DIAGNOSTIC COMPOSITION FOR THE DETECTION OF PEROXIDATIVELY ACTIVE SUBSTANCES IN BODY FLUIDS

Inventors: Walter Rittersdorf; Hans-Georg Rey; Peter Rieckmann, all of Mannheim-Waldhof, Germany

Assignee: Boehringer Mannheim GmbH, Mannheim, Germany

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References Cited
UNITED STATES PATENTS

Abstract
Test strips are provided for detecting even small amounts of blood or other peroxidatively active substances in body fluids; the strips comprising a carrier containing a hydroperoxide, at least one chromogen and, as a stabilizer, at least one phosphoric or phosphonic acid amide of the formula

R1

R2

wherein
R1 is a dimethylamino, alkoxy, aryloxy, alkyl, aryl or N-morpholine radical; and
R2 and R3, which may be the same or different, are dimethylamino or N-morpholine radicals.

23 Claims, No Drawings
DIAGNOSTIC COMPOSITION FOR THE DETECTION OF PEROXIDATIVELY ACTIVE SUBSTANCES IN BODY FLUIDS

The present invention is concerned with stable test strips for the detection of blood and other peroxidative active substances in body fluids. The detection of small amounts of blood, which are not visible to the naked eye, in urine, feces or vomit, is very important for the diagnosis of hemorrhages in the stomach, intestines and urinary tract. Such hemorrhages are caused, for example, by tumors, ulcers or inflammations of the organs in question. Furthermore, free hemoglobin can also occur in the urine and plasma due to the influence of certain hemolytic toxins. Blood and hemoglobin are peroxidative active, i.e., they liberate oxygen from hydroperoxides and transfer it to certain acceptors. Other peroxidative active substances also occur in leukocytes and bacteria. The detection of these substances is important for the diagnosis of diseases and infections of the kidneys and urinary tract. Myoglobin, which is also peroxidative active, is found, for example, in the urine after a cardiac infarct. Blood occurs especially frequently in the urine when calculi are present in the bladder or kidneys.

Their peroxidative activity is especially suitable for a sensitive detection of all of these substances. The oxygen liberated from a hydroperoxide can be transferred to a chromogen which is oxidized to a colored material and thus indicates the presence of the peroxidative active substance. This reaction has already been used for quite a long time in medicinal and forensic analysis, especially for the detection of blood. It is usually carried out in test tubes or by spot tests, hydrogen peroxide usually being used as the hydroperoxide (oxidation agent). As chromogen, there is preferably used benzidine, o-tolidine or leuco malachite green.

In view of the great importance which rapid tests have recently achieved, tests of this type have also been developed in various ways for the detection of blood in body fluids. Rapid tests are usually absorbent carriers, preferably papers, which have been impregnated with all of the reagents necessary for the detection reaction and, after simply dipping into the body fluid to be tested, show a color reaction. Test papers of this type are described, for example, in U.S. Pat. Nos. 3,012,976 and 3,092,464. There are here used, as oxidation agents, organic hydroperoxides, for example cumol hydroperoxide or p-methane hydroperoxide. These test papers are said to have a sensitive reaction and, especially with regard to blood, possess a greater specificity than rapid tests with inorganic hydroperoxides.

As is to be expected, these test papers, on which the oxidation agent and chromogen are in very close contact with one another, are not stable. In German Pat. No. 1,265,453, there is described a method of separating the two components from one another by encapsulation of the organic hydroperoxide. For the encapsulation, there can be used gum arabic and gelatin, which have been cross-linked by dialdehyde polysaccharides. This process is complicated and subject to disturbances. Thus, for example, expensive machines, such as homogenisers, are needed. Furthermore, long drying times make a continuous impregnation process considerably more difficult.

We have now found that stable test papers, which are easy to produce, are obtained in accordance with the invention.

Essentially the invention comprises test strips having hydroperoxides applied thereto together with at least one phosphoric or phosphonic acid amide of the general formula

\[
\begin{align*}
R_1 & \quad V \quad O \\
R_2 & \quad R_3
\end{align*}
\]

wherein \( R_1 \) is a dimethylamino, alkoxy, aryloxy, alkyl, aryl or N-morpholine radical and \( R_2 \) and \( R_3 \), which may be the same or different, are dimethylamino or N-morpholine radicals.

The alkoxy or alkyl radicals \( R_2 \) preferably contain up to 10 carbon atoms. The aryl or arloxy radicals can be, for example, phenyl or naphthyl radicals which are optionally substituted by halogen atoms or lower alkyl or alkoxy radicals.

The test papers according to the present invention can be produced by dissolving a hydroperoxide in a solvent, preferably in a water-alcohol mixture, together with an amide of general formula (1) and optionally together with a buffer and a wetting agent and impregnating filter paper with this solution followed by drying. When using readily volatile solvents, drying only takes a few minutes. Thus, according to this process, filter papers can be continuously impregnated and dried without difficulty.

For the detection of peroxidative active substances in feces, it is also possible to incorporate the reagents with the stabilizers used according to the present invention, into a water-stable film in the manner described in German Pat. No. 1,598,153. This has the advantage that the surface of the test strip can, for reading off the color reaction, be cleaned simply by wiping it.

Thus, the present invention provides a test strip for the detection of peroxidative active substances in body fluids, comprising a carrier containing a hydroperoxide, at least one chromogen and a compound of general formula (1), as stabilizer.

Most of the compounds of general formula (1) are known and can be prepared by known and simple processes. Some of the compounds of general formula (1) are liquids at ambient temperature and some of them are very sparingly soluble in water. It is obvious that solid and readily water-soluble compounds of general formula (1) are preferred for the stabilization of test strips, since these provide especially useful commercial products. When the compounds of general formula (1), for example in the case of hexamethyl phosphoric acid triamide, are liquid, then the test strip has an oily and somewhat unsatisfactory appearance, whereas water-insoluble compounds of general formula (1) can lead to an undesirable hydrophobing effect.

The stability of the test strips according to the present invention is most surprising since substances, the action of which, as stabilizers for hydrogen peroxide, is known, for example, urea, mannitol, acetanilide and the like, here show no stabilizing action whatsoever.
The compounds of general formula (I) are preferably used in a ratio of at least one mole per mole hydroperoxide group, a two to fourfold excess having proved to be especially useful.

Compounds of general formula (I) in which one or more of the radicals R₁, R₂ and R₃ contain an aromatic ring, somewhat reduce the reactivity of the hydroperoxides so that they are only chosen as stabilizers for especially active hydroperoxides.

As hydroperoxides, there can be used the common representatives when they are not too volatile, for example, tert-butyl hydroperoxide. The solid compounds 2,5-dimethyloxane-2,5-dihydroperoxide, tetrahydropyran hydroperoxide, and diisopropyl-benzene dihydroperoxide have proved to be especially useful but liquid compounds, such as diisopropyl-benzene-hydroperoxide, cumul hydroperoxide, p-menthene hydroperoxide and pinane hydroperoxide can also be used.

The hydroperoxides can be used in amounts of 0.5-5 g., preferably of 1-3 g., per 100 ml. of impregnation solution.

Further components of the test papers according to the present invention include chromogens, buffers, wetting agents, thickening agents and possibly activators.

As chromogens, there can be used all those which can easily be oxidized to give deep-colored compounds. These include, in particular, benzidine and its homologues, especially o-tolidine. Furthermore, the heterocyclic azines according to German Pat. No. 1,648,840 have also proved to be useful.

The chromogens can be used in amounts of from 0.05-5 g., preferably of 0.2-1.0 g, per 100 ml. impregnation solution.

As buffers, there can be used, for example, citrate, phosphate, phthalate or succinate buffers, the pH value and capacity thereof being selected in such a manner that, after dipping the test strip into the body fluid, there is obtained a pH value of 4-7 and preferably of 5-6.

It is also advantageous to add to the formulation small amounts, for example about 0.05-0.5 g. per 100 ml., of a complex former, for example sodium metaphosphate or ethylene-diamine-tetracetic acid, falsely positive reaction which can be caused by traces of metals thereby being avoided.

Since the test papers can tend to bleed due to the relatively large amounts of water-soluble substances present therein, it can be desirable to add to the formulation thickening agents, for example methyl cellulose and especially of gelatin, in amounts of about 0.5-5 g. per 100 ml.

In order to increase the sensitivity of the reaction, so-called activators can also be added. These include, for example, quinoline and derivatives thereof according to German Pat. No. 1,242,905.

As wetting agents, there are preferably used long-chain organic sulfates or sulfonates, for example sodium dodecylbenzenesulfonate, dioctyl sodium sulfosuccinate or sodium lauryl sulfate, which, as is known, stabilize radical cations, such as oxidized o-tolidine.

For the production of the test strips according to the present invention, there can be used absorbent carriers, for example filter paper, cellulose or synthetic fiber fleeces, which are impregnated with solutions of the reagents in readily volatile solvents. The impregnation is preferably carried out in two steps. First, impregnation is carried out with a solution which contains an amide of general formula (I), as well as a hydroperoxide, a wetting agent, a buffer and possibly a thickening agent. Thereafter, impregnation is carried out with a solution of the indicator and possibly of the activator. When using hydrophobic monohydroperoxides, it is advisable to apply the buffer and the hydroperoxide to the carrier in separate impregnation steps.

For the production of water-stable films, all the reagents, together with a stabilizer of general formula (I), are introduced into a solution or dispersion of a film-forming substance, for example, a polystyrene or polyamide and mixed homogeneously. The mixture is then applied in a thin layer to a substrate, for example a synthetic resin carrier, and dried.

The test strips according to the present invention are, after drying, cut up into strips and preferably sealed between a synthetic resin film and a fine-mesh material in the manner described in German Pat. No. 2,118,455.

The following Examples are given for the purpose of illustrating the present invention:

**EXAMPLE 1**

A solution is prepared containing the following components:

- dioctyl sodium sulphosuccinate: 200 g.
- ethylenediamine-tetracetic acid, disodium salt: 10 g.
- 2,5-dimethyl-hexane-2,5-dihydroperoxide: 160 g.
- phosphoric acid trimorpholide: 1270 g.
- citrate buffer (1.2 molar, pH 5.25): 3.5 liters.
- ethanol: 3.0 liters.
- distilled water: ad 10.0 liters.

The solution is placed into a trough which is provided with deflection rollers. Thereafter, a filter paper strip is drawn continuously at a speed of about 2 meters/minute through the solution and dried in a drying canal with a length of 15 meters in a current of air with a temperature of 40°C.

The paper pre-treated in this manner is further impregnated in the same manner with a 0.3% solution of o-tolidine in toluene which contains 0.2% quinine as activator.

There is obtained a pure white test paper which does not become discolored when stored in the usual manner.

When the phosphoric acid trimorpholide is omitted from the above-described formulation then, even during the second impregnation, a pale green coloration occurs which, after a few days, intensifies to a strong green-brown coloration.

Practically the same properties are shown by test papers which, instead of phosphoric acid trimorpholide, contain equimolar amounts of the following amides: phosphoric acid dimethylamide dimorpholide, phosphoric acid ethyl ester dimorpholide and ethane phosphoric acid dimorpholide.

**EXAMPLE 2**

Filter paper is impregnated with the following solution I and dried for about 15 minutes at 80°C in a drying cabinet:
Solution I

- diisopropyl-benzene hydroperoxide (50%) 3.5 g.
- benzene phosphonic acid dimorpholide 6.1 g.
- sodium dodecyl-benzene sulfonate 2.0 g.
- methanol ad 100.0 ml.

Thereafter, the filter paper was impregnated with the following solutions and dried at 40°C.

Solution II

- 1.2M citrate buffer, pH 5.25 35.0 ml.
- sodium metaphosphate 0.2 g.
- distilled water ad 100.0 ml.

Solution III

- bis-(N-ethyl-quinol-2-one)-azine 0.1 g.
- quinine 0.2 g.
- toluene ad 100.0 ml.

The test papers thus obtained were characterized by an outstanding stability and do not become discolored.

Practically the same properties are shown by test papers which, instead of benzene phosphonic acid dimorpholide, contain equimolar amounts of the following amides: benzene phosphonic acid bis-(dimethylamide) or phosphoric acid phenyl ester dimorpholide.

It will be understood that the specification and examples are illustrative but not limiting of the present invention and that other embodiments within the spirit and scope of the invention will suggest themselves to those skilled in the art.

What is claimed is:

1. Test strip for the detection of peroxidatively active substances in body fluids, comprising a carrier containing a hydroperoxide, at least one chromogen and, as a stabilizer, at least one phosphoric or phosphonic acid amide of the formula

\[ R_1 \]

\[ R_2 \]

\[ R_3 = \text{PO}=O \]

wherein

- \( R_1 \) is dimethylamino, alkoxy, aryloxy, alkyl, aryl or N-morpholino and contains not more than 10 carbon atoms;
- \( R_2 \) and \( R_3 \) are individually selected from dimethylamino and N-morpholino.

2. Test strip as claimed in claim 1, wherein \( R_1 \) in the formula is dimethylamino.

3. Test strip as claimed in claim 1, wherein \( R_1 \) in the formula is lower alkoxy.

4. Test strip as claimed in claim 1, wherein \( R_1 \) in the formula is aryloxy of up to 10 carbon atoms.

5. Test strip as claimed in claim 1, wherein \( R_1 \) in the formula is lower alkyl.

6. Test strip as claimed in claim 1, wherein \( R_1 \) in the formula is aryloxy of up to 10 carbon atoms.

7. Test strip as claimed in claim 1, wherein \( R_1 \) in the formula is N-morpholino.

8. Test strip as claimed in claim 1, wherein \( R_3 \) in the formula is dimethylamino.

9. Test strip as claimed in claim 1, wherein \( R_3 \) in the formula is N-morpholino.

10. Test strip as claimed in claim 1, wherein \( R_3 \) in the formula is dimethylamino.

11. Test strip as claimed in claim 1, wherein \( R_3 \) in the formula is N-morpholino.

12. Test strip as claimed in claim 1, wherein said acid amide is phosphoric acid trimorpholide.

13. Test strip as claimed in claim 1, wherein said acid amide is phosphoric acid dimethylamide dimorpholide.

14. Test strip as claimed in claim 1, wherein said acid amide is phosphoric acid ethyl ester dimorpholide.

15. Test strip as claimed in claim 1, wherein said acid amide is ethane phosphonic acid dimorpholide.

16. Test strip as claimed in claim 1, wherein the carrier is an absorbent material impregnated with the reagents.

17. Test strip as claimed in claim 1, wherein the carrier is a water-stable film containing the reagents.

18. Test strip as claimed in claim 1, wherein there is at least one mole of said acid amide per mole of hydroperoxide.

19. Test strip as claimed in claim 1, wherein a buffer is additionally contained in the test strip.

20. Test strip as claimed in claim 19, wherein sufficient buffer is provided to give a pH of 4 to 7 to the test strip in use.

21. Test strip as claimed in claim 1, also containing at least one of a complex former, a thickening agent, an activator and a wetting agent.

22. Method of detecting a peroxidatively active substance in a body fluid, which method comprises contacting a test sample with a test strip as claimed in claim 1.

23. Method as claimed in claim 22, wherein said peroxidatively active substance is blood.