Process and diagnostic agent for the detection of redox agents

The invention concerns processes and diagnostic agents which depend upon redox reactions which contain iodates for the avoidance of disturbances by ascorbic acid.

In clinical and pharmaceutical chemistry, biochemistry and foodstuff chemistry, redox systems are of great importance for determination methods of substrates and enzymes. For such determination methods, there exist the most varied photometric processes. However, of especial importance are so-called rapid diagnostics. They are agents in which all reagents are present in absorbent carriers or in films in dry form. The agents are brought into contact with the investigation fluid, the resultant colour can be assessed visually or with reflection photometers.

The materials to be investigated in the above-mentioned fields of chemical analysis, e.g. urine, blood, foodstuffs, medicinal preparations and the like, are frequently characterised by a more or less large content of reducing agents, the commonest of which is ascorbic acid. It is clear that redox reactions can be sensitively disturbed by strong reducing agents, such as ascorbic acid. It is known that in the case of the detection of glucose with rapid diagnostics, which depend upon the basis of the reaction of GOD-POD

redox indicators, falsely negative findings can be brought about by ascorbic acid. The H_2O_2 resulting from glucose with the help of GOD (glucose oxidase) acts, namely, with POD (peroxidase) on the ascorbic acid instead of on the indicator with oxidation and is thus removed from the determination.

It is further known that rapid diagnostics for the detection of blood in urine also react falsely negative in the presence of ascorbic acid. The ascorbic acid here presumably reduces the coloured material formed by the haemoglobin-catalysed oxidation.

As example for falsely positive findings due to ascorbic acid is to be mentioned the determination of NADH or NADPH with the help of the reduction of tetrazolium salts to give coloured formazanes. Ascorbic acid here acts in the same manner and increases the measurement signal.

Because of the especial importance and of the extent of the disturbances by reducing agents, especially ascorbic acid, attempts have, therefore, not been lacking to remove this from the investigation fluids or to develop non-disturbed processes and agents.

The following processes are known:

- oxidation with iodine solution and removal of the excess iodine with thiosulphate

- oxidation with manganese dioxide and filtering off of the unconsumed oxidation agent
- oxidation with alkaline H_2O_2
- treatment of the sample solution with anion exchangers.

All these processes require a laborious treatment of the sample solution, furthermore, especially in the case of rapid diagnostics, an integrated solution of the problem is very expensive. Test papers are also known in which urine must first be chromatographed through a zone with anion exchanger (DE-AS 15 98 008) in order then to be able to react undisturbed in the case of further running to the actual reagent zone. Tests with ion exchanger zones are commercially available for glucose and galactose. They have a complicated construction and, due to the necessary chromatography time, the analysis time is considerably increased in comparison with previously known rapid tests.

A further possibility for the removal of ascorbic acid from fluids or the interference elimination of diagnostic agents depends upon the addition of ascorbate oxidase in optical tests and rapid diagnostics (DE-AS 26 25 834).

Although this process is usable especially in the case of small ascorbic acid concentrations, the problem as a whole cannot be regarded as solved,

namely, for the following reasons:

- Ascorbate oxidase reacts only with ascorbic acid itself but not with metabolites, such as the glucuronide and the sulphate, as well as other reducing agents.
- The oxidation of ascorbic acid by ascorbate oxidase is relatively slow so that, in the case of test fluids which can contain more than 100 mg. ascorbic acid/dl., uneconomically large amounts of ascorbate oxidase must be used.
- In certain cases, the enzyme ascorbate oxidase is relatively rapidly destroyed by aggressive reagents.

Thus e.g. the cumol hydroperoxide used in a urine-blood test must be enclosed in microcapsules (USP 4 129 417).

Surprisingly, it has now been found that one can develop processes and diagnostic agents, especially rapid diagnostics, which are not disturbed by ascorbic acid and its metabolites even when these are present in relatively large amounts when to the known formulations or to the test system to be investigated one additionally adds iodate. It must be regarded as surprising that iodate admittedly oxidises the ascorbic acid sufficiently quickly but not the substrates important in clinical chemistry, furthermore many of the redox indicators usual in analysis and their particular coloured reaction products, as well as conventional adjuvants. Thus, for

example, under the usual analysis conditions (pH 5 - 9) and within the usual analysis times, there are not attacked by iodate:

- substrates:

carbohydrates (glucose, galactose and the like), cholesterol, glycerol (from triglycerides), uric acid, NADH and NADPH etc.

-substrate oxidases:

glucose oxidase, galactose oxidase, cholesterol oxidase, glycerol oxidase, uricase etc.

- indicators:

benzidine derivatives (o-tolidine, 3,3',5,5'-tetramethyl-benzidine), heterocyclic azines (azino-bis-benzo-thiazolone-sulphonic acid), formazanes as reduction products of tetrazolium salts etc.

- peroxidases:

horseradish peroxidase, haemoglobin (blood)

- adjuvants:

aryl semicarbazides which are described as stabilisers of oxidation indicators (DE-OS 27 16 060).

That this property of iodate is surprising and cannot be deduced, perhaps from the oxidation potential, is shown by a comparison with other halogen compounds. According to Cotton-Wilkinson "Anorganische Chemie" Weinheim, 1970, 2nd edition, p. 532, their standard potentials amount to:

- iodate + 0.26V
- periodate + 0.39V
-iodine + 0.54V
- bromate + 0.61V
-chlorate + 0.63V

Whereas now periodate and free iodine, besides ascorbic acid, oxidise most indicators, especially benzidine derivatives, bromate and chlorate, in spite of their higher oxidation potential, are not able to oxidise ascorbic acid and thus are completely useless for the desired purpose. Hitherto, it was also assumed of iodates that they only oxidise ascorbic acid in acetic acid solution [R. Indovina, D. Elia, Boll. Soc. Ital. Biol. sperm. 20, 390-393 (1945), C.A. 40, 6110 (1946)].

The rapid diagnostics according to the invention, practically not destroyed by ascorbic acid, are simple to produce in that one admixes an iodate with the formulations of per se known tests. Such tests are e.g.:

- test papers for the detection of blood in urine with organic hydroperoxides and o-tolidine (DE-OS 2235 152, DE-OS 26 40 211, DE-OS 12 42 905) and tetramethylbenzidine (DE-OS 24 60 903, DE-OS 27 16 060).
- test papers for the detection of glucose in urine with GOD, POD and o-tolidine (DE-OS 24 15 257, DE-AS 11 21 847, OE-PS 19 88 96), 3,3',5,5'-tetramethylbenzidine (DE-OS 24 60 903), substituted aminocarbazoles (DE-OS 22 05 733, DE-OS 23 38 932) and

heterocyclic azines (DE-OS 16 48 840)

- test papers for the detection of galactose in urine with galactose-OD, POD and o-tolidine (USP 3,362,886)
- test papers of diverse substrates with specific oxidases, POD and o-tolidine (USP 3,099,605)
- test films for the determination of glucose in blood with GOD, POD, o-tolidine (DE-OS 15 98 153) and 3,3',5,5'-tetramethylbenzidine (DE-OS 24 60 903)
- test papers for the determination of NADH or NADH-forming substrates or enzymes with tetrazolium salts and diaphorase (e.g. DE-OS 24 52 283).

Since most of the above-mentioned tests are carried out in aqueous solutions, water-soluble iodates are advantageously used. There come into question practically all water-soluble salts of iodic acid with inorganic and organic cations insofar as these in turn do not disturb the analytical process. These are, in particular, the alkali metal salts which are easy to handle and, in part, are commercially available, furthermore the alkaline earth metal salts, ammonium salts or the salts of simple amines, such as e.g. piperidine, piperazine and the like.

Only in special cases are certain modifications of the conventional formulations necessary:

- In the case of the industrial production of test papers, amongst other things, relatively long impregnation times are necessary. In these long times,

under certain circumstances, it can already result in a partial oxidation of the indicator. This is, for example, the case with substituted aminocarbazoles according to DE-OS 22 05 733. In these cases, it is expedient to bring about a certain spatial separation of the reagents in that one first impregnates the test paper with all other reagents and thereafter post-impregnates a corresponding iodate from an organic solvent which does not dissolve the other formulation components. Organo-soluble iodates are, for example, quaternary ammonium iodates, as well as salts of iodid acid with long-chained amines.

- In cases where, due to the use of iodate, stability problems arise, there can be used generally known measures of stability improvement, such as successive impregnation from different solvents, possibly with the addition of suitable separation agents, such as e.g. polymers. The use of iodate in rapid diagnostics is only expedient within the following pH limits:
- In the case of pH values below about 4.5, there increasingly results free iodine in the case of the reduction of iodate by ascorbic acid which, as already said, oxidises many indicators. Furthermore, iodate in an acidic medium possesses an oxidation potential of +1.20V (Cotton-Wilkinson, see above) and is, therefore, no longer compatible with most indicators and other formulation components.

- Above pH 7 - 8, the oxidation of ascorbic acid by iodate becomes increasingly slower so that, for an effective interference elimination, relatively large amounts of iodate are necessary, which can lead to working up difficulties and possibly to storage stability problems.

The iodates are expediently used in 2 to 20 molar excess, referred to the amounts of ascorbic acid occurring in the investigated fluids. Since the oxidation of the ascorbic acid must take place practically before the actual detection reaction, for rapidly proceeding detection reactions one requires a larger excess of iodate than for more slowly proceeding ones. Since, as already mentioned above, the oxidation of ascorbic acid slows down in the case of high pH, in these cases a larger excess must also be used. The correct amount of iodate is, in every case, easy to determine by simple serial experiments.

In comparison with known disturbance-free or low disturbance rapid diagnostics, the rapid diagnostics according to the invention display the following advantages:

- Their handling corresponds completely to the previous, in some cases long since usual rapid diagnostics.
- Their production is simple and corresponds substantially to the usual methods of fabrication.

- Compared with rapid diagnostics which contain ascorbate oxidase, they show, in part, a high degree of interference elimination, above all they are substantially more price-favourable to produce.

The use of iodate is per se not limited to the incorporation into rapid diagnostics. Thus, e.g. iodate can be added directly to the solution to be investigated and the solution thereby freed from the disturbing reducing agents further investigated in known manner photometrically or with usual rapid tests. However, it is to be noted that in such a solution, tests, the substrates or reagents of which react with iodate, can no longer be carried out.

There are, for example, in the case of urine investigation with multiple tests, the tests for nitrite and bile colouring materials (urobilinogen and bilirubin). These materials are oxidised by iodate in the acidic medium of the test paper even before their detection. Furthermore, the aromatic amines used for the usual nitrite tests are oxidised to strongly coloured compounds.

Whether a particular test is disturbed by the addition of iodate can easily be tested for in that one compares standards with and without iodate addition.

In certain cases, e.g. in the case of analysis processes which operate at pH 5 - 6, where ascorbic acid is oxidised very rapidly by iodate, it can be

advantageous to add the iodate to the reagent preparation or parts thereof and so substantially to simplify the course of the analysis. Thus, e.g. in the case of the analysis of serum, the iodate can be added to the conventional weakly acidic deproteinisation agents, such as e.g. uranyl acetate, whereby one obtains a substantially interference-eliminated test solution.

The diagnostic agents are preferably rapid tests in which the reagent system is impregnated into an absorbent carrier insoluble in the test system or is incorporated into a film swellable in the test system which is possibly fixed to a solid carrier, for example a synthetic resin foil. The reaction is then determined, after moistening with the test system, by the coloration arising.

However, the diagnostic agents can also be soluble in the test system and be present, for example, as solution, lyophilisate or reagent tablet or be incorporated into a film soluble in the test system which, in turn, is present on or in a solid carrier. The reagents are then mixed with the test system, as well as possibly further solvents and the reaction determined photometrically in a cuvette.

By test system there is thereby understood the sample to be investigated, possibly with the addition of suitable solvents. By reagent system is understood

the sum of the materials reacting with the sample and all other adjuvants, such as buffer, wetting agent, viscosity-regulating materials, stabilising agents, contrast colouring agents, as well as possibly solvents etc.

The invention is to be further explained in the following Examples without, however, limiting it to the disclosure.

Example 1

Test paper for the detection of blood (erythrocytes) in urine.

Filter paper (Schleicher & Schüll No. 23 SL) is successively impregnated with the following solutions and dried at 40°C.:

Solution 1

1.2 molar citrate buffer of pH 5.25	35.0 ml.
ethylenediaminetetraacetic acid, disodium salt	0.1 g.
dioctyl sodium sulphosuccinate	0.5 g.
2,5-dimethylhexane-2,5-dihydroperoxide (about 70%)	1.6 g.
phosphoric acid trimorpholide	12.7 g.
sodium iodate	0.5 g.
ethanol	30.0 ml.
dist. water	ad 100.0 ml.

Solution 2

3,3',5,5'-tetramethylbenzidine	0.3 g.
phenanthridine	0.2 g.

1-phenyl semicarbazide	0.02 g.
toluene/methanol (60:40)	ad 100.0 ml.

With this test paper, there can be detected the presence of 5 ery./mm^3 even in the presence of 150 - 200 mg. ascorbic acid/dl. In the case of an analogous test paper which, instead of the iodate, contains $3 \cdot 10^4$ U ascorbate oxidase, the positive reaction is still visible up to a concentration of 30 - 50 mg. ascorbic acid/dl., in the case of a paper without additions only up to about 10 mg. ascorbic acid/dl. If the test paper according to the invention is heated for 3 days to 60°C., then it retains its sensitivity. The paper with ascorbate oxidase reacts, after this stressing, only like the paper without additives.

Example 1

Test paper for the semi-quantitative determination of glucose in urine

Filter paper (Schleicher & Schüll 597 NF-Ind.) is successively impregnated with solutions of the following composition and in each case dried at 50°C.:

Solution 1

glucose oxidase (71 U/mg.)	1.2 g.
peroxidase (66 U/mg.)	0.2 g.
1.2N citrate buffer, pH 5	50.0 ml.
9-(γ-dimethylaminopropyl)-6-chloro-3-aminocarbazole dihydrochloride	2.1 g.
tartrazine	0.12 g.
laurol sarcosine	1.1 g.
water dist.	ad 100.0 ml.

Solution 2

tetramethylammonium iodate	1.4 g.
ethanol	ad 100.0 ml.

A test paper is produced in the same way which is only impregnated with Solution 1.

Urines are prepared with 100, 300 and 1000 mg. glucose/dl. into which, in each case, are weighed 0, 50, 100 and 200 mg. ascorbic acid/dl.

The test papers are dipped in and placed on an absorbent substrate. One minute after the dipping in, their reaction colours are compared, whereby that of the urine without ascorbic acid is chosen as inner standard. From the following Table, it follows that the disturbance by ascorbic acid is overcome by the addition of iodate.

indication expressed in mg. glucose/dl				
iodate	0	50	100	200 mg. ascorbic acid/dl.
-	100	neg.	neg.	neg.
+	100	100	100	100
-	300	100	neg.	neg.
+	300	300	300	300
-	1000	300	100	neg.
+	1000	1000	1000	1000

Example 3

Test paper for the detection of glucose in urine

Filter paper (Schleicher & Schüll 597 NF) is impregnated with a solution of the following composition and dried at 50°C.:

glucose oxidase (71 U/ml.)	0.38 g.
peroxidase (66 U/ml.)	0.02 g.
potassium iodate	2.00 g.
tartrazine	0.08 g.
o-tolidine	0.42 g.
ethanol	33.0 ml.
water dist.	ad 100.0 ml.

With this test paper, urines with 50 mg. glucose/dl., into which have been weighed 0, 50, 100 and 200 mh. ascorbic acid/dl., give practically the same green reaction colour.

An analogous paper without iodate gives a positive reaction only in the ascorbic acid-free urine.

Example 4

Test film for the determination of small glucose contents in blood or serum

components

polyvinyl acetate propionate dispersion (Propiofan 70 D)	45.0 g.
1.85% solution of sodium alginate in 0.5M phosphate buffer of pH 5.5	35.0 g.
sodium nonyl sulphate dissolved in 5.0 ml. water	0.75 g.

glucose oxidase (71 U/mg.)) dissolved in peroxidase (66 U/mg.)) 10 ml. water	(0.2 g. (0.25 g.
3,3',5,5'-tetramethylbenzidine dissolved in 5 ml. acetone	0.68 g.
sodium iodate	1.0 g.

The components are well mixed up, coated in a layer thickness of 200μ on to a substrate of synthetic resin and dried for 35 minutes at 60°C.

A film is produced in the same way without iodate.

Sera with 20 mg. glucose/dl. and 0, 2.5 or 5.0 mg. ascorbic acid/dl., are dropped thereon, after 1 minute wiped off and, after a further 2 minutes, the coloration is measured on a commercially available remission photometer (Reflomat ®) with the use of a linear 0 - 100 scale:

test film serum with	0	2.5	5.0 mg. ascorbic acid/dl.
without iodate	47	43	35
with iodate	46	45	45

Example 5

Test paper for the detection of NADH

Filter paper (Schleicher & Schüll 23 SL) is impregnated with a solution of the following composition and dried at 50°C.:

iodo-nitro-triphenyltetrazolium chloride	0.2 g.
sodium iodate	0.5 g.
nonyl phenol polyglycol ether	0.2 g.

diaphorase (32 U/mg.)	0.05 g.
0.15 M phosphate buffer pH 7	40.0 ml.
water dist.	ad 100.0 ml.

A test paper was produced in the same way without iodate.

With aqueous solutions of NADH, both papers react with the same red coloration. If one adds ascorbic acid to the NADH solutions, then the papers without iodate react more strongly.

Example 6

Determination of glucose in serum

Solutions

Deproteinisation solution 1:

0.16% uranyl acetate in 0.9% common salt solution

Deproteinisation solution 2:

0.16% uranyl acetate and 0.05% sodium iodate in 0.9% common salt solution

Reagent solution:

POD 0.8 U/ml., GOD 10 U/ml., azino-bis-benzthiazolone sulphonic acid, NH_4 salt 1.0 mg./ml. in phosphate buffer pH 7 (100 mmole/l.)

Sample 1:

serum with 100 mg. glucose/dl.

Sample 2:

Serum with 100 mg. glucose/dl. and 20 mg. ascorbic acid/dl.

Standard:

9.1 mg. glucose/100 ml. water

Deproteinisation

Pipette according to the following scheme (ml.) and centrifuge:

deproteinisation solution 1	1.00	1.00	-	-
deproteinisation solution 2	-	-	1.00	1.00
sample 1	0.10	-	0.10	-
sample 2	-	0.10	-	0.10
gives supernatant No.	1.1	1.2	2.1	2.2

Analysis

Pipette according to the following scheme (ml.), incubate for 30 minutes at 25°C., measure extinction at 436 nm (d = 1 cm.).

	blank	super-natant standard	1.1	1.2	2.1	2.2
dist. water	0.1	-	-	-	-	-
standard	-	0.1	-	-	-	-
supernatant	-	-	0.1	0.1	0.1	0.1
reagent solution	5.0	5.0	5.0	5.0	5.0	5.0
E	0.115	0.425	0.424	0.366	0.426	0.423
E-E (blank)	-	0.310	0.309	0.251	0.311	0.308

Result

Calculated according to C = 100.E (sample)/E (standard)

supernatant	1.1	1.2	2.1	2.2
ascorbic acid in sample	-	+	-	+
iodate in deproteinisation solution	-	-	+	+
result (mg./dl.)	99.7	89.0	100.3	99.4

Thus, the disturbance in sample 2 brought about by ascorbic acid is completely removed by iodate.

Example 7

Determination of L-glutamic acid

Solutions

Reagent solution 1:

1.2 ml. Triton X 100

30 U diaphorase

10 mg. NAD

60 mg. iodo-nitro-triphenyltetrazolium chloride in 100 ml. 0.1 M potassium phosphate/triethanolamine buffer pH 8.6

Reagent solution 2:

90,000 U glutamate dehydrogenase in 100 ml. water

Sample 1:

100 mg. L-glutamic acid in 100 ml. water

Sample 2:

100 mg. L-glutamic acid and 40 mg. ascorbic acid in 100 ml. water

Iodate solution:

200 mg. sodium iodate in 100 ml. water

Sample preparation

Pipette according to the following scheme (ml.) and leave to stand for 15 minutes at room temperature:

sample 1	1.0	1.0	-	-
sample 2	-	-	1.0	1.0
$NaIO_3$ soln.	-	1.0	-	1.0
dist. water	1.0	-	1.0	-
gives mixture No.	1.1	1.2	2.1	2.2

Analysis:

Pipette according to the following scheme (ml.), after 2 minutes measure initial extinction E1 at 492 nm (d = 1 cm.), start with reagent solution 2, after 15 minutes measure end extinction E2.

	blank	mixture 1.1	1.2	2.1	2.2
reagent solution 1	1.0	1.0	1.0	1.0	1.0
dist. water	2.0	1.8	1.8	1.8	1.8
mixture	-	0.2	0.2	0.2	0.2
E1	0.058	0.058	0.053	creep	0.041
reagent solution 2	0.03	0.03	0.03	0.03	0.03
E2	0.062	0.490	0.499	creep	0.503
E2-E1 (ΔE)	0.004	0.432	0.446	-	0.454
$\Delta E - \Delta E$ (blank)	-	0.428	0.442	-	0.452

Result

calculated according to $C = 224 \cdot (\Delta E - \Delta E \text{ (blank)})$

mixture No.	1.1	1.2	2.1	2.2
ascorbic acid in sample	-	-	+	+
iodate treatment	-	+	-	+
result (mg./dl.)	95.9	99.0	not readable	101.2

The creep of the extinction caused by ascorbic acid (slow reduction of the tetrazolium salt), which prevents an exact measurement, can be prevented by pre-incubation of the sample solution with iodate without the excess iodate disturbing the analysis.

CLAIMS

1. Process for the detection of redox reactions by the introduction of a redox reagent system into a test system, characterised in that an iodate soluble in the test system is additionally introduced in an amount which corresponds to an excess, referred to the highest amount of disturbing ascorbic acid present in the test system, and the total mixture has a pH value of 5 - 9.

2. Process according to claim 1, characterised in that the pH value of the test system is increased by the addition of the redox reagent system.

3. Process according to claim 2, characterised in that the pH value is increased from 5 - 6 to 7 - 9.

4. Diagnostic agent for the detection of redox reactions containing a redox reagent system, characterised in that an iodate soluble in the test system is additionally present in an amount which corresponds to an excess, referred to the highest amount of disturbing ascorbic acid present in the test system, and the reagent system contains a buffer which together with the test system, gives a pH value of 5 - 9.

5. Diagnostic agent according to claim 4, characterised in that 0.5 - 2 g. of iodate is present per 100 ml. of test system.

6. Diagnostic test system according to one of claims 4 - 5, characterised in that the redox reagent system consists of an oxidation indicator, a hydroperoxide,

a peroxidase and conventional adjuvants.

7. Diagnostic agent according to one of claims 4 - 5, characterised in that the redox reagent system consists of a reduction indicator, a reducing agent, as well as possibly an electron carrier.