PATENT SPECIFICATION (11) 1389368

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(54) DIAGNOSTIC AGENT

(71) We, BOEHRINGER MANNHEIM G.M.B.H. of Mannheim-Waldhof, Federal Republic of Germany, a Body Corporate organized 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 an improved diagnostic agent for the rapid and sensitive detection of bilirubin in body fluids, preferably in urine.

The detection of bilirubin is of great importance in the diagnosis of diseases of the liver and gall bladder. Thus, in the case of liver damage and of occlusion of the gall bladder duct, the early appearance of bilirubin in the urine occurs even before the bilirubin content of the serum increases and clinical symptoms of jaundice appear. On the other hand, this type of jaundice can be differentiated from haemolytic icterus in which an increased bilirubin level can only be ascertained in the serum but not in the urine.

Methods for the detection of bilirubin in the urine have been known for a long time. Of importance are hereby the methods which depend upon the evaluation of the coloured compounds formed by the coupling of bilirubin with diazonium salts. For this purpose, diazosulphanilic acid has previously been most used.

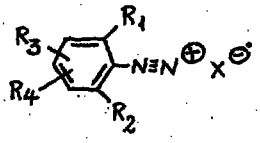
Recently, in medical practice and in clinical laboratories, the so-called rapid tests have been used more and more as a diagnostic aid. These are simple devices, usually test papers, which permit the carrying out of the detection or semi-quantitative determination of pathological components of the body in a rapid and certain manner, even by untrained personnel, such as medical auxiliaries.

Thus, for example, German Patent Specification No. 1,102,444 describes a reagent tables containing a diazonium salt and a strong acid which is placed on a spot plate moistened with a body fluid and, after moistening with water, makes the bilirubin visible as a violet ring on the substrate.

It is obvious that such a diagnostic agent is relatively laborious to use. For a wide use in medical practice and at the sick bed, it is, however, desirable that the rapid test is such that it can be read off without difficulty after simply dipping into the body fluid to be investigated. This purpose is best fulfilled by the so-called test papers; these are absorbent carriers which have been impregnated with all the reagents necessary for the detection reaction.

It is admittedly already known from German Patent Specification No. 2,007,013 to use test papers which contain certain 2,4- and 2,5-disubstituted diazonium salts for the detection of bilirubin in serum and urine. However, this method is relatively insensitive and, according to our investigations, also suffers from certain disadvantages with regard to the speed of the reaction and the sensitivity, which renders questionable its practical utility. There is, of course, the possibility of increasing the sensitivity of the detection reaction but, in this case, fresh difficulties arise. Thus, the urobilinogen which is also present in urine also reacts in the presence of these activators and simulates an increased bilirubin content. Furthermore, the normal disturbing reactions with components of the urine are hereby also activated, which can manifest themselves as false colorations or even give falsely positive indications.

We have now, surprisingly, found that when using diazonium salts of the general formula:—



(I)

wherein $X^{\ominus}$ is a stabilising anion, R_1 and R_2, which may be the same or different, are halogen atoms or lower alkyl or alkoxy radi-

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cals and R_3 and R_4, which may be the same or different, are hydrogen or halogen atoms or nitro groups or lower alkyl or alkoxy radicals, whereby the aromatic ring contains either 1—3 halogen atoms or one nitro group but not both together, the above-mentioned difficulties no longer occur, even when activators for the coupling reaction are used at the same time. Thus, these diazonium salts (I) can be used to prepare test strips which react selectively with bilirubin not only in urine but also in serum.

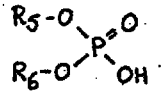
The diazonium salts of general formula (I) are either known or can be prepared from known aromatic amines by diazotisation.

The lower alkyl or alkoxy radicals in the diazonium salts (I) are to be understood to contain up to 3 carbon atoms.

The diazonium salts (I) are preferably used as the fluoborates, the stability of which is known; however, other stable salts, such as aryl-sulphonates, especially naphthalene-1,5-disulphonates, can also be used.

Diazonium salts of general formula (I) are preferred in which R_1 and R_2 are both halogen atoms, especially chlorine or bromine atoms, and the other symbols stand for hydrogen atoms.

As activators for the coupling reaction, there can be used, apart from anionic wetting agents, especially compounds of the general formula:—



(II)

wherein R_5 and R_6, which may be the same or different, are unsubstituted or substituted aliphatic, cycloaliphatic, araliphatic or aromatic hydrocarbon radicals.

The radicals R_5 and R_6 are preferably identical since phosphoric acid diesters of this type are particularly simple to prepare. The aliphatic radicals R_5 and R_6 can be straight-chained or branched and can contain up to 18 carbon atoms, the effectiveness of the esters initially increasing with an increasing number of carbon atoms but beyond a chain length of about 14 carbon atoms, the effectiveness again decreases due to the increasingly hydrophobic character of the esters. As cycloalkyl radicals, those with 5—8 carbon atoms are especially preferred. Preferred substituents in the aliphatic and cycloaliphatic radicals include, for example, halogen atoms, especially chlorine and bromine atoms, nitro groups and alkoxy radicals containing up to 8 carbon atoms. With regard to the stability of the test strips, phosphoric acid diesters of general formula (II) are preferred in which R_5 and R_6 both represent aromatic radicals. Preferred aromatic radicals include mono- and polynuclear, unsubstituted and substituted aryl radicals, for example, phenyl, xylyl, tolyl, chlorophenyl, nitrophenyl and naphthyl radicals. As araliphatic radicals, there are preferably used, for example, phenyl or naphthyl radicals which are connected to the phosphoric acid residue *via* an alkylene bridge containing up to 3 carbon atoms.

For the preparation of an especially useful test strip according to the present invention, an absorbent carrier, preferably filter paper, is impregnated with a solution which contains 0.01—2.0 g., preferably 0.05—0.5., per 100 ml. of a diazonium salt of general formula (I), as well as 3—20 g., preferably 5—15 g., per 100 ml. of a phosphoric acid diester of general formula (II) and 2—30 g. of a solid inorganic or organic acid and thereafter dried. However, it is also possible to carry out the diazotisation in the impregnation solution or on the absorbent carrier. In this case, an appropriate amine is used as starting material which is then reacted with a nitrite in the usual manner.

The bilirubin test strips according to the present invention can also contain stabilising agents for the diazonium salts, for example sodium fluoborate, magnesium sulphate, sodium metaphosphate, aryl-sulphonates or the like.

Furthermore, the test strips can also contain wetting agents in order to improve the absorbency of the test papers. In principle, all wetting agents can be used which are still surface-active in the strongly acidic medium after dipping into a body fluid to be tested, for example cationic agents (e.g. lauryl-pyridinium chloride), non-ionic agents (e.g. polyoxyethylene triglyceride) and anionic agents, especially the sulphonates and sulphates (e.g. dodecyl-benzene-sulphonate). The latter can additionally possess activating properties.

The wetting agents can be used in the impregnation solution in concentrations of 0.1—2% and preferably 0.2—0.5%.

As solvents or solvent mixtures for the impregnation, there can be used all those which do not react with the diazonium salts, in which all the components are soluble and which have a low boiling point in order that, upon drying, the diazonium salts are not subjected to too high a temperature. It has also proved to be advantageous to impregnate individual components in separated working steps. Thus, for example, a mixture of a diazonium salt (I) and an acid, for example metaphosphoric acid, can first be used for impregnation from an aqueous solution and then a further impregnation is carried out with a solution of phosphoric acid diester (II) in ethyl acetate or chloroform.

As absorbent carrier, it is preferred to use filter paper but other absorbent carriers, for example, glass fibre paper and synthetic fibre fabrics and fleeces made from acid-resistant fibres, such as polyester and polypropylene fibres, can also be used. The term "test

paper" used herein is to be understood to include these materials.

Thus, the present invention provides a test strip for the detection of bilirubin in body fluid, which comprises an absorbent carrier containing at least one diazonium salt of general formula (I) and an amount of an acid sufficient for the bilirubin-diazo coupling reaction.

Phosphoric acids of general formula (II) are known (see, for example, Houben-Weyl, Methoden d. Org. Chem., Vol. XII/2, pp 226 *et seq.*). Since the compounds (II) themselves have an acidic reaction, they can at least partially replace the acid needed for the coupling reaction. However, additional amounts of acid are usually necessary. For this purpose, a whole series of solid acids can be used. Of particular use are, for example, oxalic acid, citric acid, potassium bisulphate and, especially having regard to the stability of the diazonium salt, commercially available metaphosphoric acid, which can contain up to about 60% of its sodium salt.

The test strips according to the present invention react with bilirubin-containing urine in about 5 to 30 seconds to give red to blue colour shades. With normal urine, there is obtained a yellow reaction colour. Urobilinogen reacts, without disturbing, with a yellow colour, red shades only occurring when unusually large amounts thereof are present in the urine (more than about 8—10 mg.%). The detection of serum bilirubin is, of course, also possible with the use of the test strips according to the present invention.

The impregnated absorbent carriers can be cut up into long strips and then rolled up so that, when needed, a small piece can be torn or cut off. They can also be cut up into small rectangles and stuck or sealed on to one end of narrow strips of synthetic resins. It is of particular advantage when the test papers are sealed between two synthetic resin films according to the method described in German Patent Specification No. 1,546,307 or between a synthetic resin film and a meshwork as described in German Patent Specification No. 2,118,455, because there is then no danger that the reagents will be washed out upon dipping into a body fluid.

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 then dried at 40°C.

Solution I:

2,6-dichlorobenzene-diazonium fluoborate	0.08 g.
metaphosphoric acid	10.0 g.
citric acid	3.0 g.
distilled water	ad 100.0 ml.

Solution II:

phosphoric acid diphenyl ester	5.0 g.
dodecyl - benzene - sulphonic acid sodium salt	0.4 g.
ethyl acetate	ad 100.0 ml.

Upon dipping into bilirubin-containing urine, there are obtained, depending upon the concentration, after about 10—20 seconds, red to violet-red colorations of the test paper; the limit of sensitivity is about 0.1—0.3 mg.% bilirubin. With normal urine, yellow colorations are obtained and the same applies to urobilinogen-containing urine. If the urine contains more than about 8—10 mg., then this yellow coloration gradually changes to a red coloration after about 1 minute.

With bilirubin-containing serum, there are obtained red-violet colorations of increasing intensity, the limit of sensitivity being about 0.4 to 0.6 mg.%.

Test papers which contain 2,6 - dibromobenzene - diazonium fluorborate react in an analogous manner.

Test papers of analogous composition but which contain 2,4 - dichlorobenzene - diazonium fluorborate (cf. German Patent Specification No. 2,007,013) react with bilirubin with a blue colour. With urobilinogen-containing urine, they give a deep violet coloration after only a few seconds.

Example 2.

Test papers which react in a manner analogous to that described in Example 1 are obtained when the phosphoric acid diphenyl ester is replaced by one of the following phosphoric acid esters:

phosphoric acid di - *o* - tolyl - esters, phosphoric acid di - *p* - tolyl ester; phosphoric and bis - 3,5 - xylol ester; phophoric acid bis - *o* - chlorophenyl ester; phosphoric acid bis - *p* - chlorophenyl ester; phosphoric acid bis - *p* - nitrophenyl ester; phosphoric acid dicyclohexyl ester; or phosphoric acid dipentyl ester.

Example 3.

Filter paper is impregnated with a solution of the following composition and then dried at 40°C,:

2,6 - dichlorobenzene - diazonium fluoborate	0.16 g.
metaphosphoric acid	15.0 g.
dodecyl - benzene - sulphonic acid sodium salt	1.0 g.
methanol	20.0 ml.
distilled water	ad 100.0 ml.

This test paper reacts, after 20—30 seconds, with a violet colour with bilirubin-containing urine, the sensitivity limit being about 0.7 to 0.9 mg.%. With urine having a

high content of urobilinogen (about 8—12 mg.%), a pale red colour appears after about 1—2 minutes.

A test paper of the same composition except that it contains 2,4 - dichlorobenzene - diazonium fluorborate, reacts with bilirubin with a blue-violet colour and, with urobilinogen-containing urine, a disturbing violet coloration occurs after only a short period of time.

Example 4.

Filter paper is impregnated with a solution of the following composition and then dried at 40°C.:

2 - methoxy - 4,6 - dibromobenzene - diazonium fluoborate	0.12 g.
citric acid	10.0 g.
sodium metaphosphate	3.0 g.
distilled water	ad 100.0 ml.

This paper is then impregnated with a 5% solution of phosphoric acid diphenyl ester in ethyl acetate.

The properties of this test paper, as well as of test papers containing an equimolar amount of one of the diazonium salts set out in the following Table I, correspond to those of Example 1. The reaction colours with bilirubin are, in the case of the halogen- and alkyl- substituted compounds, more blue-violet and, in the case of the alkoxy- and nitro-substituted compounds, more grey-blue.

TABLE I

2 - methyl - 4,6 - dibromobenzene - diazonium fluoborate
2 - methyl - 4 - chloro - 6 - bromobenzene - diazonium fluoborate
2 - methyl - 4 - bromo - 6 - chlorobenzene - diazonium fluoborate
4 - methyl - 2,6 - dibromobenzene - diazonium fluoborate
4 - ethyl - 2,6 - dibromobenzene - diazonium fluoborate
4 - methyl - 2,6 - diiodobenzene - diazonium fluoborate
2,6 - dimethyl - 3 - nitrobenzene - diazonium fluoborate
2 - ethoxy - 4,6 - dibromobenzene - diazonium fluoborate
4 - methoxy - 2,6 - dibromobenzene - diazonium fluoborate
2,6 - dimethoxy - 3,5 - dibromobenzene - diazonium fluoborate
2,6 - dimethoxy - 4 - nitrobenzene - diazonium fluoborate
2,5 - dimethyl - 4,6 - dibromobenzene - diazonium fluorborate

Example 5.

A solution of the following composition is prepared at 5—10°C., then impregnated on to filter paper and dried at 40°C.:

2 - methyl - 6 - chloroaniline	0.14 g.
sodium nitrite	0.07 g.
metaphosphoric acid	10.0 g.
distilled water	ad 100.0 ml.

This test paper is thereafter impregnated with a 10% solution of phosphoric acid diphenyl ester in ethyl acetate and dried at 40°C.

Analogous test papers can be prepared with an equimolar amount of one of the following substituted aniline derivatives: 2,6 - dimethyl - 3 - bromoaniline; 2,6 - dimethyl - 4 - bromoaniline; 2,6 - dimethoxy - 3 - bromoaniline; and 2,6 - dimethoxy - 4 - chloroaniline.

The properties of the test papers obtained correspond approximately to those of the test papers according to Examples 1 and 4.

Example 6.

Filter paper is impregnated with a solution of the following composition and dried at 40°C.:

2,4,6 - trichlorobenzene - diazonium toluenesulphonate	0.3 g.
metaphosphoric acid	10.0 g.
distilled water	ad 100.0 ml.

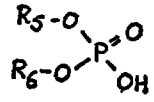
This paper is subsequently impregnated with a 10% solution of commercially available isooctyl - phosphoric acid in methylene chloride. This acid is a mixture of about 55% of the diester and 45% of the monoester of phosphoric acid with isomeric C_8 alcohols.

The properties of this test paper correspond to those of the test paper according to Example 1.

Test papers which contain an equimolar amount of one of the following diazonium salts possess practically the same properties:

2,4,6 - tribromobenzene - diazonium naphthalene - disulphonate;
2,4,6 - tribromo - 3 - methyl - benzene - diazonium fluoborate;
2 - methyl - 4,6 - dibromo - 5 - chlorobenzene - diazonium fluoborate;
2,4,6 - tribromo - 3 - methoxy - benzene - diazonium fluoborate;
2,3.6 - tribromobenzene - diazonium fluoborate.

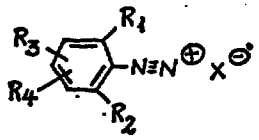
In our co-pending Application No. 38429/73 (Serial No. 1389366) there is described and claimed a test paper for the detection of bilirubin in body fluids, which contains a diazonium salt capable of coupling with bilirubin in the presence of an acid and an amount of an acid which is sufficient for the coupling reaction, as well as at least one phosphoric acid diester of the general formula:—



wherein R_5 and R_6 which can be the same or different, are unsubstituted or substituted aliphatic, cycloaliphatic, araliphatic or aromatic hydrocarbon radicals.

WHAT WE CLAIM IS:—

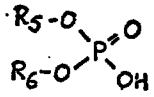
1. A test strip for the detection of bilirubin in body fluids, which comprises an absorbent carrier containing a diazonium salt of the general formula:—



wherein $X^{\ominus}$ is a stabilising anion, R_1 and R_2, which may be the same or different, are halogen atoms or lower alkyl or alkoxy radicals and R_3 and R_4, which may be the same or different, are hydrogen or halogen atoms or nitro groups or lower alkyl or alkoxy radicals, whereby the aromatic ring contains either 1—3 halogen atoms or a nitro group but not both together, and an amount of an acid sufficient for the bilirubin-diazo coupling reaction.

2. A test strip according to claim 1, which additionally contains an activator for the coupling reaction.

3. A test strip according to claim 2, wherein the activator is a phosphoric acid diester of the general formula:—



in which R_5 and R_6, which may be the same or different, are unsubstituted or substituted aliphatic, cycloaliphatic, araliphatic or aromatic hydrocarbon radicals.

4. A test strip according to any of the preceding claims, wherein the stabilising ion in the diazonium salt is a fluoborate or aryl - sulphonate ion.

5. A test strip according to claim 3 or 4, wherein the absorbent carrier has been impregnated with a solution containing, per 100 ml., 0.01—2.0 g. of the diazonium salt, 3—20 g. of the phosphoric acid diester and 2—30 g. of a solid inorganic or organic acid.

6. A test strip according to claim 5, wherein the impregnation solution contained 0.05—0.5 g. of the diazonium salt per 100 ml.

7. A test strip according to claim 5 or 6, wherein the impregnation solution contained 5—15 g. of the phosphoric acid diester per 100 ml.

8. A test strip according to any of the preceding claims, wherein a stabilising agent is also present.

9. A test strip according to claim 8, wherein the stabilising agent is sodium fluoborate, magnesium sulphate, sodium metaphosphate or an aryl - sulphonate.

10. A test strip according to any of the preceding claims, wherein a wetting agent is also present.

11. A test strip according to claim 10, wherein the wetting agent has been applied from a solution containing 0.1—2% of the wetting agent.

12. A test strip according to claim 11, wherein the wetting agent has been applied from a solution containing 0.2—0.5% of the wetting agent.

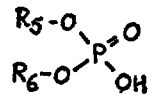
13. A test strip according to any of the preceding claims, wherein the absorbent carrier is a filter paper, glass fibre paper or a fabric or fleece made from acid-resistant synthetic fibres.

14. A test strip according to any of the preceding claims, wherein the absorbent carrier is either stuck or sealed on to one end of narrow strips of synthetic resin or is sealed between two synthetic resin films or between a synthetic resin film and a meshwork.

15. A test strip according to claim 1 for the detection of bilirubin in body fluids, substantially as hereinbefore described and exemplified.

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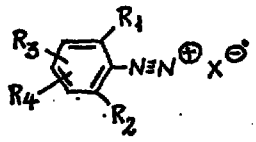
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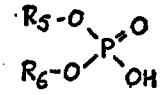
1. A test strip for the detection of bilirubin in body fluids, which comprises an absorbent carrier containing a diazonium salt of the general formula:—



wherein $X^{\ominus}$ is a stabilising anion, R_1 and R_2, which may be the same or different, are halogen atoms or lower alkyl or alkoxy radicals and R_3 and R_4, which may be the same or different, are hydrogen or halogen atoms or nitro groups or lower alkyl or alkoxy radicals, whereby the aromatic ring contains either 1—3 halogen atoms or a nitro group but not both together, and an amount of an acid sufficient for the bilirubin-diazo coupling reaction.

2. A test strip according to claim 1, which additionally contains an activator for the coupling reaction.

3. A test strip according to claim 2, wherein the activator is a phosphoric acid diester of the general formula:—



in which R_5 and R_6, which may be the same or different, are unsubstituted or substituted aliphatic, cycloaliphatic, araliphatic or aromatic hydrocarbon radicals.

4. A test strip according to any of the preceding claims, wherein the stabilising ion in the diazonium salt is a fluoborate or aryl - sulphonate ion.

5. A test strip according to claim 3 or 4, wherein the absorbent carrier has been impregnated with a solution containing, per 100 ml., 0.01—2.0 g. of the diazonium salt, 3—20 g. of the phosphoric acid diester and 2—30 g. of a solid inorganic or organic acid.

6. A test strip according to claim 5, wherein the impregnation solution contained 0.05—0.5 g. of the diazonium salt per 100 ml.

7. A test strip according to claim 5 or 6, wherein the impregnation solution contained 5—15 g. of the phosphoric acid diester per 100 ml.

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10. A test strip according to any of the preceding claims, wherein a wetting agent is also present.

11. A test strip according to claim 10, wherein the wetting agent has been applied from a solution containing 0.1—2% of the wetting agent.

12. A test strip according to claim 11, wherein the wetting agent has been applied from a solution containing 0.2—0.5% of the wetting agent.

13. A test strip according to any of the preceding claims, wherein the absorbent carrier is a filter paper, glass fibre paper or a fabric or fleece made from acid-resistant synthetic fibres.

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15. A test strip according to claim 1 for the detection of bilirubin in body fluids, substantially as hereinbefore described and exemplified.

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