



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12Q 1/26, 1/00	A1	(11) International Publication Number: WO 92/15704 (43) International Publication Date: 17 September 1992 (17.09.92)
(21) International Application Number: PCT/US92/01659 (22) International Filing Date: 27 February 1992 (27.02.92) (30) Priority data: 661,728 27 February 1991 (27.02.91) US (71) Applicant: BOEHRINGER MANNHEIM CORPORATION [US/US]; P.O. Box 50528, 9115 Hague Road, Indianapolis, IN 46250 (US). (72) Inventor: KOST, Kent, M. ; 11229 Ashley Place, Fishers, IN 46038 (US). (74) Agents: KENNEMORE, Max, J. et al.; Boehringer Mannheim Corporation, P.O. Box 50528, 9115 Hague Road, Indianapolis, IN 46250 (US).		(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent). Published <i>With international search report.</i>
(54) Title: IMPROVED METHOD AND REAGENT FOR DETERMINATION OF AN ANALYTE (57) Abstract The dynamic range of reflectance of an assay reagent which contains a reducible mediator is expanded by the addition of an oxidizing agent which will not successfully compete with the reduction of the mediator during the performance of an assay.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	ML	Mali
AU	Australia	FR	France	MN	Mongolia
BB	Barbados	GA	Gabon	MR	Mauritania
BE	Belgium	GB	United Kingdom	MW	Malawi
BF	Burkina Faso	GN	Guinea	NL	Netherlands
BG	Bulgaria	GR	Greece	NO	Norway
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	RO	Romania
CA	Canada	IT	Italy	RU	Russian Federation
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark	MG	Madagascar		
ES	Spain				

IMPROVED METHOD AND REAGENT FOR DETERMINATION OF AN ANALYTE

FIELD OF THE INVENTION

This invention relates generally to a method for determining an analyte in a sample and to a reagent composition useful in the method. More specifically, this invention relates to a reagent having improved stability and also to a reagent having an increased dynamic range of change in response characteristics or response curve.

BACKGROUND AND PRIOR ART

The central concern of clinical chemistry is the qualitative and quantitative determination of specific analytes in samples. Of special concern is the analysis of body fluid samples, such as blood, serum, urine and so forth. Determination of the presence and/or amount of various analytes, followed by comparison to established parameters determines diagnosis of diseased or abnormal states.

The literature on analytical determination of body fluid samples is large, as the art has investigated the determination of, e.g., glucose, cholesterol, creatine, sarcosine, urea and other substances in samples of blood, serum, urine and so forth.

There are many types of assays for making such analytical determinations. One type of assay is based on a sequence of reactions. The first reaction is between an analyte and an enzyme which is specific for that analyte. In

that reaction the enzyme "oxidizes" the analyte, causing it to lose an electron. In a subsequent reaction the electron is accepted by a mediator which is thus reduced. The reduced mediator can then be used in a variety of indicator systems to determine the presence and concentration of the analyte.

One such indicator system is described in commonly assigned US Patent No. 4,929,545. In that system a ferricyanide is reduced to a ferrocyanide by the electron lost by the analyte. The ferrocyanide combines with a ferric compound to form a color. The presence and degree of color change are indications of the presence and concentration, respectively, of the analyte in a specimen.

The degree of color change can be measured by a reflectance photometer. The photometer is normally a part of a meter which uses an internal "look-up" table or an algorithm to convert the change in reflectance to analyte concentration.

Other similar systems use ferrocene or one of its analogs as a mediator.

Another system for using the reduced mediator to determine the presence or concentration of an analyte is described in commonly assigned copending U.S. patent application USSN 07/451,671, filed December 15, 1989. In that system the reduced mediator is re-oxidized by the application of a current, and the decay in the current after a specific time is measured and used to determine the concentration of the analyte.

In either system increased stability of the reagent containing the mediator is always desirable. Stability is necessary to obtain a

commercially interesting shelf life. In the system described in US Patent No. 4,949,545, as in any system where change in light reflectance is measured over a range, it is desirable to increase the range. A larger range increases the size of the increments of measured reflectance and, thus, increases accuracy.

SUMMARY OF THE INVENTION

It is an object of the present invention to increase the stability of of a reagent containing a reducible mediator.

It is also an object of this invention to increase the range of change in light reflectance of a reagent containing a reducible mediator and a component which changes color in response to reduction of the mediator.

These and other objects are accomplished by a reagent composition which comprises a reducible mediator and an oxidizing agent which will improve the stability of the reagent prior to use in an assay and which will not significantly interfere with the reduction of the mediator during performance of the assay.

This invention is based on the surprising and unexpected discoveries that such an oxidizing agent can be selected and that the selected oxidizing agent increases the range of change in reflectance of an ingredient which changes color in response to reduction of the mediator.

Any oxidizing agent which will inhibit premature reduction of the reducible mediator but which will not compete successfully with the reduction of the mediator during performance of an assay is useful in the invention. In the

color-based assay described in US Patent No. 4,949,545, when the mediator is a ferricyanide, the indicator is a soluble ferric compound such as ferric chloride or ferric sulphate and the enzyme is glucose oxidase or cholesterol oxidase, it has been discovered that an alkali metal dichromate, such as sodium or potassium dichromate is suitable for use in the invention.

A preferred reagent for use in such color-based assays for glucose in blood includes potassium ferricyanide, a soluble ferric compound, glucose oxidase and potassium dichromate in a concentration of from a trace to about 17.5×10^{-4} grams per kU of glucose oxidase. Such a reagent will have a stability many times greater than the same reagent without the dichromate and will have an increased dynamic range of change in the reflectance region of interest.

The operation of the oxidizing agent with regard to improved stability is not completely understood, although it is believed that the oxidizing agent oxidizes impurities in the other reagent components which might cause premature color change in the indicator. It is also believed that the oxidizing agent stabilizes the mediator with regard to the reducing effects of light, heat and humidity but not with regard to the reducing effects of the electron provided by the reaction between the analyte and the enzyme, surprisingly all without inactivating the enzyme.

The operation of the oxidizing agent to increase the dynamic range of the reagent dose response curve is not fully understood at the present time. Although it is an observed effect, it would be expected that, in the preferred

embodiment for example, the oxidizing agent would limit the range of color change because it would act on the reduced ferrocyanide to suppress the reaction between the ferrocyanide and the ferric compound to produce Prussian blue.

It has been found through experimentation that trace quantities of a dichromate will improve the stability of the reagent, that quantities of 1.3×10^{-4} grams per kU of enzyme will increase stability about twelve-fold at elevated temperatures, and that amounts up to 17.5×10^{-4} grams per kU of enzyme can be used without hindering color development.

It has also been found that quantities of dichromate as small as 0.9×10^{-4} grams per kU of enzyme will increase the dynamic range of the reflectance region of interest by about five percentage points, which is an increase of about 10% in the size of the range.

The reagent composition of the present invention has useful commercial application in assays for components of fluid samples. A preferred use is in an assay device such as is described in a commonly assigned patent application filed on even date herewith as USSN _____, by Ralph McCroskey et al. (Attorney's Docket No. BMID 9005) where the reagent is coated on a support in a novel teststrip architecture. In that embodiment the reagent layer is contacted by whole blood which is held against the reagent layer in a predetermined volume.

DETAILED DESCRIPTION
AND EXAMPLES

The preferred embodiments of the invention are illustrated in the following examples which are intended to be illuminative, but not limiting. Other embodiments within the scope of the invention will readily occur to those of ordinary skill in the technology.

EXAMPLE 1

Test strips were prepared and used in order to show the use of the invention in reflectance assays.

A coating mass was prepared which contained, per kilogram of the coating mass, 30 g. 4-amino butyric acid, 3.9 g. of ferric sulfate, 36 g. of ferricyanide salt and 1,000 kU of glucose oxidase. Nonreactive ingredients which made up the remainder of the mass included TiO_2 as a white pigment, TWEEN-20 (nonionic surfactant), PROPIOFAN-70D (film former) and NATROSOL (thickening agent). The pH of this mass was 4.1.

Potassium dichromate was added to aliquots of the coating mass in concentrations ranging from a trace to 17.52×10^{-4} grams as shown in Tables 1a and 1b, below. Some of the aliquots were then subjected to accelerated degradation testing in a thermal stress chamber. The accelerated testing subjects the reagent to elevated temperatures in order to predict a normal shelf life.

After stressing, the aliquots were examined for stability. Color change in the reagent before contact with a fluid sample containing an analyte specific for the enzyme indicates instability and diminished shelf life. Concentrations of dichromate in the various

aliquots, the duration of the test, the elevated temperature of the stress chamber and the resulting increase in shelf life over the aliquot with no dichromate is shown in Table 1a, below. The observations indicate that the addition of a dichromate in even trace amounts can improve significantly, while greater amounts will improve stability for longer times.

Other aliquots were subjected to samples of a glucose-containing control solution which contained glucose in the same range as normal human blood. Table 1b shows the dichromate concentrations of the various aliquots and indicates whether color development is acceptable or hindered. The results indicate that the dichromate does not hinder color development of the reagent until relatively high concentrations are reached.

TABLE 1a

Dichromate Concentration (grams/kU of enzyme)	Stability (normalized)
0	x @ 25° C. y @ 35° C.
trace	3x 3y
0.4 x 10 ⁻⁴	5x 8y
1.3 x 10 ⁻⁴	6x 12y

TABLE 1b

Dichromate grams/ kU enzyme	Color Performance
4.83×10^{-4}	Acceptable
8.76×10^{-4}	Acceptable
13.14×10^{-4}	Acceptable
17.14×10^{-4}	Color Development Slowed.

This experiment shows that the addition of a soluble dichromate to a reagent containing a mediator, an enzyme specific for an analyte and an indicator which reacts with the reduced form of the mediator to produce a color will improve the stability of the reagent without inactivating the enzyme and without suppressing color development over a useful range of concentrations.

EXAMPLE 2

Teststrips were prepared and used in order to show the improvement in the range of change in reflectance when the dichromate oxidizer is added to the reagent.

Two batches of test strip reagent is prepared as in Example 1. One batch contains no dichromate, and the other batch contains about 0.9×10^{-4} grams of dichromate per kU of enzyme. Each batch is divided into aliquots and coated

onto a transparent carrier layers which are part of test strips. Test strips made with each batch of reagent are subjected to samples of a glucose solution containing differing concentrations of glucose. The layers were then interrogated from the side opposite the coated side by a reflectance photometer. The ranges of reflectance of light at 660 nM for reagent containing the dichromate and for reagent not containing the dichromate are shown In Table 2, below.

TABLE 2

Reagent	% Reflectance at 0 mg/dl glucose	% Reflectance at 500 mg/dl glucose
Without dichromate	53	18
With dichromate	58	18

This experiment shows that the addition of the dichromate to the reagent increases the dynamic range of change in reflectance by five percentage points, which is an increase of about 10 percent in the size of the range.

The present invention has been disclosed in the above teachings and examples with sufficient clarity and conciseness to enable one skilled in the art to make and use the invention, to know the best mode for carrying out the invention and to distinguish it from other inventions and from what is old. Many variations and obvious adaptations of the invention will readily come to

mind, and these are intended to be contained within the scope of the invention as claimed below.

What is claimed is:

1. An reagent composition having an improved dynamic range of change in light absorbance useful for the determination of the presence or concentration of an analyte in a liquid sample, the composition comprising an enzyme, a mediator which is reducible in response to a reaction between an the analyte and the enzyme, a reactant which reacts with the reduced form of the mediator to change the light reflectance of the composition over a range, and an oxidizing agent which will not successfully compete with the reduction of the mediator in response to said reaction.
2. The reagent composition of claim 1 wherein the mediator is a soluble ferricyanide compound, the enzyme is glucose oxidase or cholesterol oxidase and the oxidizing agent is an alkali metal dichromate.
3. The reagent composition of claim 2 wherein the alkali metal dichromate is sodium dichromate or potassium dichromate.
4. The reagent composition of claim 3 wherein the enzyme is glucose oxidase and the alkali metal dichromate is potassium dichromate present in a concentration of from a trace to about 17.5×10^{-4} grams per Ku of enzyme.
5. A composition having an improved dynamic range of change in light absorbance useful in determining an analyte in a sample, comprising:
 - (a) an enzyme which specifically reacts with the analyte;

- (b) a soluble ferricyanide compound which is reducible in the presence of the reaction between the enzyme and the analyte to produce a ferrocyanide compound;
 - (c) a soluble ferric compound which reacts with a ferrocyanide compound to form a visually or optically detectable reaction product therebetween which reaction product changes the light absorbance of the reagent composition relative to the concentration of the analyte;
 - (d) a buffer which does not prevent formation of the reaction product, wherein the composition has a pH of from about 3.0 to about 7.0; and
 - (e) an alkali metal dichromate oxidizing agent which is present in a concentration at which it will not successfully compete with the reduction of the ferricyanide compound.
6. The composition of claim 5 wherein the alkali metal dichromate is potassium dichromate or sodium dichromate.
7. The composition of claim 6 wherein the enzyme is glucose oxidase or cholesterol oxidase.
8. The composition of claim 7 wherein the enzyme is glucose oxidase and the alkali metal dichromate is potassium dichromate present in a concentration of from a trace to about 17.5×10^{-4} grams per Ku of enzyme.
9. The composition of claims 1 or 5 coated onto a carrier.

10. Method for improving the dynamic range of light absorbance of a composition useful for the determination of the presence or concentration of an analyte in a liquid sample, the composition comprising an enzyme, a mediator which is reducible in response to a reaction between an the analyte and the enzyme and a reactant which reacts with the reduced form of the mediator to change the light absorbance of the composition over a range, the method comprising adding an oxidizing agent which will not successfully compete with the reduction of the mediator in response to said reaction, whereby the dynamic range over which the change in light absorbance occurs is increased.

11. The method of claim 10 wherein the mediator is a soluble ferricyanide compound, the enzyme is glucose oxidase or cholesterol oxidase and the oxidizing agent is an alkali metal dichromate.

12. The method of claim 11 wherein the alkali metal dichromate is sodium dichromate or potassium dichromate.

13. The method of claim 12 wherein the enzyme is glucose oxidase and the alkali metal dichromate is potassium dichromate present in a concentration of from a trace to about 17.5×10^{-4} grams per Ku of enzyme.

14. A method for improving the dynamic range of light absorbance of a composition comprising (a) an enzyme which specifically reacts with an analyte; (b) a soluble ferricyanide compound which is reducible in the presence of the

reaction between the enzyme and the analyte to produce a ferrocyanide compound; (c) a soluble ferric compound which reacts with a ferrocyanide compound to form a visually or optically detectable reaction product; and (d) a buffer which does not prevent formation of the reaction product, wherein the composition has a pH of from about 3.0 to about 7.0; the method comprising adding to the composition an alkali metal dichromate oxidizing agent which is present in a concentration at which it will not successfully compete with the reduction of the ferricyanide compound.

15. The method of claim 14 wherein the alkali metal dichromate is selected from the group consisting of potassium dichromate and sodium dichromate.

16. The method of claim 15 wherein the enzyme is glucose oxidase or cholesterol oxidase.

17. The method of claim 16 wherein the enzyme is glucose oxidase and the alkali metal dichromate is potassium dichromate present in a concentration of from a trace to about 17.5×10^{-4} grams per Ku of enzyme.

18. Method for determining an analyte which reacts with the enzyme of the composition of claims 1 or 5 in a liquid sample, the method comprising contacting the liquid sample with the composition of claims 1 or 5 and determining formation of said reaction product as a measure of the analyte.

19. The method of claim 18 wherein the liquid sample is a body fluid.

20. The method of claim 18 wherein the analyte is glucose or cholesterol.

21. Method for determining the presence of an analyte in a liquid sample, the method comprising contacting the liquid sample to the composition of claims 1 or 5 wherein said composition contains an enzyme which reacts with the analyte and determining formation of the reaction product wherein formation of the reaction product is indicative of the presence of the analyte in the sample.

22. An analytical element useful in determining an analyte in a sample, comprising

- (a) a support carrier;
- (b) a reagent layer applied to the support carrier, the reagent layer comprising the composition of claims 1 or 5.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/01659

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³ According to International Patent Classification (IPC) or to both National Classification and IPC IPC (5): C12Q 1/26, 1/00 US CL : 435/25, 4, 969; 436/805, 905											
II. FIELDS SEARCHED Minimum Documentation Searched⁴ <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 25%;">Classification System</th> <th style="width: 75%;">Classification Symbols</th> </tr> <tr> <td style="text-align: center; padding: 5px;">U.S.</td> <td style="padding: 5px;">US CL. 435/25, 4, 969; 436/805, 905</td> </tr> </table> Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched⁵ Online search conducted on APS, CA, Biosis and Dialog			Classification System	Classification Symbols	U.S.	US CL. 435/25, 4, 969; 436/805, 905					
Classification System	Classification Symbols										
U.S.	US CL. 435/25, 4, 969; 436/805, 905										
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴ <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%;">Category*</th> <th style="width: 60%;">Citation of Document,¹⁶ with indication, where appropriate, of the relevant passages¹⁷</th> <th style="width: 30%;">Relevant to Claim No. ¹⁸</th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td style="vertical-align: top;">US, A, 4,929,545 (Freitag) 29 May 1990, see the entire document.</td> <td style="text-align: center; vertical-align: top;">1-22</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">A</td> <td style="vertical-align: top;">US, A, 4,576,913 (Adachi et al) 18 March 1986, see column 5, lines 1-25.</td> <td style="text-align: center; vertical-align: top;">1-22</td> </tr> </tbody> </table>			Category*	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸	Y	US, A, 4,929,545 (Freitag) 29 May 1990, see the entire document.	1-22	A	US, A, 4,576,913 (Adachi et al) 18 March 1986, see column 5, lines 1-25.	1-22
Category*	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸									
Y	US, A, 4,929,545 (Freitag) 29 May 1990, see the entire document.	1-22									
A	US, A, 4,576,913 (Adachi et al) 18 March 1986, see column 5, lines 1-25.	1-22									
* Special categories of cited documents:¹⁵ "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family											
IV. CERTIFICATION <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; padding: 5px;"> Date of the Actual Completion of the International Search² 27 MAY 1992 </td> <td style="width: 50%; padding: 5px;"> Date of Mailing of this International Search Report² 05 JUN 1992 </td> </tr> <tr> <td style="width: 50%; padding: 5px;"> International Searching Authority¹ ISA/US </td> <td style="width: 50%; padding: 5px;"> Signature of Authorized Officer²⁰ ABDEL A. MOHAMED </td> </tr> </table>			Date of the Actual Completion of the International Search ² 27 MAY 1992	Date of Mailing of this International Search Report ² 05 JUN 1992	International Searching Authority ¹ ISA/US	Signature of Authorized Officer ²⁰ ABDEL A. MOHAMED					
Date of the Actual Completion of the International Search ² 27 MAY 1992	Date of Mailing of this International Search Report ² 05 JUN 1992										
International Searching Authority ¹ ISA/US	Signature of Authorized Officer ²⁰ ABDEL A. MOHAMED										