 is selected from the group consisting of lower alkyl and hydrogen and water soluble salts thereof.

Examples of benzodiazepine compounds of Formula I above which are particularly suitable in the practice of the 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)-1,3-dihydro-2-oxo-2H-1,4-benzodiazepin-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;
- 7-bromo-1,3-dihydro-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;
- 7-bromo-1,3-dihydro-1-(β -hydroxypropyl-2-(2-pyridyl)-2H-1,4-benzodiazepin-2-one; and
- 7-bromo-5-(2-pyridyl)-1,3-dihydro-1-[3-(N-cyanomethylamino)propyl]-2H-1,4-benzodiazepin-2-one whose preparation is disclosed in Early et al. U.S. Pat. No. 3,464,978 issued Sept. 2, 1969.

The term "lower alkyl" as used throughout this specification includes both straight and branched chain alkyl groups having from 1 to 7 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, propionyloxy, butyryloxy 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. Any conventional water soluble acid addition salt of the compounds of Formula I may be

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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 Formula I with organic or inorganic acids such as, for example, hydrochloric acid, hydrobromic acid, nitric acid, sulfuric acid, acetic acid, formic acid, succinic acid, maleic acid, p-toluene sulfonic acid and the like.

The quantity of the compound of Formula I which is utilized in the second reagent of the diagnostic kit as contemplated herein or which is added in a continuous flow diagnostic system for each sample or specimen is variable. In all instances, however, a sufficient quantity of the compound of Formula I should be provided to react with all of the ferrous ions theoretically present in the sample. In order to insure that all of the ferrous ions present in the sample or specimen have been complexed, it is preferred to add a quantity of the color-forming compound of Formula I which is in excess of that actually required to complex all of the ferrous ions which are available for complexing. Advantageously, when quantitating the iron content of whole blood, there can be provided, for example, from about 0.7×10^{-6} moles to about 7.0×10^{-6} moles of a compound of Formula I per ml. of whole blood utilized, preferably from about 2.8×10^{-6} moles to about 3.5×10^{-6} moles of a compound of Formula I per ml. of whole blood utilized. When quantitating the iron content of blood serum, there can be provided, for example, from about 0.7×10^{-6} moles to about 7.0×10^{-6} moles of a compound of Formula I per cc. of blood serum utilized, preferably from about 1.4×10^{-6} moles to about 3.5×10^{-6} moles of a compound of Formula I per cc. of blood serum utilized where the soluble salts of the compounds of Formula I are employed, a sufficient amount of these salts is utilized to provide the desired molar quantity of the compound of Formula I.

As the buffer for the compounds of Formula I, it is desirable to employ a compound or combination of compounds which will maintain the pH of the test sample in the range of from about 4.5 to about 5.5. The selection of the buffering agent or combination of agents in the practice of the invention is largely dependent on the pH of the solution at the time the buffer agent-Formula I compound combination is added. For example, where a quantitative determination of the iron content of a sample of whole blood is carried out, the alkali metal hypochlorite utilized to liberate the iron as ferric ions creates a pH of about 11.0. It is therefore necessary for optimum color development with the compound of Formula I to utilize a buffer pair comprising an acid such as, for example, ascorbic acid or acetic acid and a water soluble salt of acetic acid. In general, any water soluble salt of acetic acid can be employed in the practice of the invention with the ammonium or alkali metal/salt, e.g. sodium or potassium acetate preferred. Other water-soluble salts can be used however, if desired. The most preferred salt is sodium acetate. The quantity of the acetic acid salt used in the solution can be varied within certain limits. Further, the amount of acetic acid, or in lieu thereof, ascorbic acid employed is also variable.

In a quantitative determination of the iron content of serum, the use of a conventional acid deproteinizing agent creates a pH of about 2.0. In this instance, a water-soluble salt of acetic acid, preferably sodium acetate is utilized as the buffer to achieve the desired pH. In its broadest embodiment the invention contemplates the use of a sufficient quantity of a buffering agent to provide a final test sample, i.e., a solution in a test tube or a continuous flow system, containing the benzodiazepine color reagent, the chemical reducing agent and the buffer which has a pH within the range of from about 4.5 to about 5.5.

The buffer components may be utilized either in the dry form in admixture with the compound of Formula I or as an aqueous solution for the continuous flow system.

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In general, where a buffer pair is utilized, there is contemplated the use of a quantity of the buffer pair such that an aqueous solution thereof would contain, per liter, a ratio of from about 0.1 mole to about 2.0 moles of the acetic acid salt. Where acetic acid is employed as the acid component of the pair a final product having a pH within the range of from about 4.5 to about 5.5 is obtained by the use of a quantity of acetic acid salt such that a solution thereof contains per liter a ratio of from about 0.1 mole to about 2.0 moles of acetic acid. Where, however, ascorbic acid is used in combination with the water-soluble acetic acid salt, a final solution having a pH range of 4.5 to 5.5 is obtained by using with the quantity of acetic acid salt described above a quantity of ascorbic acid such that a liter of solution would contain from about 0.056 mole to about 0.283 mole.

Where an acid deproteinizing agent has been employed and the buffer comprises a water-soluble acetic acid salt such as, for example, sodium acetate, a final solution having a pH range of 4.5 to 5.5 is obtained by using a sufficient quantity of such a salt so that a liter of solution would contain from about 2.6 mole to about 3.3 mole.

From the foregoing description it is evident that the composition of the second reagent may be packaged in a dry state or made into aqueous stock solutions which are added into a continuous flow diagnostic system in measured amounts. The actual amounts of the reagents utilized in a specific instance may be easily calculated in relation to the specimen being tested from the molar quantities given herein. These calculations are considered to be well within the purview of a person skilled in the art.

A representative diagnostic kit for the quantitative determination of serum iron comprises, for example:

Reagent I:	Per unit test, mg.
Ascorbic acid	22.0
Reagent II:	
Formula I compound	2.1
Sodium acetate	507.9

A representative diagnostic kit for the quantitative determination of the iron content of hemoglobin comprises, for example:

Reagent I:	Per unit test mg.
Ascorbic acid	13.2
Reagent II:	Per unit test
Formula I compound	mg-- 120.0
Sodium acetate (anhydrous)	mg-- 36.0
Glacial acetic acid	ml-- 0.26

Where the compositions of the present are utilized in a continuous flow system, aqueous solutions of the reagents advantageously contain the following concentrations:

Solution I:	Percent by weight
Solution II:	
Ascorbic acid	1.00-5.00
Acetic acid water soluble salt	0.82-16.40
Acetic acid	0.60-12.00
Formula I compound	0.10-5.00

Where ascorbic acid is utilized both as a component of the buffer pair and as the chemical reducing agent, the total concentration in both solutions will advantageously be from about 1.98% to about 10.00% by weight of which from about 0.98% to about 5.00% by weight serves as a component of the buffer pair.

It is within the purview of this invention to combine the diagnostic kit reagent materials set forth above into a single solution prior to combining with the specimen to be tested. It is further within the purview of the invention to package the buffer materials as a separate reagent. The separate packaging of the buffer material permits a single diagnostic kit to be utilized for both manual and continuous flow determinations, the latter requiring less buffering material per test. Separate packaging of the buf-

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fering material also allows the user, if desired, to add buffer in increments to obtain the optimum pH.

In accordance with this invention the hemoglobin iron content of a whole blood sample can be quantitatively determined by first extracting the iron into an aqueous solution in the ferric state from the hemoglobin by addition of, for example, sodium hypochlorite, thereafter adding the reducing agent reagent in aqueous solution thereby reducing the ferric ions to ferrous ions, reacting the ferrous ions with the reagent containing the compound of Formula I and the buffer in aqueous solution to produce a brilliant deep purple color and colorimetrically quantitating the iron present in the solution. Alternatively, the reduction and the reaction with the compound of Formula I can be effected simultaneously. If desired, the quantity of hemoglobin present in the whole blood sample can be calculated utilizing the following formula:

$$\frac{\text{Hemoglobin in Sample (grams/100 ml.)}}{\text{Hemoglobin in Standard (grams/100 ml.)}} \times \frac{\text{Absorbance Sample}}{\text{Absorbance Standard}}$$

The hemoglobin in the standard is calculated by the following formula:

$$\frac{\text{Hemoglobin in Standard (grams/100 ml.)}}{\text{Iron in Standard (milligrams/100 ml.)}} = 3.47$$

The constant 3.47 is used for the conversion since it is now accepted that hemoglobin contains 0.347 percent of iron.

The above procedure provides a simple and quick method for quantitatively determining the hemoglobin content of whole blood, which is ideally suited for routine diagnostic use.

In general, the hemoglobin iron from the whole blood sample can be liberated in the ferric state by any convenient and conventional method. However, in accordance with the preferred method of this invention, the liberation of the iron is accomplished by treating the blood at room temperature with an alkali metal hypochlorite salt, preferably sodium hypochlorite. The use of a hypochlorite salt serves to liberate the iron in the form of ferric ions which, when reduced from the ferric state to the ferrous state will react with the compound of Formula I in the presence of a buffer to produce a color that can be quantitated thereby enabling the amount of iron in the blood to be determined easily and rapidly.

The quantity of alkali metal hypochlorite salt which is used in the preferred embodiment of this invention is variable. However, a sufficient quantity of hypochlorite salt should be used to liberate all of the hemoglobin iron which is present in the blood. An excess of hypochlorite salt is used to insure that all of the iron present in this sample is liberated. The precise manner in which the hypochlorite salt is incorporated into the sample is not particularly critical. Generally, it will be introduced into the sample in the form of a dilute aqueous solution. In the preferred practice of this invention, the salt is added to the sample in the form of an aqueous solution containing from about 2.5 percent to about 25 percent by weight of a hypochlorite salt, e.g. sodium hypochlorite.

In the case of blood serum, the specimen is first treated with an acidified, reducing agent-containing reagent to convert the iron present to the ferrous state and liberate it from the iron-carrier proteins. The ferrous iron is then separated from the serum proteins by extraction with a conventional acid deproteinizing agent such as, for example, trichloroacetic acid. Alternatively, the ferric iron is first liberated from the serum in aqueous solution with the deproteinizing agent and thereafter reduced to the ferrous

state. In either case, the resulting ferrous iron in the aqueous solution is reacted with the reagent containing the compound of Formula I and a water-soluble salt of acetic acid to produce a brilliant purple color. The iron content in the resulting purple solution is then quantitatively determined by standard colorimetric means.

In another aspect the analytical compositions of the present invention are utilized in a method of analyzing serum iron in an automatic or continuous flow system which consists essentially of mixing the specimen with a sufficient amount of an acidified aqueous solution of a reducing agent to convert all of the iron in the specimen to the ferrous state, dialyzing the mixture to form an aqueous solution of ferrous ions which is passed into and mixed with an aqueous solution of the above-described benzodiazepine color-forming reagent at a constant pH of from about 4.5 to about 5.0 and quantitating the iron present by photometric means.

FIG. 1 is a schematic flow diagram illustrating a continuous flow automated system for analyzing serum iron in biological fluids utilizing the diagnostic combination 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 optical density of the photometric responses 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 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 to 40 specimen samples with a 2:1 wash ratio per hour. A sample, so drawn, is mixed with an acidified reducing agent, preferably acidified ascorbic acid, which separates iron in the ferric state from the transferrin (iron-carrying protein) and reduces it to the ferrous state. The mixture is passed through glass mixing coils of conventional design. The mixture is next pumped through a dialyzer module that is provided with a cellophane membrane or the like through which the iron in the ferrous state passes by dialysis into a recipient stream comprising the 5-(2-pyridyl)-2H-1,4-benzodiazepine color reagent, preferably 7-bromo - 1,3 - dihydro - 1 - (3 - dimethylaminopropyl) - 5 - (2 - pyridyl) - 2H - 1,4 - benzodiazepin - 2 - one, maintained at a pH of about 4.5 to about 5.5, preferably at about 5.0. The dialyzer module is maintained at a constant temperature of 37° C. The residual, non-diffusable portion of the sample serum mixture is discarded. The color reagent and the iron in the ferrous state is passed through a second mixing coil. Color develops during transit through this mixing coil. 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 measured absorbances are recorded on a conventional recording mechanism.

The continuous flow system illustrated in FIG. 1 aspirates at a rate of 20 to 40 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 an adaptation in accordance with the system illustrated in FIG. 1 of the manifold system of the Technicon Auto Analyzer.

The reagents utilized in connection with the automated procedure consist of a solution of a reducing agent and a solution of the buffered color-forming compound of Formula I. The reducing reagent solution comprises sufficient reducing agent in solution so that each aliquot introduced into the system contains enough reducing agent

to convert all of the iron in the sample to the ferrous state. A preferred reducing agent solution contains, for example 0.040 g. of ascorbic acid in 99 ml. of 1.5 N-2 N hydrochloric acid and 1 ml. of a wetting agent such as, for example, a non-ionic detergent such as Actionox, Sterox, Brij 35 and the like. The color-forming reagent comprises sufficient color-forming compound so that each measured portion introduced into the system has enough to react with the ferrous iron in the sample, for example, 1 g. of a compound of Formula I, 50 g. of ammonium acetate and approximately 25 ml. of glacial acetic acid in a liter of water. The pH of the solution is maintained between about 5.1 and 5.3 (25° C.).

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

The standards in the sample tray are aspirated at 20 to 40 samples per hour (2:1 wash ratio). A cup containing iron-free water and a preliminary serum specimen are sampled. These are followed by the specimens to be analyzed.

As indicated heretofore, the present invention provides an extremely important diagnostic and analytical tool. The described diagnostic kit can be used to determine the iron in various materials such as body fluids, rapidly and accurately. More particularly, compositions contained in the diagnostic kits of the present invention can be used to quantitate the iron in blood serum or plasma and further consequently to utilize the iron content results as a basis for a rapid, convenient determination of the hemoglobin content of fresh whole blood. In addition to being a rapid and accurate method for making the determinations, the results obtained by the diagnostic compositions of the present invention are characterized by a high degree of reproducibility.

In utilizing the compositions of the present invention, the addition of the compound of Formula I to the test system produces the desired brilliant purple coloration immediately. The color deepens as the reaction proceeds to completion. In utilizing the compositions of the present invention in manual diagnosis, the mixture of specimen and reagents is allowed to stand at room temperature until color changes are no longer discernible to the naked eye and the color has become constant. In general, it has been found that full development of the purple color will occur over a period of from about 5 to 20 minutes. In the utilization of the compositions of the present invention in a continuous flow system, the use of apparatus such as, for example, double mixing coils to insure repeated thorough mixing compensates for this waiting period.

To determine the absorbance maximum, color is developed in a sample containing 6.0 µg. of iron. The absorbance of this mixture is then measured at wave lengths ranging from 450 nm. to 700 nm. in a Beckman Spectrophotometer, Model DBG, using a 1 cm. cuvette. With increasing wave length above 450 nm. there is a progressive rise in absorbance which reaches a maximum at about 580 nm. The absorbance will diminish at higher wave lengths.

The quantitative determination of iron in the sample of serum is carried out by any conventional colorimetric method, preferably as follows the absorbance of the purple colored sample is measured against a reagent blank at 580 nm. using a standard spectrophotometer, e.g., a Coleman Junior Spectrophotometer, employing a cuvette with 19 mm. light path. The quantity of iron in the specimen is determined in the conventional manner by reference to a calibration curve prepared by plotting the corrected absorbances of standards against concentrations in µg. iron/100 ml. serum. The calculation may be done

automatically utilizing conventional devices or manually according to the following formula:

$$\text{Iron Content of Sample} = \frac{\text{Absorbance of Sample}}{\text{Absorbance of Standard}} \times \text{Iron Concentration of Standard}$$

Iron Concentration
× of Standard
($\mu\text{g}/100 \text{ ml.}$)

Table I sets forth a comparison of results obtained when 13 randomly selected sera were analyzed utilizing the automated procedure of the invention and the manual procedure.

TABLE I

Comparison of Manual and Automated Serum Iron Analysis—($\mu\text{g}/100 \text{ ml.}$)

Specimen number	Manual	Automated	Difference
1-----	60	57	-3
2-----	60	57	-3
3-----	95	96	+1
4-----	85	80	-5
5-----	160	153	-7
6-----	69	73	+4
7-----	66	62	-4
8-----	77	73	-4
9-----	99	102	+3
10-----	96	96	+0
11-----	77	75	-2
12-----	130	133	+3
13-----	107	108	+1

For a fuller understanding of the nature and objects of this invention, reference may be had to the following examples which are given solely as further illustrations of the invention.

EXAMPLE 1

A stirred solution of 22.0 g. of 7-bromo-1,3-dihydro-4-(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 NaOCH_3) 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 100.0 ml. of dichloromethane. The resultant solution was washed with water, dried and evaporated. The oil was next dissolved in 100.0 ml. of ethyl acetate and filtered over 100.0 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 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. $181^\circ\text{--}183^\circ \text{dec.}$

EXAMPLE 3

This example demonstrates the applicability of the test method to a blood hemoglobin determination.

In the method, a 20.0 $\mu\text{l.}$ volume of whole blood was added to 2.0 ml. of an aqueous solution containing 17.0% by weight of sodium hypochlorite. To the mixture thus obtained was added 6.5 ml. of 2.7 percent thioglycolic acid prepared in 1 M sodium acetate. Subsequently, 1.5 ml. of an aqueous solution containing 0.33 percent of

7-bromo-1,3-dihydro-1-(3-dimethylaminopropyl)-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one dihydrochloride formed in Example 2 was added to the mixture. Almost immediately a purple coloration was noted. The reaction mixture was allowed to stand for a period of about ten minutes to allow the color to fully develop. The absorbance of the mixture was measured against a reagent blank at 580 nm. in a Coleman Spectrophotometer using a cuvette with 19 mm. light path. Using the data thus obtained, the hemoglobin content of the blood sample was calculated.

Test procedures utilizing the benzodiazepine, i.e., 7-bromo-1,3-dihydro-1-(3-dimethylaminopropyl)-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one dihydrochloride, described in the preceding paragraph were performed on blood samples obtained from healthy individuals and hospitalized patients. For comparative purposes, the hemoglobin content of the same blood samples was also determined by the cyanmethemoglobin method. In the latter procedure, a 20.0 $\mu\text{l.}$ volume of whole blood was added to 5.0 ml. of an aqueous solution containing 1.0 gram per liter of sodium bicarbonate, 50.0 mg. per liter of potassium cyanide and 200.0 mg. per liter of potassium ferricyanide. After allowing a period of ten minutes for complete color formation, the absorbance of each sample was measured against a reagent blank at 540 nm. in a Coleman Junior Spectrophotometer using a cuvette with a 19 mm. light path. The net absorbance was converted into hemoglobin concentrations by means of a standard curve constructed with dilutions of cyanmethemoglobin of known concentration.

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

TABLE.—DETERMINATION OF HEMOGLOBIN CONTENT
[Grams/100 ml.]

Sample number	Benzodiazepine test (grams per 100 ml.)	Cyanmethemoglobin (grams per 100 ml.)
1-----	15.5	15.2
2-----	10.9	11.0
3-----	14.2	13.9
4-----	10.0	10.3
5-----	14.8	15.2
6-----	15.2	15.2
7-----	13.3	13.4
8-----	4.6	4.8
9-----	14.4	14.3
10-----	5.8	5.6
11-----	13.0	13.0
12-----	16.0	16.2
13-----	11.3	11.6
14-----	12.0	12.0
15-----	15.1	15.5
16-----	15.8	15.8
17-----	13.2	13.2
18-----	14.4	14.4
19-----	14.4	14.4
20-----	12.0	12.0
21-----	10.4	10.2
22-----	12.5	12.2
23-----	12.2	12.1
24-----	11.8	11.4
25-----	11.2	10.9

EXAMPLE 4

This example demonstrates the applicability of the test method to a serum iron determination.

The iron content in five different samples of serum was determined by utilizing the following procedure.

A mixture of 2 ml. of clear unhemolyzed serum and 1.0 ml. 2 N hydrochloric acid were allowed to stand for 20 minutes and 3.0 ml. 10 percent by weight aqueous trichloroacetic acid was added under constant stirring to the serum. After standing 10 minutes the trichloroacetic acid serum mixture was centrifuged at 2,500 r.p.m. for 15 minutes. A 4.0 ml. of aliquot of the clear supernatant liquid was treated with 2.0 ml. of a 25.0% by weight aqueous solution of ammonium acetate which contained 5.0% by weight ascorbic acid and 0.1 percent by weight of 7-bromo-1,3-dihydro-1-(3-dimethylaminopropyl)-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one dihydrochloride. The absorbance of the solution was measured against a reagent

blank at 580 nm. in a Coleman Spectrophotometer using a cuvette with a 19 mm. light path. The iron content of the serum sample was calculated in the manner hereinbefore mentioned.

The iron content of these 5 samples was also determined by the method given by D. S. Fisher et al. utilizing s-tri-pyridyltriazine as described in Clin. Chem., 10: 21, (1964).

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

DETERMINATION OF SERUM IRON CONTENT

[$\mu\text{g.}/100\text{ ml.}$]		
Sample number	Ben zodiazepin test	Fisher et al.
1.....	83	78
2.....	107	111
3.....	95	99
4.....	75	79
5.....	110	118

EXAMPLE 5

This example demonstrates the applicability of the test procedure to a whole blood iron determination utilizing a buffer comprising sodium acetate and glacial acetic acid.

(a) In this example 82.0 grams of sodium acetate were dissolved in 800 ml. of deionized water. Thereafter, 60 grams of glacial acetic acid were added to and dissolved in the solution. The solution was then diluted to one liter with deionized water. The solution thus obtained had a pH of 4.8.

(b) To 80 ml. of the solution produced as described in paragraph (a) of this example there was added 3.0 grams of ascorbic acid and 0.33 gram of 7-bromo-1,3-dihydro-1-(3-dimethylaminopropyl)-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one dihydrochloride. The solution which was thus obtained was diluted to 100 ml. using the aqueous sodium acetate-acetic acid solution described in paragraph (a).

The solution produced as described in paragraph (b) of this example, was utilized in determining the iron content of fresh whole blood. From its iron content, the hemoglobin content of the blood was calculated. In the procedure a 20- $\mu\text{l.}$ sample of well-mixed whole blood was added to 2 ml. of a 2.5 percent aqueous solution of sodium hypochlorite. The liquid mixture was thoroughly mixed, following which it was allowed to stand at room temperature for a period of about three minutes. Thereafter, 4 ml. of the solution described in paragraph (b) of this example was added to the hypochlorite-blood mixture with vigorous mixing. A solution having a violet color was obtained. The color was permitted to develop by allowing the solution to stand at room temperature for a period of about five minutes. The absorbance of the solution was measured at 580 nm. in a Coleman Spectrophotometer using a cuvette with 19 mm. light path against a reagent blank set at zero absorbance. The reagent blank was prepared by carrying 20- $\mu\text{l.}$ deionized water through the entire procedure. Additionally, duplicate 20- $\mu\text{l.}$ aliquots of several dilute standard iron solutions were carried through the entire procedure. In producing the standard solution, pure iron wire (0.100 gram) was dissolved in 50 ml. of boiling 10 percent nitric acid. When the iron wire had completely dissolved, the solution was cooled and transferred quantitatively to a 100 ml. volumetric flask. It was then diluted to 100 ml. volume with deionized water to provide a stock standard. Working standard solutions were obtained by diluting the stock standard so that 1 ml. of solution contained, respectively, 0.2, 0.4, 0.5, 0.6 mg. of iron. Under the conditions of the analysis, i.e., using a 20 $\mu\text{l.}$ sample, these standards were equivalent to 5.8, 11.6, 14.5 and 17.4 grams of hemoglobin, respectively. Using the data thus obtained, the hemoglobin content of the blood sample was calculated.

The determination was made on 25 randomly selected blood samples obtained from healthy individuals and hos-

pitalized patients. For comparative purposes, the hemoglobin content of the same blood sample was also determined by the hemoglobincyanide method. This method is described in the publication, A. Hainline, "Hemoglobin" in D. Seligson, Ed., Standard Methods of Clinical Chemistry, Academic Press, Inc., New York, 1958, volume 2, page 52.

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

TABLE.—COMPARISON OF HEMOGLOBIN ANALYSIS

Specimen	[Grams per 100 ml.]	
	Test method	
	Benzodiazepine reagent (This example)	Hemoglobincyanide method
1.....	13.3	13.1
2.....	11.4	11.3
3.....	12.1	11.8
4.....	13.5	13.3
5.....	11.6	11.5
6.....	14.6	14.5
7.....	14.6	14.6
8.....	11.1	11.1
9.....	14.7	14.5
10.....	11.1	11.2
11.....	12.1	11.9
12.....	15.4	15.7
13.....	10.3	10.1
14.....	14.6	14.6
15.....	14.9	14.7
16.....	10.6	10.5
17.....	10.2	10.1
18.....	12.2	12.1
19.....	8.5	8.5
20.....	9.3	9.4
21.....	11.2	11.0
22.....	12.2	12.1
23.....	8.6	8.7
24.....	9.7	9.5
25.....	10.0	10.1

The foregoing table demonstrates the close correlation between the test method carried out using the composition of this example and the hemoglobincyanide method.

EXAMPLE 6

This example demonstrates the applicability of the test procedure to a whole blood iron determination utilizing a buffer comprising sodium acetate and ascorbic acid.

(a) In this example 82.0 grams of sodium acetate were added to and dissolved in 800 ml. of deionized water. Thereafter, 50.0 grams of ascorbic acid was added with stirring to and dissolved in the aqueous sodium acetate solution. Subsequently, 3.3 grams of 7-bromo-1,3-dihydro-1-(3-dimethylaminopropyl)-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one dihydrochloride was added to the solution. The solution thus obtained was diluted to one liter with deionized water.

(b) The solution produced as described in paragraph (a) of this example was used to determine the iron content of fresh whole blood. From its iron content the hemoglobin content of the blood was calculated. In the procedure a 20 $\mu\text{l.}$ sample of well-mixed whole blood was added to 2 ml. of a 2.5 percent aqueous solution of sodium hypochlorite. The liquid mixture was thoroughly mixed, following which it was allowed to stand at room temperature for a period of three minutes. Thereafter, 4 ml. of the solution described in paragraph (a) of this example was added to the hypochlorite-blood mixture with vigorous mixing. A solution having a violet color was obtained. The color was permitted to develop by allowing the solution to stand at room temperature for a period of about five minutes. The absorbance of the solution was measured at 580 nm. in the Coleman 6D Spectrophotometer using a cuvette with 19 mm. light path against a reagent blank. The reagent blank was prepared by carrying 20 $\mu\text{l.}$ aliquots of several dilute standard iron solutions were carried through the entire procedure. The standard iron solutions were prepared in the manner described in Example 5.

A specimen of fresh blood was analyzed by the procedure described using the above reagent solution and

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found to contain 4.96 $\mu\text{g. iron}/10 \mu\text{l.}$ (14.3 grams hemoglobin/100 ml.). To 2.0 ml. sodium hypochlorite solution of 10 $\mu\text{l.}$ of a series of solutions containing increasing quantities of iron was added and the solutions reanalyzed for iron content by the described procedure. The results obtained are given in the table.

Experiment number	Blood Fe, $\mu\text{g.}/\text{aliquot}$	Fe added, $\mu\text{g.}/\text{aliquot}$	Fe (calcd.), $\mu\text{g.}$	Fe (found), $\mu\text{g.}$	Recovery, percent
1-----	4.96	0.68	5.64	5.55	98.4
2-----	4.96	1.35	6.31	6.31	100.0
3-----	4.96	2.03	6.99	7.07	101.0

EXAMPLE 7

This example demonstrates the applicability of the test procedure to a serum iron determination.

(a) In this example 50.0 grams of ascorbic acid were added to and dissolved in a solution of 164.0 grams of sodium acetate in 800 ml. of deionized water. Thereafter, 2.0 grams of 7-bromo-1,3-dihydro-1-(3-dimethylamino-propyl)-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one, dihydrochloride were dissolved in the solution. The solution was then diluted to one liter with deionized water.

(b) The reagent solution produced as described in paragraph (a) of this example was used in the determination of the iron content of blood serum. In the procedure, 2.0 ml. of clear, unhemolyzed serum was diluted with 2.5 ml. of deionized water and the solution thus obtained was treated with 1.5 ml. of 20 percent trichloroacetic acid. The mixture was heated in a boiling water bath for fifteen minutes, cooled rapidly to room temperature and centrifuged at 2500 r.p.m. for ten minutes. A 4.0 ml. aliquot of the clear supernatant liquid was mixed with 0.35 ml. of 50 percent ammonium acetate solution. Thereafter, 1.0 ml. of the solution described in paragraph (a) of this example was added with good mixing. The absorbance of the violet solution which was thus produced was measured after twenty minutes in a Coleman 6D Spectrophotometer at 580 nm. against a reagent blank prepared by carrying 2.0 ml. of deionized water through the entire procedure. The iron content of the serum was determined by reference to a standard iron solution carried through the identical procedure.

Four serum specimens were analyzed for iron content by the described procedure. For comparative purposes, four additional specimens of the same serum were quantitated for iron content by a known procedure using bathophenanthroline sulfonate (R. J. Henry, C. Sobel and N. Chiamori, Clin. Chem. Acta 3: 523, 1958). The results are set forth in the table which follows.

Serum number	Serum iron ($\mu\text{g.}/100 \text{ ml.}$)	
	Example 7 procedure	R. J. Henry et al. procedure
1-----	81	86
2-----	78	72
3-----	91	94
4-----	78	81

The foregoing table shows that there is a close correlation between the results of serum iron determinations carried out by the method of R. J. Henry et al. and those of serum iron determinations carried out using the compositions of the present invention.

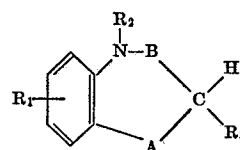
I claim:

1. A method for the quantitative determination of the iron content of serum consisting essentially of providing in continuous flow, the sequential steps comprising:

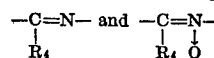
- combining a measured specimen of serum with a reagent comprising an acidified aqueous solution of a reducing agent;
- mixing said reagent composition with said specimen in continuous flow, said reducing agent converting said iron to the ferrous state;

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- passing said mixture through a separating zone, thereby separating by dialysis in said zone from said mixture an aqueous solution containing ferrous ions;
- mixing said aqueous solution containing ferrous ions by concurrent flow with a color-forming reagent comprising a buffered aqueous solution of a color-forming compound selected from the group consisting of the formula:



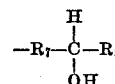
wherein A is selected from the group consisting of



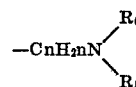
B is selected from



and $\text{---CH}_2\text{---}$; R_1 is selected from the group consisting of halogen, hydrogen, trifluoromethyl, nitro and amino; R_2 is selected from the group consisting of



hydrogen, lower alkyl and



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 and hydrogen; R_6 is selected from the group consisting of lower alkyl, hydrogen,



and $\text{---C}\equiv\text{N}$; 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_7 is lower alkyl; and R_8 is selected from the group consisting of lower alkyl and hydrogen

and water soluble acid addition salts thereof, thereby forming a colored solution; and

- flowing said colored solution to an analyzing zone and photometrically determining quantitatively the amount of iron in said colored solution during its flow through said analyzing zone.

2. The method according to claim 1 wherein said reducing agent is ascorbic acid and said color-forming compound is buffered to a pH of from about 4.5 to about 5.5 with sodium acetate.

3. The method according to claim 1 wherein said color-forming compound is 7-bromo-1,3-dihydro-1-(3-dimethylaminopropyl)-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one dihydrochloride.

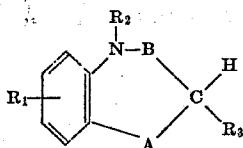
4. The method according to claim 1 wherein said color-forming compound is 7-bromo-1,3-dihydro-1-[4-(4-methyl-1-piperazinyl)butyl]-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one dihydrobromide.

5. In a process of quantitating the iron content of whole blood comprising:

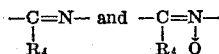
- extracting the iron from said whole blood into an aqueous solution in the ferric state with an alkali metal hypochlorite;

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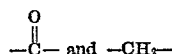
- (b) treating the ferric ions in said aqueous solution with a reducing agent to reduce said ferric ions to ferrous ions;
- (c) reacting said ferrous ions with a color-forming compound selected from the group consisting of compounds of the formula



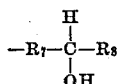
wherein A is selected from the group consisting of



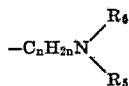
B is selected from



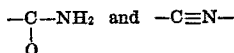
R₁ is selected from the group consisting of halogen, hydrogen, trifluoromethyl, nitro and amino; R₂ is selected from the group consisting of



hydrogen, lower alkyl and



n is an integer from 2 to 7; R₃ is selected from the group consisting of hydrogen, hydroxy, lower alkyl, lower alkoxy and lower alkanoyloxy; R₄ is 2-pyridyl; R₅ is selected from the group consisting of lower alkyl and hydrogen; R₆ is selected from the group consisting of lower alkyl, hydrogen,



and R₅ and R₆ 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; R₇ is lower alkyl; and R₈ is selected from the group consisting of lower alkyl and hydrogen; and water soluble acid addition salts thereof to produce a colored solution; and

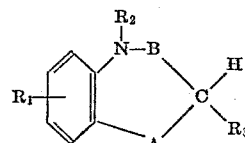
- (d) Colorimetrically quantitating the iron present in said solution by means of said color, the improvement which comprises buffering the reaction solution to a pH of from about 4.5 to about 5.5 with a buffering agent comprising sodium acetate.

6. In a process for quantitating the iron content in serum comprising:

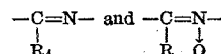
- (a) extracting the iron from said serum into an aqueous solution in a ferric state with an acid deproteinizing agent;
- (b) treating the ferric ions in said aqueous solution with a reducing agent to reduce said ferric ions to ferrous ions;

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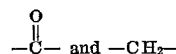
- (c) reacting said ferrous ions with a color-forming compound selected from the group consisting of compounds of the formula



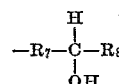
wherein A is selected from the group consisting of



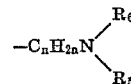
B is selected from



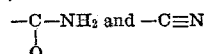
R₁ is selected from the group consisting of halogen, hydrogen, trifluoromethyl, nitro and amino; R₂ is selected from the group consisting of



hydrogen, lower alkyl and



n is an integer from 2 to 7; R₃ is selected from the group consisting of hydrogen, hydroxy, lower alkyl, lower alkoxy and lower alkanoyloxy; R₄ is 2-pyridyl; R₅ is selected from the group consisting of lower alkyl and hydrogen; R₆ is selected from the group consisting of lower alkyl, hydrogen,



and R₅ and R₆ 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; R₇ is lower alkyl; and R₈ is selected from the group consisting of lower alkyl and hydrogen; and water soluble acid addition salts thereof,

the improvement which comprises buffering the reaction solution to a pH from about 4.5 to about 5.5 with a buffering agent comprising a buffer pair consisting of a water soluble salt of acetic acid and an acid selected from the group consisting of ascorbic acid and acetic acid.

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JOSEPH SCOVORONEK, Primary Examiner

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U.S. Cl. X.R.

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