

PATENT SPECIFICATION

(11) 1390 900

1390 900

- (21) Application No. 33731/73 (22) Filed 16 July 1973
 (31) Convention Application No. 2235152 (32) Filed 18 July 1972 in (19)
 (33) Germany (DT)
 (44) Complete Specification published 16 April 1975
 (51) INT CL² G01N 33/16
 (52) Index at acceptance
 G1B 11T 11X 17AX 22A 22X



(54) TEST STRIPS

(71) We, **BOEHRINGER MANN-HEIM G.M.B.H.**, of Mannheim-Waldhof, Federal Republic of Germany, a Body-Corporate organised under the laws of the Federal Republic of Germany, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

The present invention is concerned with a test strip for the sensitive and rapid detection of very small amounts of blood and of other peroxidate-active substances in body fluids.

The detection of small amounts of blood, which are invisible to the naked eye, in urine, faeces or vomit is very important for the diagnosis of haemorrhages in the stomach, intestines and urinary tract. Such haemorrhages are caused, for example, by tumors, ulcers and inflammations of the corresponding organs. Furthermore, free haemoglobin can also occur in the urine and plasma due to the influence of certain haemolytic toxins in the blood. Blood and haemoglobin are peroxidative-active, i.e. they liberate oxygen from hydroperoxides and transfer it to certain acceptors. Other peroxidative-active substances occur in leucocytes and bacteria. The detection of these substances is important for the diagnosis of diseases and infections of the kidney and urinary tract. Myoglobin, which is also peroxidative-active, is found in the urine, for example, after a cardiac infarct. Furthermore, blood occurs especially frequently in the urine when calculi are present in the bladder or kidneys.

Their peroxidate action is especially suitable for a sensitive detection of all of these substances. The oxygen liberated from a hydroperoxide is hereby transferred to a chromogen which is oxidised to a coloured substance and thus the presence of the peroxidate-active substance is indicated. This reaction has been used for quite a long time in medicinal and forensic analysis, especially

for the detection of blood. The reaction is, as a rule, carried out in a test tube or as a spot test, hydrogen peroxide usually being employed as the hydroperoxide. As chromogen, there is preferably used benzidine, o-tolidine or leuco malachite green.

Rapid tests are usually carried out using absorbent carriers, preferably papers, which have been impregnated with all of the reagents necessary for the detection reaction. After simply dipping them into a body fluid, they show a colour reaction. Because of the great importance which rapid tests have recently achieved, they have been developed in various ways for the detection of blood in body fluids.

Since, for the detection of blood, the sensitivity of the rapid test is of decisive importance and, furthermore, it is also desirable, if possible, to include leucocytes and bacteria which are less peroxidate-active, various attempts have already been made to increase the sensitivity of the known detection reactions by means of additives. Thus, for example, German Patent Specification No. 1,242,905 describes test papers which, as activating additives, contain certain quinoline derivatives, preferably quinine. It is certainly asserted that the sensitivity can be considerably increased with the mentioned additives but, according to our own investigations, it is practically impossible also to detect leucocytes and bacteria with the test strips improved in this manner.

The activating action of quinoline *per se* on the blood detection reaction has been known for a long time (see Zeitschrift f. gerichtl. Med., 12, 216/1928); however, this, as well as its simple derivatives, are liquid or volatile and, therefore, cannot be used for a rapid test.

We have now found that test strips for the detection of peroxidate-active substances with a hitherto unachievably high sensitivity are obtained when, as activator, there is used an aromatic compound of the general formula:—

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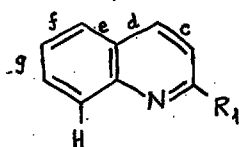
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(I),

wherein R_1 is a hydrogen atom or a methyl radical and benzene and/or pyridine rings are fused on at least one of the positions indicated with c, (d,e), f and g, provided that two adjacent rings must not simultaneously each contain a cyclic nitrogen atom, and wherein the aromatic compounds (I), apart from the positions indicated by H and R_1 , can be substituted by lower alkyl radicals (as hereinafter defined), two of which together can also form an alkylene bridge containing 3 or 4 carbon atoms.

Those compounds of general formula (I) are preferred in which R_1 is a hydrogen atom. By lower alkyl radicals, there are to be understood alkyl radicals containing up to 4 carbon atoms.

In the following, there are given examples of the most important groups of compounds coming within the scope of general formula (I); the individual compounds are all known from the literature:

1. Benzoquinolines.

1.1 benzo[c]quinoline (phenanthridine)

2-methylphenanthridine

6-methylphenanthridine

2-ethylphenanthridine

1.2 benzo[f]quinoline

3-methylbenzo[f]quinoline

1,3-dimethylbenzo[f]quinoline

1,2-tetramethylenebenzo[f]quinoline

1,2-trimethylenebenzo[f]quinoline

1.3 benzo[g]quinoline

4-methylbenzo[g]quinoline

2,4-dimethylbenzo[g]quinoline

2. Dibenzoquinolines.

2.1 dibenzo[c,f]quinoline (benzo[α]phenanthridine)

2.2 dibenzo[c,d,e]quinoline (4 - azapyrene, thebenidine)

3. Pyridoquinolines

3.1 pyrido[2,3-f]quinoline (1,7-phenanthroline)

2 - methyl - 1,7 - phenanthroline

2,8 - dimethyl - 1,7 - phenanthroline

3.2 pyrido[3,2-f]quinoline (4,7-phenanthroline)

3 - methyl - 4,7 - phenanthroline

3,8 - dimethyl - 4,7 - phenanthroline

1,3,4,8 - tetramethyl - 4,7 - phenanthroline

3.3 pyrido[2,3-g]quinoline (1,6-anthrazoline)

2,7 - dimethyl - 1,6 - anthrazoline.

Consequently, according to the present in-

vention, there is provided a test strip for the detection of peroxidate-active substances in body fluids, comprising a carrier containing a hydroperoxide, at least one chromogen and, as activator, a compound of general formula (I).

It was not to have been foreseen that the modification according to the present invention, of the known activator quinoline would lead to such an extraordinary increase in effectiveness. It is important for the present invention that only anelation of certain positions of the quinoline system enables an increase of activity to be achieved. Thus, for example, in the case of anelation of the [b]- and [h]-positions of the quinoline, i.e. in the case of acridine or of benzo[h]quinoline, the activity is reduced in comparison with quinoline.

The effectiveness of the compounds used as activators according to the present invention is difficult to explain; if, as might be regarded as being obvious, it were due to complex formation with the peroxidate-active substances, then, for example, *o*-phenanthroline, which is known to be a strong complex former, should also have a strong activating effect. However, this is not the case, whereas *m*- and *p*-phenanthroline are very effective activators.

Surprisingly, the compounds of general formula (I) do not increase the sensitivity of peroxidates of vegetable origin, for example horse radish peroxidase, but act specifically on peroxidate-active substances of human and animal origin. It is thus possible selectively to detect leucocytes, blood and blood components and bacteria in faeces or in vomit in the presence of vegetable peroxidases. In this case, myoglobin is detected with about the same degree of sensitivity as haemoglobin.

The sensitivity of the detection reaction is increased by some of the compounds of general formula (I) to such an extent that even in urine it is possible to detect individual erythrocytes which are visible on the test paper as coloured dots. Thus, for example, by means of phenanthridine, it is possible to produce a test paper with which it is still possible clearly to detect 5 erythrocytes per mm³ urine. This corresponds to a blood dilution of 1:1,000,000.

It is, of course, obvious that not all of the compounds coming within the scope of general formula (I) possess activating properties of the same degree. Thus, it is possible to adjust the sensitivity of, for example, a blood test in accordance with practical requirements. For example, test strips of increasing activity are obtained when, as activator, there are used the compounds set out in the following and in the given order: *m*-phenanthroline < *p*-phenanthroline < benzo(f)quinoline < phenanthridine.

The sensitivity can be further modified by alkyl substitution; thus, for example, by

means of a methyl substituent in the α -position to a cyclic nitrogen atom, the activity is somewhat reduced, whereas otherwise it usually leads to an increase of the activation.

Thus, for example, the sensitivity limit of a test strip which contains a weak activator, for example 2,8 - dimethyl - 1,7 - phenanthroline, is about 50 erythrocytes/mm³ urine.

The activation agents according to the present invention can be used in amounts of about 0.05—1.0 g., preferably of 0.2—0.5 g., per 100 ml. impregnation solution.

Further components of a rapid test for blood are an organic hydroperoxide, an oxidation indicator (chromogen), a buffer and a surface-active agent, as well as, if desired, a phosphoramidate, for example phosphoric acid trimorpholide, for stabilisation, as well as other conventional adjuvants.

As hydroperoxides, there can be used, for example, cumol hydroperoxide or 2,5 - dimethyl - hexane - 2,5 - dihydroperoxide, and as indicators those of the benzidine series, for example *o*-tolidine, or those of the heterocyclic azines series, for example bis - (N - ethyl - quinol - 2 - one) - azine or (N - methylbenzthiazol - 2 - one) - (1 - ethyl - 3 - phenyl - 5 - methyltriazol - 2 - one) - azine (see German Patent Specification No. 1,648,840).

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

As buffer, there can be used, for example, a citrate, phosphate, phthalate or succinate buffer, the pH value and capacity being so chosen that, after dipping the test strip into a body fluid, a pH value of 4—7, preferably of 5—6, is obtained thereon.

It is also advantageous to add to the formulation small amounts of about 0.05—0.5 g. per 100 ml. of a chelating agent, for example sodium metaphosphate or ethylenediamine-tetraacetic acid, whereby falsely positive reactions, which could be due to traces of metals, are avoided.

Since the test strips, due to the relatively large amounts of water-soluble substances present therein, could tend to bleed, it is of practical importance to add a thickening agent to the formulation, for example methyl cellulose and, in particular, gelatine, preferably in an amount of about 0.5—5 g. per 100 ml.

As wetting agent, there is preferably used a long-chained organic sulphate or sulphonate, for example sodium dodecyl-benzene sulphonate, dioctyl sodium sulphosuccinate or sodium lauryl sulphate, which, as is known, stabilises radical cations, such as oxidised *o*-tolidine. The wetting agents can be added to the impregnation solution in amounts of 0.5 to 5%, preferably of 1—3%.

For the production of the test strips according to the present invention, absorbent

carriers, for example filter paper, cellulose or synthetic resin fleeces, can be impregnated with solutions of the reagents in readily volatile solvents. This is preferably carried out in two separate steps. First, impregnation is carried out with a solution which contains a hydroperoxide, wetting agent, buffer and optionally a thickening agent. Thereafter, impregnation is carried out with a solution of an indicator and of an activator of general formula (I).

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-meshed material in the manner described in German Patent Specification No. 2,118,455.

For the detection of peroxidate-active substances in faeces, it is also possible to incorporate the activators according to the present invention, together with the reagents, in a water-stable film in the manner described in German Patent Specification No. 1,598,153. This has the advantage that the surface of the test strip can, for reading off the colour reaction, be cleaned simply by wiping it.

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

Example 1.

Filter paper is successively impregnated with the following solutions and dried at 40°C.:

Solution 1.		100
1.2M citrate buffer, pH 5.25	35.0 ml.	
ethylenediamine - tetraacetic acid, disodium salt	0.1 g.	
dioctyl sodium sulphosuccinate	2.0 g.	105
2,5 - dimethylhexane - 2,5 - dihydroperoxide (about 70%)	1.6 g.	
phosphoric acid trimorpholide	12.7 g.	110
ethanol	30.0 ml.	
distilled water	ad 100.0 ml.	
Solution 2		
<i>o</i> -tolidine	0.3 g.	
phenanthridine	0.2 g.	115
toluene	ad 100.0 ml.	

A white test paper is obtained which, upon dipping into a blood-containing urine, becomes green coloured after about 5 to 20 seconds. If the erythrocytes are intact, then the paper is green flecked. If haemolysis has taken place or if free haemoglobin is present in the urine, then the paper becomes uniformly green coloured. The sensitivity is about 5 erythrocytes/mm³ or the corresponding amount of haemoglobin. A smaller number of intact erythrocytes can, under certain

circumstances, still bring about individual green dots on the test paper. The sensitivity with regard to myoglobin corresponds to that for haemoglobin.

- 5 Leucocytes and bacteria are also detected when intact, by flecking, or when lysed, by a uniform coloration.

Example 2.

- When, in Solution 1 of Example 1, instead of 2,5 - dimethyl - hexane - 2,5 - dihydroperoxide, there is used an equimolar amount of diisopropyl-benzene hydroperoxide and the phenanthridine in Solution 2 is replaced by one activator listed hereinafter, then test papers are obtained, the sensitivity of which towards blood, leucocytes and bacteria, is of approximately the same order as the test paper of Example 1: 2 - methyl - or 2 - ethyl - phenanthridine; benzo[f]quinoline; 20 1,2 - tetramethylenebenzo[f]quinoline; benzo[g]quinoline; 4 - methyl - benzo[g]quinoline; dibenzo[c,d,e]quinoline; dibenzo[c,f]quinoline; pyrido[3,2-f]quinoline; 3 - methyl - 4,7 - phenanthroline; or pyrido[2,3-f]quinoline.

Example 3.

Filter paper is impregnated with the following solutions and dried at 40°C.:

Solution 1

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|----|--|--------------|
| 30 | 1.2M citrate buffer, pH 5.25 | 40.0 ml. |
| | ethylenediamine-tetraacetic acid, disodium salt | 0.1 g. |
| | dioctyl sodium sulphosuccinate | 2.0 g. |
| 35 | gelatine | 2.0 g. |
| | 2,5 - dimethylhexane - 2,5 - dihydroperoxide (about 70%) | 1.6 g. |
| | ethanol | 30.0 ml. |
| 40 | distilled water | ad 100.0 ml. |

Solution 2

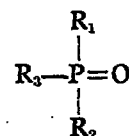
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|--|-----------------------------|--------------|
| | o-tolidine | 0.3 g. |
| | 3 - methylbenzo[f]quinoline | 0.2 g. |
| | toluene | ad 100.0 ml. |

- 45 The test paper obtained is about ten times less sensitive than the test papers according to Examples 1 and 2 (about 50—100 erythrocytes/mm³ in 30—60 seconds).

- 50 Test papers of similar sensitivity are obtained when, instead of 3 - methylbenzo[f]quinoline, there is used an equimolar amount of one of the following activators: 1,3 - dimethylbenzo[f]quinoline; 2,4 - dimethylbenzo[g]quinoline; 6 - methylphenanthridine; 55 3,8 - dimethyl - 4,7 - phenanthroline; 2,8 - dimethyl - 1,7 - phenanthroline; or 2,7 - dimethyl - 1,6 - anthrazoline.

- 60 In our co-pending Application No. 33730/73, (Serial No. 1390899) there is described and claimed a test strip for the detection of peroxidate-active substances in body

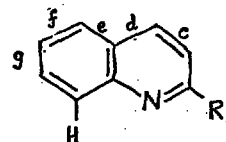
fluids, comprising a carrier containing a hydroperoxide, at least one chromogen and a compound of the general formula:—



wherein R₁ is a dimethylamine, alkoxy, aryl-oxy, alkyl, aryl or N-morpholino radical and R₂ and R₃, which may be the same or different, are dimethylamino or N-morpholino radicals, as stabiliser.

WHAT WE CLAIM IS:—

1. Test strip for the detection of peroxidate-active substances in body fluids, comprising a carrier containing a hydroperoxide, at least one chromogen and, as activator, a compound of the general formula:—



wherein R₁ is a hydrogen atom or a methyl radical and benzene and/or pyridine rings are fused on at least one of the positions indicated with c, (d,e), f and g, provided that two adjacent rings must not simultaneously contain a cyclic nitrogen atom, and wherein said activator compound can be substituted, other than in the positions indicated by H and R₁, by lower alkyl radicals (as hereinbefore defined), two of which together can also form an alkylene bridge containing 3 or 4 carbon atoms.

2. Test strip according to claim 1, wherein the carrier is an absorbent material impregnated with the reagents.

3. Test strip according to claim 2, wherein the carrier is a water-stable film with the reagents incorporated therein.

4. Test strip according to any of the preceding claims, wherein the reagent solution used for the production thereof contains 0.05—1 g. of activator per 100 ml. of solution.

5. Test strip according to claim 4, wherein the reagent solution used for the production thereof contains 0.2—0.5 g. of activator per 100 ml. of solution.

6. Test strip according to any of the preceding claims, wherein the reagent solution used for the production thereof contains 0.05—5 g. of chromogen per 100 ml. of solution.

7. Test strip according to claim 6, wherein the reagent solution used for the production thereof contains 0.2—1.0 g. of chromogen per 100 ml. of solution.



* C L M S *

circumstances, still bring about individual green dots on the test paper. The sensitivity with regard to myoglobin corresponds to that for haemoglobin.

- 5 Leucocytes and bacteria are also detected when intact, by flecking, or when lysed, by a uniform coloration.

Example 2.

- When, in Solution 1 of Example 1, instead of 2,5 - dimethyl - hexane - 2,5 - dihydroperoxide, there is used an equimolar amount of diisopropyl-benzene hydroperoxide and the phenanthridine in Solution 2 is replaced by one activator listed hereinafter, then test papers are obtained, the sensitivity of which towards blood, leucocytes and bacteria, is of approximately the same order as the test paper of Example 1: 2 - methyl - or 2 - ethyl - phenanthridine; benzo[f]quinoline; 1,2 - tetramethylenebenzo[f]quinoline; benzo[g]quinoline; 4 - methyl - benzo[g]quinoline; dibenzo[c,d,e]quinoline; dibenzo[c,f]quinoline; pyrido[3,2-f]quinoline; 3 - methyl - 4,7 - phenanthroline; or pyrido[2,3-f]quinoline.

Example 3.

Filter paper is impregnated with the following solutions and dried at 40°C.:

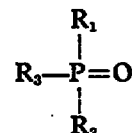
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|--|--------------|--|
| Solution 1 | | |
| 1.2M citrate buffer, pH 5.25 | 40.0 ml. | |
| ethylenediamine-tetraacetic acid, disodium salt | 0.1 g. | |
| dioctyl sodium sulphosuccinate | 2.0 g. | |
| gelatine | 2.0 g. | |
| 2,5 - dimethylhexane - 2,5 - dihydroperoxide (about 70%) | 1.6 g. | |
| ethanol | 30.0 ml. | |
| distilled water | ad 100.0 ml. | |
| Solution 2 | | |
| o-tolidine | 0.3 g. | |
| 3 - methylbenzo[f]quinoline | 0.2 g. | |
| toluene | ad 100.0 ml. | |

- 45 The test paper obtained is about ten times less sensitive than the test papers according to Examples 1 and 2 (about 50—100 erythrocytes/mm² in 30—60 seconds).

- 50 Test papers of similar sensitivity are obtained when, instead of 3 - methylbenzo[f]quinoline, there is used an equimolar amount of one of the following activators: 1,3 - dimethylbenzo[f]quinoline; 2,4 - dimethylbenzo[g]quinoline; 6 - methylphenanthridine; 3,8 - dimethyl - 4,7 - phenanthroline; 2,8 - dimethyl - 1,7 - phenanthroline; or 2,7 - dimethyl - 1,6 - anthrazoline.

- 60 In our co-pending Application No. 33730/73, (Serial No. 1390899) there is described and claimed a test strip for the detection of peroxidate-active substances in body

fluids, comprising a carrier containing a hydroperoxide, at least one chromogen and a compound of the general formula:—



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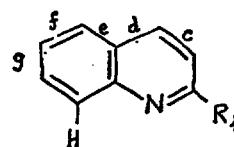
wherein R₁ is a dimethylamine, alkoxy, aryl-oxy, alkyl, aryl or N-morpholino radical and R₂ and R₃, which may be the same or different, are dimethylamino or N-morpholino radicals, as stabiliser.

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WHAT WE CLAIM IS:—

1. Test strip for the detection of peroxidate-active substances in body fluids, comprising a carrier containing a hydroperoxide, at least one chromogen and, as activator, a compound of the general formula:—

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wherein R₁ is a hydrogen atom or a methyl radical and benzene and/or pyridine rings are fused on at least one of the positions indicated with c, (d,e), f and g, provided that two adjacent rings must not simultaneously contain a cyclic nitrogen atom, and wherein said activator compound can be substituted, other than in the positions indicated by H and R₁, by lower alkyl radicals (as hereinbefore defined), two of which together can also form an alkylene bridge containing 3 or 4 carbon atoms.

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2. Test strip according to claim 1, wherein the carrier is an absorbent material impregnated with the reagents.

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3. Test strip according to claim 2, wherein the carrier is a water-stable film with the reagents incorporated therein.

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4. Test strip according to any of the preceding claims, wherein the reagent solution used for the production thereof contains 0.05—1 g. of activator per 100 ml. of solution.

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5. Test strip according to claim 4, wherein the reagent solution used for the production thereof contains 0.2—0.5 g. of activator per 100 ml. of solution.

6. Test strip according to any of the preceding claims, wherein the reagent solution used for the production thereof contains 0.05—5 g. of chromogen per 100 ml. of solution.

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7. Test strip according to claim 6, wherein the reagent solution used for the production thereof contains 0.2—1.0 g. of chromogen per 100 ml. of solution.

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8. Test strip according to any of the preceding claims, wherein the reagent solution used for the production thereof contains a buffer.
- 5 9. Test strip according to any of the preceding claims, wherein the reagent solution used for the production thereof contains a chelating agent.
- 10 10. Test strip according to claim 9, wherein the chelating agent is present in the reagent solution in an amount of from 0.05—0.5 g. per 100 ml. of solution.
- 15 11. Test strip according to any of the preceding claims, wherein the reagent solution used for the production thereof contains a thickener.
- 20 12. Test strip according to claim 11, wherein the thickener is present in the reagent solution in an amount of from 0.5—5 g. per 100 ml. of solution.
13. Test strip according to any of the preceding claims, wherein the reagent solution used for the production thereof contains a wetting agent.
14. Test strip according to claim 13, wherein the wetting agent is present in the reagent solution in an amount of from 0.5 to 5%.
15. Test strip according to claim 14, wherein the wetting agent is present in the reagent solution in an amount of from 1—3%.
- 30 16. Test strip according to any of the preceding claims, wherein the solution used for the production thereof contains a stabiliser.
- 35 17. Test strip according to claim 1, substantially as hereinbefore described and exemplified.
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Printed for Her Majesty's Stationery Office, by the Courier Press, Leamington Spa, 1975.
Published by The Patent Office, 25 Southampton Buildings, London, WC2A 1AY, from which copies may be obtained.