The application of water-dispersible hydrophobic dyes as labels in immunoassays.
The present invention relates to a process for the qualitative and/or quantitative determination of an immunochromatically reactive component such as a hapten, antigen or antibody in an aqueous test medium using the immunochromatically reactive components with respect to each other, whereby one or more labelled immunochromatically reactive components are employed and whereby during or after a certain reaction period for the immunochromatical reaction, optionally after separation of the free and bound immunochromatically reactive component(s), in the test medium or in one of the fractions obtained after separation, the nature and/or the quantity of the label is determined using methods known for this purpose.

A large number of methods are known whereby substances can be determined in a qualitative and/or quantitative manner, based on the formation of specific immuno complexes. A variety of analytical techniques are available for the direct or indirect detection of the finally-formed immuno complexes. Apart from reading off with the naked eye, physical methods are often widely used, such as spectrophotometry, fluorimetry, nephelometry and electron–dark field microscopy. These methods can be combined with the use of a label or tracer. Instead of the actual immuno complex, the label, coupled with one of the components of the complex, is then detected, so that a considerably lower detection limit can be attained.

As examples of qualitative immunochromatical techniques we can mention the classical precipitin reaction (Heidelberger and Kendall, 1930) and immunodiffusion — similarly based on immuno-precipitation — (Ouchterlony, 1948) followed in 1953 by immuno-electrophoresis developed by Grabar. In the latter-mentioned two methods antigen and antibody encounter each other via diffusion in an agar gel. The result is precipitation line can be, whether after prior colouring or not, be perceived by the naked eye. One disadvantage of these, as such simple methods is that diffusion takes a rather long time and that the detection limit is relatively high.

Quantitative methods of determination based on the principle of immuno-precipitation were developed by Mancini (1965; radial immuno-diffusion) and Laurell (1966; rocket-electrophoresis). Disadvantages of these methods are similarly a rather long determination period and/or a relatively high detection limit.

Apart from these non-labelled immunochromatical techniques, in the course of years a number of labelled techniques have been developed, among which we can mention the haemagglutination test where one of the components is attached to the surface of erythrocytes; the technique of immunofluorescence where one of the components is labelled with a fluorescent compound (fluorophore), the radio-immunoassay developed by Yalow and Berson around 1959 whereby, instead of a fluorophore a radio-active atom or radio-active gurup is used as the tracer; and the most recent technique of enzyme-immunoassay, on which the first publications appeared in 1971 from two groups working independently of each other, these being the Swedish investigators Engvall and Perlmann and the Dutchmen Schuurs and van Weemen. In principle the latter assay is analogous with the known radio-immunoassays with the distinction that instead of radioactive tracing, an enzyme is used as label.

In the U.S. patent No. 4,108,972 a label is described which consists of a particle with a size of 100–200 mesh in which is embedded a small amount of a detectable compound such as a radio-active tag or a fluorescent compound. These labels are not suited for use in a Sandwich-type of assay. In an agglutination-type of assay the particles already sediment even without aggregation. Hence they are useful only in agglutination tests wherein aggregation of the particles is detected by microscopic examination or by counting the particles — both methods being unsuiting for routine use in clinical laboratories. Furthermore the amount of the detectable compound in the particles is too low to enable sensitive assay to be carried out with this label.

Radio-immunoassays which are widely used have undoubtedly shown great value, but it is afflicted by a number of significant shortcomings such as the risk factor because of working with radioactive materials, the high costs of reagents and equipment, the short stability of radioactively-labelled reagents, and the requirement that only qualified personnel may perform such assays.

The enzyme-immunoassay does not suffer from these disadvantages, but nevertheless it is desirable that new assay techniques be developed which are even more sensitive, can be performed more rapidly, can be more easily automated, and/or enable the simultaneous determination of several immuno components.

The present invention relates to an immunoassay which is characterised in that the labelled immunochromatically reactive component(s) has (have) been obtained by direct or indirect attachment of such a component or components to particles of an aqueous sol of a hydrophobic organic dye or pigment. The "dispersed dye immunoassay" (DIA) developed in accordance with the present invention is considerably simpler than the "Radio-Immunoassay" (RIA), because during the final detection use can be made of a simple reading by eye and/or of a simple colorimeter. As compared with the "Enzyme-Immunoassay" (EIA, ELISA, EMIT) the determination is simpler and more rapid because the enzyme/substrate incubation can be omitted. Furthermore, it is possible to determine two or more components simultaneously by application of such chromophores as labels, that are clearly distinguishable spectrophotometrically. Finally the advantage of a dye as label which can be synthesized reproducibly, which can be characterised exactly by analytical/chemical methods and which is stable (in the form of a colloidal particle) is evident as compared with radioactive and enzyme labels of limited
stability, and/or variable batch quality; whereas detection limits are at least equivalent. Sols of dispersed
dyes have advantages to metal sols (e.g. gold), in colorimetric assays, due to the considerably higher molar
absorbances of the dye sols as compared to the metal sols; for example:
  gold sol (particle size 50 nm): 3300 l. mole\(^{-1}\). cm\(^{-1}\).
disperse dyes (5.000–80.000 l. mole\(^{-1}\). cm\(^{-1}\); cf. K. Venkataraman: “The Analytical Chemistry of

In addition, the colour can be intensified (increase in absorbance) during the final determination of the
dye label by dissolving the dye sol particles into an organic solvent (e.g. ethanol, methanol, iso-propanol).
For example:

<table>
<thead>
<tr>
<th>label</th>
<th>(\lambda_{\text{max}}) (sol) (nm)</th>
<th>(\lambda_{\text{max}}) (EtOH) (nm)</th>
<th>(A_{\lambda_{\text{max}}}) (\text{cm}^{-1})</th>
<th>(\varepsilon^{**})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palanil(^{R})</td>
<td>496</td>
<td>70,77</td>
<td>28300</td>
<td></td>
</tr>
<tr>
<td>Luminous Yellow G</td>
<td>464</td>
<td>110,36</td>
<td>44100</td>
<td></td>
</tr>
<tr>
<td>Palanil(^{R})</td>
<td>520</td>
<td>48,33</td>
<td>19300</td>
<td></td>
</tr>
<tr>
<td>Luminous Red G</td>
<td>544</td>
<td>88,27</td>
<td>35300</td>
<td></td>
</tr>
</tbody>
</table>

\(\ast\) l. g\(^{-1}\). cm\(^{-1}\),
\(\ast\) l. mole\(^{-1}\). cm\(^{-1}\)

To the group of coloured organic compounds which are applicable in the form of a hydrophobic sol in the
invention described here, belong all the hydrophobic organic dyes and pigments which are insoluble in
water or soluble only to a very limited extent.

Among these we should also include the water-soluble organic dyes, insofar as these in suitable
concentrations form association colloids which, whether after prior cross-linking or not, can be stabilised.
Furthermore it is also possible to use the leuco-vat dyes which are soluble in alkaline aqueous medium, and
which can be converted by oxidation into their original coloured and water-insoluble form; these also
include the leuco-vat dyes which are water soluble and stabilised in the form of a sulphate half-ester.
Another useful group is the group of dye components which, soluble as such in water and whether or not
coloured, after coupling to each other in situ, for example via oxidation or diazo coupling, can be converted
into water-insoluble dyes. The following groups can be mentioned as examples of the above-mentioned
dyes, using for this purpose the official Colour Index nomenclature: “disperse dyes, solvent dyes,
pigments, vat dyes, sulphur dyes, mordant dyes, solubilised (leuco) vat dyes, solubilised (leuco) sulphur
dyes, azoic dyes, oxidation bases, ingrain dyes” and “transfer dyes” which have not yet been officially
named.

The colloidal dye particles to be applied as labels can be prepared by a large number of methods which
are already known; see for example: Kruty (Ed.) (1952) “Colloid Science”, Vol. I, Elsevier, Amsterdam;
Ursachen der Agglomeration von Dispersionsfarbstoffen durch Farbstoffhilfsmittel beim Färben”,
Forschungsbericht Neue Serie No. 2, Institut für Chemiefasern der Institute für Textil- und Faserforschung

The procedure in accordance with the present invention is particularly suitable for the qualitative
and/or quantitative determination of an immunochemically reactive component, such as a hapten, antigen
or antibody present in an aqueous test medium, but can also be employed for the histological or cytological
determinations of such components.

For this reason the invention similarly relates to a new immunochemical reagent obtained by the direct
or indirect attachment of an immunochemically reactive component to particles of an aqueous sol of a
hydrophobic organic dye or pigment.

Advantageously this immunochemical reagent can be freeze-dried or spray-dried in order to provide
better stability upon storage. Hence, the invention similarly relates to such a freeze-dried or spray-dried
immunochemical reagent.

The invention similarly relates to new test kits containing such an immunochemical reagent.

By coupling the immunochemically reactive component to the particle, directly or indirectly, we mean
any chemical, physical or physicochemical bonding, such as a chemical covalent bond, via hydrogen bridges, polar attraction, or adsorption including also bio-specific adsorption.

The particles of the aqueous sol of a hydrophobic organic dye or pigment have a particle size of at least 5 nm, and preferably from 10 to 500 nm.

The dye sol particles carry a charge, which gives a stabilising effect by mutual repulsion. By adding mainly strong electrolytes, the charge pattern is modified, so that aggregation and flocculation take place. This can be prevented by coating the particles with macromolecules which possess polar groups, such as proteins, polysaccharides, polyethylene glycols, polyvinyl alcohols etc.

As protective proteins it is possible to use antigens, antibodies or polypeptide fragments thereof which are still immunochemically active. Furthermore it is possible to envisage hapten attached to macromolecules (e.g. proteins, polysaccharides) which during the pertinent immunoassay do not give rise to any interfering reaction with the other components. During this the dyestuff sol-labelled, immunochemically-active component is simultaneously obtained.

It may occur that, in order to stabilise the dyestuff sol, such a high concentration of, for example, antibody is required on the surface of the colloidal particles that the effective immunochemical activity of this immobilised protein is affected, for example by steric hindrance. In such a case the coating can be performed in two stages:

1) coating with an optimum quantity, to be determined, of for example an antibody, followed by
2) coating with a macromolecular compound (e.g. a protein, a polysaccharide, polyethylene glycol, polyvinyl alcohol) which, during the pertinent immunoassay, does not give rise to an interfering reaction with the other components. This “subsequent coating”, e.g. with bovine serum albumine, can at the same time serve to reduce possible non-specific adsorption effects.

Another possibility of a protective protein can be protein A or the group of lectines (e.g. Con A). After an initial coating of the sol particles with these proteins it is possible, due to the specific affinity of the said proteins, to apply a second layer selectively by adsorption of immunoglobulins (via the Fc part, and glycoproteins) also including immunoglobulins, via the sugar residue(s) present.

Another possibility is that the dyestuff sol particles are first coated by a polymer or copolymer which is inert in the final immunoassay, after which subsequently by adsorption and/or covalent attachment, an immunochemically-active component can be attached to the layer of coating material. During the coating of the sol particles by the inert polymer or copolymer each particle can be enveloped separately, but it is also possible for several colloid particles to be included inside one and the same polymeric layer.

The covering of the dyestuff sol particles by the inert polymer can take place in two ways: by bringing the dyestuff sol in contact with the polymer, followed by adsorption and/or covalent attachment to the sol particles, or by bringing the sol into an environment of a monomer, or different monomers, and polymerising or co-polymerising the latter in situ. Polymerisation can be undertaken for example by radiation or by the addition of a suitable initiator, such as for example a persulphate. The envelopment of a dyestuff sol particle by in situ polymerisation of the monomeric solution, in which the particle is located, under the influence of an inorganic initiator such as a persulphate, involves practical difficulties, because the sol — when such an initiator is added — flocculates out. It was however established that such coating is nevertheless possible by first of all protecting the sol particles, and then placing the protected particles in the monomeric solution, polymerisation only being initiated subsequently. The compounds mentioned above can be employed as protective agents for this purpose.

Coating of the colloidal dyestuff particles with the following aim(s): stabilisation of the sol, the application of an immunochemically-reactive component, elimination of non-specific adsorption effects, and/or the application of an intermediary polymer or co-polymer layer respectively, can be performed via direct/indirect adsorption at the colloidal dyestuff particles, but also by covalent chemical attachment. The latter is governed by the presence of suitable functional groups in the coating material and in the dyestuff. For example one can envisage the diazotization of aromatic amino groups followed by diazo attachment to an activated aromatic ring system; carboxyl groups can be activated by a carbodi-imide and then, possibly via an active ester, be attached to a primary amino component. Aliphatic primary amino groups and hydroxyl groups can be activated for example by cyanogen bromide or halogen-substituted di- or tri-azines, after which attachment with a primary amino component or with for example a component containing a —SH, —OH or imidazolyl group can take place. Use can also be made of bifunctional reactive compounds. For example glutaraldehyde can be used for the mutual coupling of primary amino components, whilst for example a hetero-functional reagent such as N-succinimidyl 3-(2-pyridyldithio) propionate can be employed for the coupling of a primary amino component to a component containing a thiol group.

In this context mention can also be made of the reactive dispersiv-, and other reactive dyestuffs not soluble in water, where the dye consists of a chromophore covalently coupled with a group which as such is already reactive, such as for example halogen-substituted di- and triazines, epoxy groups, vinyl-sulphonic groups and dihaloquinoxalines (see Siegel, (1972) in: Venkataraman (Ed.): “The Chemistry of Synthetic Dyes”, Academic Press, New York, Vol. VI; Harms, 1979 in: Banks (Ed.): “Organofluorine Chemicals and their Industrial Applications”, Ellis Horwood Ltd., Chichester; pp. 188—204).

Usually the immunochemically-reactive components labelled with colloidal dye particles are employed as reagent in combination with other reagents, for the detection and/or quantitative determination of for
example happens, antigens and antibodies, for which all types of immunochemical techniques can be considered such as those used for RIA and EIA.

Hence the invention also relates to "test kits" for use in such immunochemical techniques, which as their most important component contain an immunochemically-reactive component labelled with a dyestuff sol, consisting of a dyestuff sol, the particles of which are coated directly or indirectly, adsorptively and/or covalently, with the immunochemically-reactive component.

One of the conventional immunochemical techniques is competitive immunoassay, which can be used for the demonstration and/or determination of any immunochemically reactive component. For the demonstration, for example, of a certain antigen, this method consists of bringing a test sample, containing an unknown quantity of antigen, into contact either with a certain quantity of the corresponding antigen labelled with a dye sol and an antibody attached to an insoluble carrier, which is directed against this antigen, or a certain quantity of antigen attached to an insoluble carrier and an antibody labelled with a dyestuff sol directed against this antigen.

After the reaction has terminated the nature and/or the quantity of the dyestuff is determined in the bound and/or the free fraction, which provides a qualitative and/or quantitative indication for the antigen to be determined. Mutatis mutandis, an analogous procedure applies to the determination of other immunochemically reactive components.

Widespread use is also made of the "sandwich technique" which is also appropriate for use with immunochemically-reactive components labelled with colloidal dyestuff particles. Using this technique such a component, e.g. an antibody in cases where an antigen has to be determined, is immobilised on an insoluble carrier material. This carrier material can for example be the interior surface of the reaction vessel in which the immunochemical reaction is performed; it is also possible to employ carrier materials in the form of beads or small rods. After initial incubation with the specimen containing the antigen, possibly followed by a washing step, a second incubation takes place with an antibody labelled with a dyestuff sol, after which the dyestuff is determined in the bound and/or the free phase.

Apart from the techniques mentioned for this purpose there are also innumerable other immunochemical techniques where the immunochemically-reactive component labelled with the dyestuff sol can be employed as reagent. We are thinking here especially of an immunochemical test based on the agglutination principle. Here for example an antibody labelled with a dyestuff sol is added to a specimen of a liquid containing the antigen to be determined. The separation of the bound and free fractions of the labelled components can be dispensed with here, because the detection is based on a visual assessment of the dyestuff sol or on a spectrophotometric/colorimetric determination.

The present invention also renders it possible to demonstrate the presence in a test specimen of different immunochemically reactive components, such as for example haptenes, antigens and antibodies or combinations thereof, simultaneously by employing, for each of the components to be demonstrated, a corresponding immunochemically reactive component which has been labelled with a colloidal dyestuff particle which is characteristic for that component.

Determination of the nature and/or the concentration of the dyestuff at the end of the test can be performed using various known techniques. As examples of these we can mention visual assessment which is excellently suitable for a qualitative determination precisely when employing dyestuffs; for quantitative determination use can for example be made of colorimetry/spectrophotometry. The said methods are also suitable for the ultimate detection in the agglutination test, where importance attaches not so much to the concentration of the dyestuff, but instead to the external appearance of the dyestuff sol (a greater or lesser degree of aggregation, possible flocculation, spectral modifications caused by this).

Furthermore for the quantitative determination of the dyestuff (or dyestuffs, during simultaneous determination) though can be given to fluorimetry and — in the case of metal complex dyestuffs and/or pigments, to "normal" and/or flameless atomic absorption spectrophotometry. The invention will now be described in greater detail with the aid of the following examples.

Example 1
Colorimetric and/or visual determination of human chorionic gonadotropin (HCG) in accordance with the DIA principle described ("Sandwich test").

1.1. Preparation of the dye sol
Palani's red BF (BASF, 7 g) was dispersed in distilled water (140 ml). The dispersion was stirred for 45 minutes at room temperature and then centrifuged (30 min, 125 g=1.225 N/kg). The supernatant was transferred into other centrifuge tubes and centrifuged (30 min, 7 500 g=73 500 N/kg). The supernatant was removed and the pellet was washed three times with distilled water (2×140 ml, centrifuge: 30 min, 7 500 g=73 500 N/kg). The pellet was resuspended in distilled water (70 ml), and subsequently so many glass beads (dia.≈3 mm and dia.≈4 mm, mixture 1:2) are added that the liquid level is the same as that of the beads. Then rolling was carried out for 5 days at room temperature on a roller bench. The liquid was decanted and centrifuged (30 min, 300 g=2 940 N/kg). The supernatant was then transferred into other centrifuge tubes and again centrifuged (30 min, 1 000 g=9 800 N/kg). From this last supernatant 3/4 (52.5 ml) was carefully aspirated and this concentrated dyestuff sol was stored at room temperature. The extinction at 533 (λ_max) nm of a 20×diluted sample of this sol was 1.57.
0 032 270

1.2. Preparation of the rabbit anti-HCG immunoglobulin/dye sol conjugate

A sample (0.8 ml) of the dye sol described in 1.1 above was diluted with distilled water (4.2 ml) and the pH was adjusted to 7.0 using 0.1 mol/l NaOH or HCl. The extinction of this sol is 5.0 at 533 nm (λₑₓ). Subsequently rabbit anti-HCG immunoglobulin solution (0.1 ml) was added; the reaction mixture was shaken every 15 minutes during one hour at room temperature, after which a solution of bovine serum albumin (BSA) was added (1 ml; 307.2 g BSA+0.1 g sodium metholiate+5 mmol NaCl/l, pH 7.0). The dispersion was shaken every 15 minutes during one hour at room temperature and was then centrifuged (30 min, 4,000 g=39 200 N/kg). The supernatant was removed and the pellet was resuspended up to a volume of 5 ml in a solution having the following composition: 51.2 g BSA+0.1 g sodium metholiate+5 mmol NaCl/l (pH adjusted to 7.0 with 0.1 mol/l NaOH).

* The solution of rabbit anti-HCG immunoglobulin was prepared as follows: The immunoglobulin fraction of rabbit anti-HCG serum was isolated via the known Na₂SO₄ precipitation method. The precipitate was dissolved in and dialysed against an aqueous solution of 5 mmol/l NaCl, the pH of which has been adjusted to 7.0 with solid Na₂CO₃. The dialysed solution was finally diluted to a protein concentration of 1 mg/ml (in accordance with the Warburg-Kackar formula: protein concentration (mg/ml)=1.45×A₁₆₀cm⁻¹×0.75×A₁₁₀cm⁻¹). or 0.65 mg/ml (according to A₁₁₀cm⁻¹, 1%=14.5 for IgG).

1.3. "Coating" of Microelisa® plates with rabbit anti-HCG immunoglobulin

Phosphate buffer (FFB)

0.04 mol/l Na₂HPO₄ and 0.04 mol/l NaH₂PO₄ were mixed to give a solution with pH 7.4; then add NaCl up to a concentration of 0.15 mol/l.

Solution A

Rabbit anti-HCG immunoglobulin (see 1.2) dissolved in FFB up to a concentration of 30 mg/l.

Solution B

20 g BSA+0.1 g sodium metholiate/l FFB.

Solution A (0.11 ml) was pipetted into the wells of Microelisa® plates, after which the plates were incubated for 16 hours at 0-4°C. After aspiration, solution B (0.11 ml) was added to all the wells, after which the plates were incubated for 30 minutes at room temperature. Finally the wells were aspirated and washed three times with distilled water, after which the plates were dried (16 hours at room temperature over pre-dried silicagel), packaged in aluminium laminate bags (with silica gel sachet) and stored at 4°C.

1.4. Determination of a standard curve for HCG in phosphate buffer and in blank urine

Phosphate buffer (FFB)

As described in 1.3, but with 1 g BSA/l, and 1 g sodium metholiate/l.

Washing buffer I

Phosphate buffer.

Washing buffer II

0.1 mol TRIS (HOC₆H₅CN₃H₅)+0.1 mol NaCl+0.5 g Tween®-20+0.1 g sodium metholiate/l, adjusted to pH 7.4 with 4 mol/l HCl.

Ethanol

P.A., 96% (v/v).

HCG

A solution of human chorionic gonadotropin with a content of 1,000 IU (immunoassay)/ml FFB.

Blank urine

Urine from non-pregnant women, filtered over Hyflo® and subsequently frozen; prior to use filtered through folding filter.

Conjugate

The dye sol/anti-HCG conjugate, prepared as described in 1.2, (9 ml) was mixed with concentrated phosphate buffer (1 ml; 10×concentrated FFB).

Procedure

1. The following dilution series of the HCG solution were made in FFB and urine respectively: 4,000, 1,000, 250, 62.5, 15.6, 3.9 m IU (immunoassay)/ml.

2. The 0.1 ml of these solutions, and of blank FFB and urine, was pipetted into the wells of a Microelisa® plate which has previously been "coated" as described in 1.3 with rabbit anti-HCG immunoglobulin (see 1.3). All this is done in duplicate.

3. Close the plate with a suitable cover, and incubate for 3.5 hours at 37°C in an atmosphere saturated with water vapour.
4. Aspirate the wells, pipette wash buffer I (0.3 ml) into each well and aspirate again. 
5. Pipette the conjugate (0.1 ml) into each well. 
6. Incubate as described in item 3, but now for 18 hours. 
7. Aspirate the wells, assess the colour in the wells visually and/or pipette wash buffer II (0.3 ml) into the wells. Aspirate and repeat this procedure twice further. 
8. Add ethanol (0.12 ml) to all the dry wells, shake gently and assess the colour by eye and/or measure the extinction at 516 nm (=\(\lambda_{\text{max}}\)).

If, using the procedure described, samples having an unknown HCG content are also included, the HCG concentration can easily be estimated by eye; a more accurate determination can if required be performed by extinction measurement and comparison with the standard curve. Standard curves for HCG in urine and FFB determined in this manner are shown in Fig. 1.

The detection limit, defined as (blank+2×standard deviation) is for FFB 3.9 m IU (immunoassay)/ml, and for urine 15.6 IU (immunoassay)/ml.

1.5. Determination of HCG in the urine of women in order to detect pregnancy
Reagents: see section 1.4.
Procedure
As described in 1.4., but now with the inclusion of the corresponding urine specimens, which may or may not be diluted. The results are shown in the following table:

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Dilution</th>
<th>Pregnosticon(^{R}) &quot;All-in&quot;</th>
<th>Visual</th>
<th>(A_{0.56})</th>
<th>HCG conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1374</td>
<td>Undiluted</td>
<td>+</td>
<td>+</td>
<td>0.588</td>
<td>4 000</td>
</tr>
<tr>
<td>1374</td>
<td>1:10</td>
<td>+</td>
<td>+</td>
<td>0.595</td>
<td>40 000*</td>
</tr>
<tr>
<td>1130</td>
<td>Undiluted</td>
<td>+</td>
<td>+</td>
<td>0.659</td>
<td>4 000</td>
</tr>
<tr>
<td>305</td>
<td>Undiluted</td>
<td>+</td>
<td>+</td>
<td>0.707</td>
<td>4 000</td>
</tr>
<tr>
<td>726</td>
<td>Undiluted</td>
<td>-</td>
<td>-</td>
<td>0.057</td>
<td>60</td>
</tr>
<tr>
<td>546</td>
<td>Undiluted</td>
<td>-</td>
<td>-</td>
<td>0.043</td>
<td>65</td>
</tr>
<tr>
<td>1123</td>
<td>Undiluted</td>
<td>-</td>
<td>-</td>
<td>0.047</td>
<td>65</td>
</tr>
</tbody>
</table>

*in the undiluted specimen.

The conclusion as to whether or not pregnancy exists corresponds with the results of the pregnancy test Pregnosticon\(^{R}\) "All-in". In this latter test "+" coincides with \(\geq 1000\) IU/ml and "+" with \(\leq 500\) IU/ml.

Example 2
As example 1, but disperse dys/anti-HCG conjugates were prepared using Resolin\(^{R}\) Brilliant Blue RRL and the fluorescent disperse dyes Samaron\(^{R}\) Brilliant Red H6GF Samaron\(^{R}\) Brilliant Yellow H10GF, Palanil\(^{R}\) Luminous Red G and Palanil\(^{R}\) Luminous Yellow G as colloidal labels. (For corresponding \(\lambda_{\text{max}}\) values see Example 9).

Dilution series of HCG were made in buffer. Reduction of the incubation periods as compared to the standard procedure was investigated: HCG incubation 2.5 h and conjugate incubation 2.5 h instead of 6.5 and 18 h, respectively. A detection limit of 0.02—0.25 mIU HCG/ml was obtained. This compares very well with other test systems:

---RIA: 2 mIU HCG/ml (DL:HCG concentration at (90% binding+2×SD))
---EIA: 20 mIU HCG/ml (DL: as for RIA)
---SPIA: 0.25—1 mIU/ml (DL:blank+2×SD) (colorimetric detection)
---rev-HA: 10—20 mIU HCG/ml (DL: HCG concentration giving a significant change in pattern).

Additionally, the total test period was decreased from 24.5 h to 5 h, with only a limited effect on the detection limit (in the case of Samaron Brilliant Red H6GF).

The fluorescent disperse dyes were investigated in order to improve the detection limit by measuring fluorescence instead of absorbance. A significant improvement was obtained in the case of Samaron\(^{R}\) Brilliant Yellow H10GF and Palanil\(^{R}\) Luminous Yellow G, whereas no effect was found in the case of Samaron\(^{R}\) Brilliant Red H6GF, and Palanil\(^{R}\) Luminous Red G.

7
Example 3
DIA for Hepatitis B surface Antigen (HBsAg); sandwich system.

3.1. Preparation of dye sol
See section 1.1.; the disperse dyes Palanil® Red BF and Samaron® Brilliant Red H6GF were used as colloidal labels. (For corresponding \( \lambda_{\text{max}} \) values see Example 9).

3.2. Preparation of the sheep-(anti-HBsAg) IgG/dye conjugate
See section 1.2.; but use sheep-(anti-HBsAg) immunoglobulin instead of rabbit anti-HCG IgG.

3.3. Coating of Microelisa® plates with sheep-(anti-HBsAg) IgG.
See section 1.3.; use the sheep instead of the rabbit immunoglobulins.

3.4. Determination of standard curves for HBsAg (subtype ad and ay).
Dilution series of HBsAg (ad and ay) were made using human negative control serum as diluent, in the range 4—1000 ng/ml. Samples (0.1 ml) of these dilutions, and of the negative control serum, were assayed according to the procedure described in section 1.4. (steps 3—8); for \( \lambda_{\text{max}} \) (ethanol) see Example 9.
Detection limits of 16—23 ng/ml (ad) and 24—38 ng/ml (ay) were obtained with the Samaron® dye/conjugate. For comparison:

- EIA (Hepanostika®): 3 ng/ml
- EIA (Hepanostika-T®): 0.7 ng/ml (DL: mean negative value + 5 × SD)
- SPIA: 20—40 ng/ml (DL: blank + 2 × SD) (colorimetric detection).

The DIA/sandwich system was also used to compare several samples of monoclonal antibodies, with a standard preparation of heterogeneous sheep anti-HBsAg IgG. Three samples gave dose response curves similar to the standard, whereas the other preparations were of a distinctly poorer quality.

Example 4
DIA for Human Placental Lactogen (HPL); sandwich system.

4.1. Preparation of dye sol.
See section 1.1.; the disperse dyes Palanil® Red BF and Palanil® Yellow 3G were used as colloidal labels (for corresponding \( \lambda_{\text{max}} \) values see Example 9).

4.2. Preparation of the rabbit (anti-HPL) IgG/dye conjugate.
See section 1.2.; but use anti-HPL instead of anti-HCG.

4.3. Coating of Microelisa® plates with rabbit anti-HPL IgG.
See section 1.3.; but use anti-HPL instead of anti-HCG.

4.4. Determination of Standard curves for HPL.
Dilution series of HPL were made in FFB (see section 1.4) in the range 0.4—100 ng/ml. Samples (0.1 ml) of these dilutions, and of FFB, were assayed according to the procedure described in section 1.4. (steps 3—8); for \( \lambda_{\text{max}} \) (ethanol) see Example 9.
A detection limit of 1.2—1.7 ng HPL/ml was obtained. For comparison:

- RIA: 0.03—0.14 ng/ml
- EIA: 2 ng/ml
- SPIA: 0.12 ng/ml (colorimetry).

Example 5
DIA for anti-Rubella; sandwich system.

5.1. Preparation of dye sol.
See section 1.1.; the disperse dyes Palanil® Red BF and Resolin® Brilliant Blue RRL were used as colloidal labels (for corresponding \( \lambda_{\text{max}} \) values see Example 9).

5.2. Preparation of the sheep anti-(human IgG) IgG/dye conjugate.
See section 1.2.; but use the sheep immunoglobulin instead of the rabbit material.

5.3. Coating of Microelisa® plates with inactivated Rubella viral antigen (obtained from tissue culture).
See section 1.3.; but use the Rubella antigen instead of the immunoglobulin.
5.4. Determination of standard curves for human anti-Rubella.

Dilution series of human anti-Rubella were made using sheep negative control serum as diluent, in the range 0.4—320 IU/ml. Samples (0.1 ml) of these dilutions, and of the negative control serum, were assayed according to the procedure described in section 1.4. (steps 3—8); for \( \lambda_{\text{max}} \) (ethanol) see Example 9.

The detection limit, defined as (BL+2×SD), was 2.5 IU/ml, which compares favourably with an estimation of the detection limit of Rubenostika of \( \sim 10 \) IU/ml.

Example 6

DIA for human Prolactin (PRL); sandwich system.

6.1. Preparation of dye sol.

See section 1.1.; the disperse dyes Palanil\textsuperscript{R} Luminous Red G and Palanil\textsuperscript{R} Luminous Yellow G were used as colloidal labels (for corresponding \( \lambda_{\text{max}} \) values, see Example 9).

6.2. Preparation of the monoclonal (anti-PRL) IgG/dye conjugate.

See section 1.2.; but use the monoclonal IgG instead of the rabbit material.

6.3. Coating of the Microelisa\textsuperscript{R} plates/strips with monoclonal (anti-PRL) IgG.

See section 1.3.; but use the monoclonal IgG instead of the rabbit material.

NOTE: Immunoglobulins from different clones were used for the preparation of the conjugate (6.2.) and for the coating of the plates/strips.

6.4. Determination of standard curves for PRL.

Dilution series of PRL were made in FFB (see section 1.4.) in the range 0.4—100 ng/ml. Samples (0.2 ml) of these dilutions, and of FFB, were assayed according to the procedure described in section 1.4. (steps 3—8), with the following modifications:

— incubation of antigen (PRL): 20 h, room temperature
— incubation of conjugate: 20 h, room temperature.

For \( \lambda_{\text{max}} \) (ethanol) see Example 9.

A detection limit of 1—4 ng/ml was obtained.

For comparison: EIA 1—4 ng/ml

SPIA 6—10 ng/ml.

Example 7

Simultaneous determination of HCG and HPL, according to the DIA principle; sandwich system.

7.1. Preparation of the dye sols.

See section 1.1.; the disperse dyes Resolin\textsuperscript{R} Brilliant Blue RRL and Palanil\textsuperscript{R} Yellow 3G, were used as colloidal labels (for corresponding \( \lambda_{\text{max}} \) values see Example 9).

7.2. Preparation of the rabbit (anti-HCG) IgG/ and rabbit (anti-HPL) IgG/dye conjugates.

See section 1.2.; prepare the following combinations:

— Resolin\textsuperscript{R} Brilliant Blue RRL/anti-HCG
— Palanil\textsuperscript{R} Yellow 3G/anti-HPL.

The combined conjugate is prepared by mixing equal volumes of the two conjugates, yielding a final absorbance of 5 (at \( \lambda_{\text{max}} \)) for each dye-conjugate.

7.3. Coating of Microelisa\textsuperscript{R} plates with rabbit anti-HCG, rabbit anti-HPL, and with a mixture of both.

See section 1.3.; plates for the simultaneous assay were prepared using the following coating mixture: rabbit anti-HCG 15 ng/l

rabbit anti-HPL 15 ng/l.

7.4. Simultaneous determination of HCG and HPL.

Generally, the assay procedure described in section 1.4. was used (for \( \lambda_{\text{max}} \) values in ethanol, see Example 9):

a) The single and combined conjugates were tested in microtitre plates which were coated only with rabbit anti-HCG or anti-HPL. The combined conjugate and the anti-HCG conjugate gave an equal response in a dilution series of HCG in FFB (see section 1.4.), whereas the anti-HPL conjugate did not react. The anti-HPL conjugate gave a higher response than the combined conjugate in a dilution series of HPL in FFB, whereas the anti-HCG conjugate did not react.

b) Finally, samples of HCG, HPL and (HCG+HPL) in FFB were incubated in the walls of a microtitre plate coated with rabbit anti-HCG and anti-HPL simultaneously. The second incubation was performed with the
combined conjugate. The results are presented in Figure 2, which demonstrates the possibility of a simultaneous DIA/sandwich.

Example 8
DIA for testosterone.

This method is based on the detection of free anti-testosterone on the solid phase, after incubation with the testosterone containing sample.

8.1.1. Preparation of the dye sol
See section 1.1.

8.1.2. Preparation of testosterone-11\alpha-hemisuccinyl-BSA/dye sol conjugate
Testosterone-11\alpha-hemisuccinate is dissolved in 2 ml dimethylformamide (DMF) and then the solution is cooled to \(-15^\circ\text{C}\).
Bovine serum albumin (BSA, 140 mg) is dissolved in distilled water (3 ml), after which 40 μl of 4 mol/l NaOH and 2 ml DMF is added. The solution is then cooled to \(-15^\circ\text{C}\).
Now 12.5 μl of N-methylmorpholine and 12.5 μl of isobutyl chloroformate is added to the solution of testosterone derivative. After three minutes this reaction mixture is added to the BSA solution. Whilst stirring the reaction mixture is held for 1 hour at \(-15^\circ\text{C}\) and then for 3 hours at 0°C. Then the reaction mixture is dialysed against distilled water for 16 hours at 4°C, with the distilled water being regularly renewed. The dialysed solution is then centrifuged and the clear supernatant is freeze-dried.
The testosterone-11\alpha-hemisuccinyl-BSA/dye sol conjugate is now prepared using the method described for the immobilisation of rabbit anti-HCG immunoglobulin on a Pellanil\textsuperscript{R} Red BF sol whilst using the same concentration for the testosterong-BSA derivative as for the rabbit anti-HCG immunoglobulin.

8.1.3. “Coating” of Microelia\textsuperscript{R} plates with rabbit anti-testosterone immunoglobulins
The coating was carried out as described in section 1.3, using the immunoglobulin fraction isolated from a rabbit antisera, obtained by immunisation with testosterone-11\alpha-hemisuccinyl-BSA.

8.1.4. Determination of testosterone
Using the reagents and the test procedure as described for HCG in section 1.4., a standard curve was determined for testosterone by means of which subsequently the testosterone concentration of unknown samples was calculated via the \(A_{\max}\) of the samples, obtained in accordance with the said test procedure. The detection limit of this determination is 1 ng/ml. It is easy to estimate the testosterone contents by reading with the naked eye (comparison of the colour intensity of standard and unknown samples after stopping the reaction).

This method is based on a competitive binding of testosterone (T) and T\textsuperscript{3}-BSA to anti-(T\textsuperscript{11}-BSA), which was immobilized onto the solid phase (e.g. polystyrene tube, or microtitre plate). Detection followed by a second incubation with an anti-(T\textsuperscript{11}-BSA)/dye conjugate.

Note:
T\textsuperscript{3}-BSA: testosterone-3-O-carboxymethoxime, covalently coupled to a NH\textsubscript{2}-function of BSA (amide bond).
T\textsuperscript{11}-BSA: testosterone-11-hemisuccinate, covalently coupled to a NH\textsubscript{2} function of BSA (amide bond).

8.2.1. Preparation of dye sol.
See section 1.1.; the disperse dyes Samaron\textsuperscript{R} Brilliant Red H6GF and Samaron\textsuperscript{R} Brilliant Yellow H10GF were used as colloidal labels (for corresponding \(\lambda_{\max}\) values, see Example 9).

8.2.2. Preparation of the rabbit (anti-T\textsuperscript{11}-BSA) IgG/dye conjugate.
See section 1.2.; but use the rabbit anti-T\textsuperscript{11}-BSA instead of anti-HCG.

8.2.3. Coating of Microelia\textsuperscript{R} plates and polystyrene tubes with rabbit (anti-T\textsuperscript{11}-BSA) IgG.
See section 1.3.; but use the rabbit (anti-T\textsuperscript{11}-BSA) instead of the anti-HCG.

Note:
1 ml of solutions A and B are used in case of coating of the tubes.

8.2.4. Determination of standard curves for testosterone.
Solutions of T and T\textsuperscript{3}-BSA were made in FFB (see section 1.4.).
a) The concentration of T\textsuperscript{3}-BSA to be used in the competitive assay, was investigated by incubation of a dilution series of T\textsuperscript{3}-BSA only, followed by the conjugate; 1 ml volumes per tube, or 0.1 ml volumes per microtitre plate well. Procedure as described in section 1.4. (steps 3—8): for \(\lambda_{\max}\) (ethanol) see Example 9.
For the total competitive assay concentrations of T³-BSA corresponding with 2 and 16 pmol T/ml total test volume, were chosen.

b) The competitive assay was performed using constant concentrations of T³-BSA corresponding with 2 and 16 pmol T/ml total test volume, and a dilution series of 0—64 pmol T/ml sample; for the tubes: 0.9 ml of testosterone containing sample and 0.1 ml of a T³-BSA solution (corresponding with 20, respectively, 160 pmol T/ml); for the microtitre plate the respective volume are changed to 0.09 and 0.01 ml. Further procedure as described in section 1.4. (steps 3—8); for λ_max (ethanol) see Example 9.

The detection limit, defined as (BL—2·SD), was 0.2—0.4 pmol T/ml sample, using a T³-BSA concentration corresponding with 2 pmol T/ml total test volume. The total detection range was about 0—8 pmol T/ml sample. In RIA and EIA, 50% binding is reached at, respectively, 1 pmol T/ml and 0.7 pmol T/ml.

Example 9
Alternative methods for the preparation of dye sols.

9.1. Disperse dyes.

Instead of the Palanil® Red BF (BASF) mentioned in section 1.1., other disperse dyes have also been used for preparing dyestuff sols, including:

<table>
<thead>
<tr>
<th>Disperse Dye</th>
<th>λ_max (nm) water&lt;sup&gt;a&lt;/sup&gt;</th>
<th>λ_max (nm) ethanol&lt;sup&gt;b&lt;/sup&gt;</th>
<th>λ_measuring (nm)&lt;sup&gt;c&lt;/sup&gt; ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palanil® Violet 8R BASF</td>
<td>623</td>
<td>571</td>
<td>—</td>
</tr>
<tr>
<td>Palanil® Yellow 3G BASF</td>
<td>415</td>
<td>443</td>
<td>443</td>
</tr>
<tr>
<td>Palanil® Luminous Yellow G&lt;sup&gt;d&lt;/sup&gt; BASF</td>
<td>496</td>
<td>464</td>
<td>443</td>
</tr>
<tr>
<td>Palanil® Luminous Red G&lt;sup&gt;d&lt;/sup&gt; BASF</td>
<td>520</td>
<td>544</td>
<td>540</td>
</tr>
<tr>
<td>Terasil® Brilliant Flavin 8GF&lt;sup&gt;e&lt;/sup&gt; Ciba-Geigy</td>
<td>488</td>
<td>461</td>
<td>443</td>
</tr>
<tr>
<td>Terasil® Brilliant Pink 4BN Ciba-Geigy</td>
<td>571</td>
<td>571</td>
<td>540</td>
</tr>
<tr>
<td>Cibacet® Violet 2R Ciba-Geigy</td>
<td>538</td>
<td>592</td>
<td>549</td>
</tr>
<tr>
<td>Foron® Brilliant Flavin S8GF&lt;sup&gt;f&lt;/sup&gt; Sandoz</td>
<td>433</td>
<td>427</td>
<td>—</td>
</tr>
<tr>
<td>Resolin® Blue R&lt;sup&gt;g&lt;/sup&gt; Blue RRL Bayer</td>
<td>670</td>
<td>578</td>
<td>600</td>
</tr>
<tr>
<td>Procinyl® Blue R&lt;sup&gt;h&lt;/sup&gt; ICI</td>
<td>672</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Samarón® Brilliant Red H6GF&lt;sup&gt;i&lt;/sup&gt; Hoechst</td>
<td>512</td>
<td>510</td>
<td>510</td>
</tr>
<tr>
<td>Samarón® Brilliant Yellow H10GF&lt;sup&gt;i&lt;/sup&gt; Hoechst</td>
<td>451</td>
<td>458</td>
<td>443</td>
</tr>
<tr>
<td>Samarón® Brilliant Orange HFR&lt;sup&gt;i&lt;/sup&gt; Hoechst</td>
<td>508</td>
<td>499</td>
<td>492</td>
</tr>
<tr>
<td>Samarón® Violet HFRL Hoechst</td>
<td>566</td>
<td>543</td>
<td>540</td>
</tr>
</tbody>
</table>

<sup>a</sup> as colloidal solution.
<sup>b</sup> as molecular solution.
<sup>c</sup> these values were used, due to the presently available filters.
<sup>d</sup> representatives of disperse dyes which can also be detected by fluorometry.
<sup>e</sup> a representative of “reactive” disperse dyes.
Sols were prepared from the commercial dyestuffs, starting with a 5% (w/v) dispersion of the dyestuff in distilled water, in case of dry, powdered products; in case of liquid preparations experiments were started with a 5% (v/v) dispersion in distilled water.

Fractionation of the dye dispersion in water was carried out by centrifuging as described in 1.1. Fractionation to particle size has also been carried out with the aid of filters having a defined pore size; in this way usable sols are obtained, but the yield of dyestuff was considerably less than with centrifuging, whilst furthermore the method is extremely time-consuming.

Hydrodynamic chromatography and the use of gradients during centrifuging form a useful supplement to the methods mentioned above.

9.2. Transfer dyes

Transfer dyes are dyes which are used during transfer printing, whereby a coloured pattern is transferred from one surface to another, generally from paper to textile. The “sublimation-, sublimation-, dry heat-, or thermopriniting process” makes use of sublimable organic dyes which are generally insoluble in water and soluble in organic environments. Sols of this type of dye have also been made up in water, via the “condensation method” (see J. Th. G. Overbeek in: H. R. Kruyt (Ed.) “Colloid Science”, Vol. I, pp. 59-60; 1962, Elsevier, Amsterdam).

9.2.1. Lurafix® Blue FFR (BASF)

Solutions (1 ml) of Lurafix® Blue FFR in acetone with the following concentrations: 2, 1.5, 1.0, 0.8, 0.6, 0.4, and 0.2 g/l were added, during intensive stirring, always to 49 ml distilled water. The suspensions obtained were centrifuged (30 minutes, 1000 g=9 800 N/kg) and the pellets were washed with distilled water (50 ml) and again centrifuged under the above-mentioned conditions. Then the pellets are suspended in such a volume of distilled water that the final concentration is 0.1 mg/ml. The final dye sols were obtained by subjecting the various suspensions to an ultrasonic treatment (Branson Sonifier B-12, 2 minutes, 70 Watts). The absorption spectrum of these dye sols was recorded in the region 750—360 nm. The value of λ_{max} dropped from 716 to 617 nm, starting from the sol corresponding to an original dye/acetone concentration of 2 g/l down to 0.2 g/l. These spectral changes are indicative of a decreasing particle size (see H. R. Kruyt: “Colloids”, P. 132; 1930, Wiley, New York; G. H. Jonker in H. R. Kruyt (Ed.): “Colloid Science”, Vol. I, p. 102; 1982, Elsevier, Amsterdam; F. B. Gribnau, Dissertation, Utrecht 1935).

9.2.2. Lurafix® Red BF (BASF)

a. A solution of Lurafix® Red BF in acetone (1 ml, 0.5 g/l) is added, under vigorous stirring, to distilled water (24 ml). Then, under vacuum and at room temperature, the acetone is gently evaporated. Via an initially stable sol we finally get a precipitate. The suspension is centrifuged (30 minutes, 1000 g=9800 N/kg) and the pellet is resuspended in distilled water (25 ml) followed by an ultrasonic treatment (Branson Sonifier B-12, 2 minutes, 70 Watts).
b. A solution of Lurafix® Red BF in acetone (1 ml, 5 g/l) is added under vigorous stirring to distilled water (249 ml, 50°C). After 1 minute at 50°C the mixture is cooled down to room temperature. After standing for one day at room temperature the initially stable sol starts to partially flocculate.

Photographs were taken of both freshly prepared sols by means of a scanning electron microscope, see photographs 1 and 2.

9.3. Fat dyes (solvent dyes)

A group of hydrophobic organic dyes which are insoluble in water but soluble in organic solvents or mixtures thereof. Using condensation methods as described in 9.2. sols can be made from these dyes in aqueous media.

9.4. Vat dyes

These water-insoluble anthraquinoid or indigoid dyes can be converted by reduction in alkaline medium into the corresponding, water-soluble leuco compounds. From these it is then possible to prepare dye sols by controlled oxidation. The leuco compounds (solubilised vat dyes) stabilised as sulphate ester can also be used for this purpose.

9.5. Organic pigments

These compounds which by definition are insoluble in water and organic media can be converted via a dispersion method (see P. Nylen and E. Sunderland: “Modern Surface Coatings”, Interscience Publishers, London 1965) into colloidal “solutions”.

Example 10

Variations on the preparation of dye sol/immunoglobulin conjugates, as described in Example 1 (1.2.).

10.1. Immobilisation of immunoglobulin on colloidal dye particles

10.1.1. Lurafix® Blue FFR (BASF)

A solution of Lurafix® Blue FFR in acetone (5 ml, 1 g/l) is added under vigorous stirring to distilled water.
(245 ml). After centrifuging (30 minutes, 1000 g = 9800 N/kg) the supernatant is removed and the pellet is washed with distilled water (50 ml). The pellet is resuspended in distilled water (up to 50 ml) and the suspension is treated ultrasonically (Branson Sonifer B-12, 2 minutes, 70 Watt). The sol obtained is diluted to give a A492nm of 1.0.

Rabbit anti-HCG immunoglobulin solution (0.2 ml, 1 mg/ml in a solution of 5 mmol NaCl/l, pH 7.0) is added to 10 ml of this sol and the mixture is stored for 16 hours at room temperature. Then a solution of Carbowax®-20M (0.2 ml, 10 g/l) in a solution of 5 mmol NaCl/l, pH 7.0) is added and after being kept for 30 minutes at room temperature the sol is centrifuged (30 min, 4000 g = 39,200 N/kg). The pellet is washed twice with a Carbowax®-20M solution (0.2 g/l in a solution of 5 mmol NaCl/l, pH 7.0) and subsequently resuspended therein to give an end volume of 5 ml.

The procedure described above is repeated, but now using normal rabbit immunoglobulin. The immuno-activity of the conjugates is established in the following manner:

Specimens (2 ml) of each conjugate are diluted using the latter-mentioned Carbowax®-20M solution. To this 0.1 ml of a solution of HCG labelled with horse-radish peroxidase HRP is added, and the reaction mixture is incubated for two hours at room temperature. Then this is centrifuged (30 mins, 4000 g = 39,200 N/kg), the pellets are washed twice with the latter-mentioned Carbowax®-20M solution and resuspended in a solution of chromogen/substrate (o-phenylene-diamine/urea peroxide). After one hour the reaction temperature the enzyme reaction is stopped with 4 mol/l sulphuric acid, and the A492 of the supernatants is measured after centrifuging (30 mins, 4000 g = 39,200 N/kg).

Dye/anti-HCG Ig conjugate: A492=0.556.
Dye/normal Ig conjugate: A492=0.275.

10.1.2. Palani® Red BF (BASF)

Immobilisation of for example proteins on solid carrier materials can be obtained via adsorption and via, direct or indirect, covalent coupling. The latter depends on the presence of suitable functional groups in the chemical structure of the dye. For example it is possible to use aromatic amino groups in a diazo coupling, whilst carboxyl groups can be activated by means of a carboxilimide. Aliphatic primary amino groups and hydroxy groups can be activated by means of cyanogen bromide. Use can also be made of bi-functional compounds; thus it is possible to use glutaraldehyde for the coupling of amino components.

It is also possible in this context to use reactive dispersion dyes, these being dyes in which the chromophore is attached to a group which is already reactive as such, e.g. halotriazines and halo-pyrimidines.

The following examples have been performed with the dispersion dye Palani® Red BF (BASF) using a fraction which was isolated in the following manner:

A dispersion of this dye in water (62.5 g/l) is centrifuged (30 min, 750 g = 7,350 N/kg). The pellet is washed five times with distilled water and twice with a solution of poly (vinyl alcohol) (PVA) in water (1 g PVA/l; PVA: Mowiol® 28-99, Hoechst). Finally the pellet is resuspended to a final volume of 2 l in a PVA solution (0.1 g PVA/l in a solution of 5 mmol NaCl/l, pH 7.0). Samples (25 ml) of this dye sol, optionally after processing as described in greater detail in the following, were mixed with a solution of sheep anti-HCG immunoglobulin (0.65 ml, 40 mg/ml) in a solution of 5 mmol NaCl/l, pH 7.0).

Adsorption and/or attachment of this protein to the colloidal dye particles was carried out for 16 hours under the specified conditions:

10.1.2.1. Adsorption

a. Adsorption at pH 4.0 and 0 mol NaCl/l
b. Adsorption at pH 4.0 and 0.1 mol NaCl/l
c. Adsorption at pH 7.0 and 0 mol NaCl/l
d. Adsorption at pH 7.0 and 0.1 mol NaCl/l
e. Adsorption at pH 6.0 and 0 mol NaCl/l (sol and protein were mixed at pH 4.0)
f. Adsorption at pH 6.0 and 0.1 mol NaCl/l (sol and protein were mixed at pH 4.0).

10.1.2.2. Diaz coupling

The dye was diazotised at 4°C using NaNO2/HCl and subsequently the pH of the reaction mixture was adjusted to 8.6 using solid Na2CO3. Finally protein was added; the coupling was performed at 4°C.

a. NaNO2: 0.1 mol/l HCl: 0.1 mol/l
b. NaNO2: 0.05 mol/l HCl: 0.05 mol/l
c. NaNO2: 0.01 mol/l HCl: 0.01 mol/l

10.1.2.3. Cross-linking by means of glutaraldehyde

The glutaraldehyde concentration of the dye sol was set at 1 and 10 mmol/l respectively, after which the pH was adjusted to 7.4 and 10.0 respectively. After 1 hour at room temperature the pH 10 was reduced to 8.0. Finally protein was added. The reaction was performed at room temperature.

a. Glutaraldehyde: 1 mmol/l pH 7.4
b. Glutaraldehyde: 10 mmol/l pH 7.4
c. Glutaraldehyde: 1 mmol/l pH 10.0
10.1.2.4. CNBr activation

The dye sol was centrifuged and the pellet was washed with a solution of 5 mmol NaCl/l, pH 7.0. This was followed by resuspension in the same solution, or in a solution of PVA (10 g/l in a solution of 5 mmol NaCl/l, pH 7.0). The CNBr concentration of the various sols was adjusted to 0.045 and 0.005 mol/l respectively, after which the pH was adjusted to 11.0 using 4 mol/l NaOH. After 12.5 min at room temperature the reaction mixtures were centrifuged and the pellets were washed twice with 0.1 mol/l NaHCO₃, pH 8.5 (4°C). This was followed by resuspension to the initial volume in 0.025 mol/l NaHCO₃, pH 8.5. The protein coupling was performed at 4°C.

a. PVA concentration: 0 g/l CNBr conc.: 0.045 mol/l
b. PVA concentration: 10 g/l CNBr conc.: 0.045 mol/l
c. PVA concentration: 10 g/l CNBr conc.: 0.005 mol/l

After 16 hours, the various reaction mixtures were centrifuged (30 min, 1000 g=9,800 N/kg). The supernatants were removed and the pellets were washed twice with a solution of 5 mmol NaCl/l, pH 7.0 and resuspended to a final volume of 20 ml in the same solution.

The immuno-activity of the various conjugates was determined by incubating 5 ml of each conjugate (16 hours, 4°C, in the dark) with HCG, labelled with horse-radish peroxidase (HRP). Then the reaction mixtures are centrifuged (1000 g=9,800 N/kg, 30 min). The pellets are washed twice with 3 ml of a solution of 5 mmol NaCl/l, pH 7.0 and subsequently re-suspended in a solution of chromogen/substrate (o-phenylene-diamine/urea peroxide). After one hour reaction time at room temperature (in the dark) the enzyme reaction is stopped with 4 mol/l sulphuric acid and after centrifuging (30 min, 1000 g=9,800 N/kg) the A₄₉₂ of the supernatants is measured.

The above-mentioned procedure was also performed using HRP alone, to check specificity.

### Table

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>A₄₉₂ (HCG-HP*)</th>
<th>A₄₉₂ (HRP)*</th>
<th>A₄₉₂ (HCG-HP)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.1.2.1-a.</td>
<td>0.195</td>
<td>0.094</td>
<td>0.750</td>
</tr>
<tr>
<td>b.</td>
<td>0.420</td>
<td>0.098</td>
<td>1.970</td>
</tr>
<tr>
<td>c.</td>
<td>1.738</td>
<td>0.109</td>
<td>1.280</td>
</tr>
<tr>
<td>d.</td>
<td>0.942</td>
<td>0.109</td>
<td>1.496</td>
</tr>
<tr>
<td>e.</td>
<td>0.230</td>
<td>0.109</td>
<td>1.496</td>
</tr>
<tr>
<td>f.</td>
<td>1.129</td>
<td>0.103</td>
<td>1.496</td>
</tr>
<tr>
<td>10.1.2.2-a.</td>
<td>0.738</td>
<td>0.120</td>
<td>1.260</td>
</tr>
<tr>
<td>b.</td>
<td>0.568</td>
<td>0.113</td>
<td>1.316</td>
</tr>
<tr>
<td>c.</td>
<td>0.777</td>
<td>0.086</td>
<td>1.505</td>
</tr>
<tr>
<td>10.1.2.3-a.</td>
<td>1.097</td>
<td>0.062</td>
<td>1.920</td>
</tr>
<tr>
<td>b.</td>
<td>0.489</td>
<td>0.073</td>
<td>1.920</td>
</tr>
<tr>
<td>c.</td>
<td>0.443</td>
<td>0.065</td>
<td>1.920</td>
</tr>
<tr>
<td>d.</td>
<td>0.436</td>
<td>0.118</td>
<td>1.920</td>
</tr>
<tr>
<td>10.1.2.4-a.</td>
<td>1.900</td>
<td>0.088</td>
<td>2.153</td>
</tr>
<tr>
<td>b.</td>
<td>1.300</td>
<td>0.137</td>
<td>2.020</td>
</tr>
<tr>
<td>c.</td>
<td>1.022</td>
<td>0.118</td>
<td>1.671</td>
</tr>
</tbody>
</table>

*) The conjugates were tested on three different days:
Day 1: 10.1.2.1-a/f
Day 2: 10.1.2.2-a/c and 10.1.2.3-a/c
Day 3: 10.1.2.4-a/c

**) These conjugates were tested on the same day.

10.2. The effect of “subsequent coating” of dye sol/immunoglobulin conjugates on immuno-activity

The experiments were performed using as example Palanil® Red BF (BASF), using the type of sol already prepared in accordance with the method described in 1.1. above. This sol was adjusted to pH 7.0 using 0.1 mol/l NaOH or HCl and the extinction (at 533 nm) was set at 5.0.

Samples (37 ml) of this sol are mixed with a solution of rabbit anti-HCG immunoglobulin (0.74 ml, 1 g/l in a solution of 5 mmol NaCl/l, pH 7.0). The Ig concentration in the reaction mixture is then 20 mg/l. After 1 hour incubation at room temperature the following are added to the sols:

a. Nothing
b. Carbowaax®-20M up to a concentration of 0.2 g/l
c. BSA up to a concentration of 0.2 g/l.
After a further 1 hour incubation at room temperature the sols are centrifuged (30 min, 4000 g = 39,200 N/kg). The pellets are re-suspended to a final volume of 37 ml in a solution of 5 mmol NaCl/l with either no extra additive, 0.2 g Carbowax®20M/l, or 0.2 g BSA/l.

The various conjugates and the blank dye sol were tested in a DIA ("Sandwich test") using the procedure described in section 1.4. The dilution series for HCG employed was 0, 250, 1000 and 4000 IU (immunoassay)/l phosphate buffer.

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>A_{515}</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCG (IU/l)</td>
<td>0</td>
</tr>
<tr>
<td>10.2-a</td>
<td>0.579</td>
</tr>
<tr>
<td>b</td>
<td>0.165</td>
</tr>
<tr>
<td>c</td>
<td>0.621</td>
</tr>
</tbody>
</table>

10.3. Purification methods for isolation of the conjugate after preparation
Inter alia the following techniques can be considered as methods of purification:

- centrifugation
- gel chromatography
- affinity chromatography
- membrane filtration
- partial precipitation (flocculation, followed by washing and reconstitution of the sol).

Centrifugation and gel chromatography were investigated in detail and the results were compared with those for a conjugate which had not been purified but otherwise identically prepared. For this purpose we used non-purified conjugate 10.2-c.

10.3-a Gel chromatography
4 ml conjugate (A_{370} = 5.0) is passed through a Sepharose® CL 2B column (Pharmacia K 16/20, bed volume 35 ml; equilibrated with a solution of 5 mmol NaCl + 0.2 g BSA + 1.0 g NaNO_3/l, pH 7.0). The column is eluted with equilibration buffer (room temperature, 30 ml/hour); detection of the eluate by measuring the A_{360}. Fractions of 1.3 ml are collected; the main fractions from the dye peak are mixed.

10.3-b Centrifugation
4 ml conjugate (A_{370} = 5.0) is centrifuged (30 min, 4000 g = 39,200 N/kg) and re-suspended to give a final volume of 4 ml using a solution of 5 mmol NaCl + 0.2 g BSA + 1.0 g NaNO_3/l, pH 7.0.

10.3-c No purification
The unpurified conjugate 10.2-c is used for this purpose.

The three conjugates are tested in a DIA (Sandwich test) using the procedure described in section 1.4. The HCG dilution series in section 10.2. was employed.

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>A_{515}</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCG (IU/l)</td>
<td>0</td>
</tr>
<tr>
<td>10.3-a</td>
<td>0.228</td>
</tr>
<tr>
<td>b</td>
<td>0.218</td>
</tr>
<tr>
<td>c</td>
<td>0.234</td>
</tr>
</tbody>
</table>

10.4. Effect of the immunoglobulin concentration used during the preparation of the conjugate on the final immuno-activity
Samples (5 ml) of a dye sol prepared from Palanil® Red BF (BASF) in accordance with the method described in section 1.1. are mixed with 0.1 ml of a rabbit anti-HCG immunoglobulin solution (5 mmol NaCl/l, pH 7.0) resulting in final concentrations of:

- 10.4-a 10 mg/l
- b 20 mg/l
- c 40 mg/l
- d 80 mg/l

After incubation for one hour at room temperature, 0.1 mol of a BSA solution (5 mmol NaCl + 20 g BSA/l, pH 7.0) is added, resulting in a final concentration of 0.4 g BSA/l. After incubation for 1 hour at room
temperature the soils are centrifuged (30 min, 4000 g=39,200 N/kg), the supernatants are removed and the pellets are re-suspended up to a final volume of 5 ml using a solution of 5 mmol NaCl+0.4 g BSA+0.1 g sodium merthiolate/l, pH 7.0.

The immuno-activity of the conjugates is ascertained by a DIA (Sandwich test) in accordance with the procedure described in section 1.4.; the HCG dilution series from section 10.2. was employed.

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>( A_{510} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCG (IU/l)</td>
<td>0</td>
</tr>
<tr>
<td>10.4-a</td>
<td>0.268</td>
</tr>
<tr>
<td>b</td>
<td>0.362</td>
</tr>
<tr>
<td>c</td>
<td>0.511</td>
</tr>
<tr>
<td>d</td>
<td>0.507</td>
</tr>
</tbody>
</table>

10.5. Effect of the type of anti-HCG immunoglobulin on the immuno-activity of the conjugate; the use of anti-HCG immunoglobulin isolated from sheep and rabbit anti-HCG serum and of monoclonal mouse anti-HCG immunoglobulin

The anti-HCG immunoglobulin/dye sol conjugates were prepared by coating samples (5 ml \( A_{510}^{1000}=5.0 \)) of a Palanil\textsuperscript{R} Red BF sol, made in accordance with the method described in section 1.1., in the following manner:

10.5-a Sheep anti-HCG immunoglobulin
The protein solution (0.1 ml, 1 g/l) in a solution of 5 mmol NaCl/l, pH 7.0) is added to the sol and the reaction mixture is incubated for 1 hour at room temperature. Then a BSA solution (0.1 ml, 20 g/l in a solution of 5 mmol NaCl/l, pH 7.0) is added and after incubation for 1 hour at room temperature the sol is centrifuged (30 min, 4000 g=39,200 N/kg). The supernatant is removed and the pellet is re-suspended up to a final volume of 5 ml in a solution of 5 mmol NaCl+0.4 g BSA+0.1 g sodium merthiolate/l, pH 7.0.

10.5-b Rabbit anti-HCG immunoglobulin
The same as 10.5-a, but now with the rabbit immunoglobulin.

10.5-c Monoclonal mouse anti-HCG immunoglobulin
As 10.5-a, but now with the mouse immunoglobulin and using the following quantities:
10.5-c-1: 0.1 ml immunoglobulin solution (1 g/l, 5 mmol NaCl/l, pH 7.0).
10.5-c-2: 0.1 ml immunoglobulin solution (0.25 g/l, 5 mmol NaCl/l, pH 7.0).
The immuno-activity of the conjugates was ascertained using a DIA (Sandwich test) in accordance with the procedure described in section 1.4. The following HCG dilution series was used: 0, 15.6, 62.5, 250, 1000 and 4000 IU (immunoassay/l).

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>( A_{510} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCG (IU/l)</td>
<td>0</td>
</tr>
<tr>
<td>10.5-a</td>
<td>0.483</td>
</tr>
<tr>
<td>b</td>
<td>0.556</td>
</tr>
<tr>
<td>c-1</td>
<td>0.406</td>
</tr>
<tr>
<td>c-2</td>
<td>0.437</td>
</tr>
</tbody>
</table>

10.6 Effect of the BSA concentration offered during “subsequent coating” on the immuno-activity of the conjugate
Conjugates of the dispersion dye Palanil\textsuperscript{R} Red BF (BASF) were made in accordance with the procedure described in sections 1.1. and 1.2., with however the following variations in the BSA concentrations:
The conjugates were isolated as described in section 1.2, and finally re-suspended in a solution having the following composition: 5 mmol NaCl + 0.1 g sodium methillolate + x g BSA/l (pH adjusted to 7.0 with 0.1 mol/l NaOH); the BSA concentration is always equal to that during the “subsequent coating”, and is thus respectively: x=1.6, 3.2, 5.1, 12.8, 25.6, 51.2 g/l (see table). The immuno-activity of the conjugates was ascertained by a DIA (“Sandwich test”) in accordance with the procedure described in section 1.4. During this the following HCG dilution series was used: 0, 3.9, 15.6, 62.5, 250, 1000, 4000, 16000 IU immunoassay/l.

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>BSA conc. in the added solution (g/l)</th>
<th>BSA conc. in the reaction mixture (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.6-a</td>
<td>9.8</td>
<td>1.6</td>
</tr>
<tr>
<td>b</td>
<td>19.2</td>
<td>3.2</td>
</tr>
<tr>
<td>c</td>
<td>38.4</td>
<td>6.4</td>
</tr>
<tr>
<td>d</td>
<td>76.8</td>
<td>12.8</td>
</tr>
<tr>
<td>e</td>
<td>153.6</td>
<td>25.6</td>
</tr>
<tr>
<td>f</td>
<td>307.2</td>
<td>51.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>(A_{510})</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCG (IU/l)</td>
<td></td>
</tr>
<tr>
<td>10.6-a</td>
<td>0.322</td>
</tr>
<tr>
<td></td>
<td>0.397</td>
</tr>
<tr>
<td></td>
<td>0.378</td>
</tr>
<tr>
<td></td>
<td>0.436</td>
</tr>
<tr>
<td></td>
<td>0.565</td>
</tr>
<tr>
<td></td>
<td>0.773</td>
</tr>
<tr>
<td></td>
<td>0.954</td>
</tr>
<tr>
<td></td>
<td>1.034</td>
</tr>
<tr>
<td>b</td>
<td>0.254</td>
</tr>
<tr>
<td></td>
<td>0.282</td>
</tr>
<tr>
<td></td>
<td>0.286</td>
</tr>
<tr>
<td></td>
<td>0.344</td>
</tr>
<tr>
<td></td>
<td>0.489</td>
</tr>
<tr>
<td></td>
<td>0.707</td>
</tr>
<tr>
<td></td>
<td>0.892</td>
</tr>
<tr>
<td></td>
<td>0.960</td>
</tr>
<tr>
<td>c</td>
<td>0.137</td>
</tr>
<tr>
<td></td>
<td>0.154</td>
</tr>
<tr>
<td></td>
<td>0.175</td>
</tr>
<tr>
<td></td>
<td>0.235</td>
</tr>
<tr>
<td></td>
<td>0.397</td>
</tr>
<tr>
<td></td>
<td>0.661</td>
</tr>
<tr>
<td></td>
<td>0.812</td>
</tr>
<tr>
<td></td>
<td>0.906</td>
</tr>
<tr>
<td>d</td>
<td>0.062</td>
</tr>
<tr>
<td></td>
<td>0.078</td>
</tr>
<tr>
<td></td>
<td>0.095</td>
</tr>
<tr>
<td></td>
<td>0.147</td>
</tr>
<tr>
<td></td>
<td>0.297</td>
</tr>
<tr>
<td></td>
<td>0.594</td>
</tr>
<tr>
<td></td>
<td>0.778</td>
</tr>
<tr>
<td></td>
<td>0.852</td>
</tr>
<tr>
<td>e</td>
<td>0.039</td>
</tr>
<tr>
<td></td>
<td>0.040</td>
</tr>
<tr>
<td></td>
<td>0.054</td>
</tr>
<tr>
<td></td>
<td>0.110</td>
</tr>
<tr>
<td></td>
<td>0.283</td>
</tr>
<tr>
<td></td>
<td>0.572</td>
</tr>
<tr>
<td></td>
<td>0.759</td>
</tr>
<tr>
<td></td>
<td>0.843</td>
</tr>
<tr>
<td>f</td>
<td>0.021</td>
</tr>
<tr>
<td></td>
<td>0.029</td>
</tr>
<tr>
<td></td>
<td>0.044</td>
</tr>
<tr>
<td></td>
<td>0.111</td>
</tr>
<tr>
<td></td>
<td>0.310</td>
</tr>
<tr>
<td></td>
<td>0.629</td>
</tr>
<tr>
<td></td>
<td>0.803</td>
</tr>
<tr>
<td></td>
<td>0.882</td>
</tr>
</tbody>
</table>

Example 11
Colorimetric and/or visual determination of human chorionic gonadotropin (HCG) according to the DIA principle (agglutination test; method 1).

11.1. Preparation of the dye sol
See 1.1.

11.2. Preparation of the rabbit anti-HCG immunoglobulin/dye sol conjugate
See 1.2.; in this case however the BSA solution contains 2.4 g/l, because the pellet is resuspended in a solution having the described composition, but with 0.4 g BSA/l. The conjugate is finally further diluted up to a value of \(A_{930} = 2.2\).

11.3. Determination of HCG in phosphate buffer or urine TRIS buffer
1 mol TRIS+1 mol NaCl+10 g BSA/l, adjusted to pH 7.4 with 4 mol/l HCl. HCG See 1.4.

Urine
See 1.4.

Phosphate buffer (FBF)
See 1.4.

Conjugate
See 11.2.

Procedure
1. HCG solution of 5 000 and 1 000 IU (immunoassay)/l are made up by diluting the standard HCG solution with FBF or urine.

2. Pipette into a cell (1 cm light path): conjugate (1 ml), TRIS buffer (0.1 ml) and HCG solution (0.11 ml). Mix well and immediately scan the absorption spectrum in the region 750—360 nm, with FFB and urine respectively as reference.

3. Allow the cells to stand at room temperature for 20 hours. Then assess the contents by eye and shake the cells and then once again establish an absorption spectrum in the region 750—360 nm.

Representative spectra are given in Fig. 3, while numerical data are provided in the following table:
HCG conc. (IU/l) | Visual* | \(\left( A_{532} - A_{533}\right)_{0} \) | \(\left( A_{673} - A_{673}\right)_{x} \)
---|---|---|---
0 | − | 0 | 0
1000 | + | 0.15 | 0.14
5000 | ++ | 0.40 | 0.21

* ) "−": no agglutination/aggregation. Dye sol stable.

"+": incipient agglutination/aggregation. The supernatant is lighter in colour than with the corresponding blank (0 IU HCG/l) due to sedimentation.

"
++": complete agglutination/aggregation. The dye sol has flocculated completely and the dye is precipitated.

**) O: 0 IU HCG/l.

x: 1 and 5 respectively, IU HCG/l.

Example 12
Colorimetric determination of human chorionic gonadotropin (HCG) according to the DIA principle (agglutination test; method 2).

12.1. Preparation of dye sol
See section 1.1.

12.2. General test procedure for DIA/agglutination
The dye-immunoglobulin conjugate (2 ml) was pipetted into a glass or polystyrene cuvette and mixed with 0.2 ml of the antigen sample dissolved in FFB (see section 1.4.), or 0.2 ml FFB only, and 0.2 ml of distilled water or of a solution of MgSO₄ in distilled water. The cuvettes were maintained at room temperature (without agitation) and the absorbances were determined (without prior agitation) after regular time intervals. The antigen causes agglutination of the dye sol particles, which yields a decrease in absorbance due to the increased effective particle size per se and due to the concomitant sedimentation of the aggregates.

All further experiments are concerned with the determination of HCG.

12.3. Preparation of the rabbit (anti HCG) IgG/dye conjugate
Standard procedure: see section 11.2.
Optimization was performed by investigating the following parameters:

12.3.1. Secondary coating of conjugates with BSA
Conjugates for DIA/sandwich are secondarily coated with BSA in order to reduce non-specific effects; this extra coating of the conjugate will certainly also improve its stability and will be disadvantageous therefore for conjugates to be used in DIA/agglutination. Conjugates coated with varying amounts of BSA, and also without BSA, were compared in the agglutination assay. The conjugate without BSA yielded the highest sensitivity and lowest detection limit in the shortest test period, and was still sufficiently stable to give a constant blank value.

12.3.2. Optimal IgG concentration during conjugate preparation
Palanil® Red B/anti-HCG conjugates were prepared using different IgG concentrations during the coating, and were screened in the agglutination assay using a constant HCG concentration of 5 IU/ml sample (or: 0.42 IU/ml total test volume). The optimal IgG concentration during conjugate preparation appeared to be 0.033 mg/ml reaction mixture at a sol concentration corresponding with \( A_{532}^{1%} = 5 \).

12.3.3. Effect of incubation of IgG at pH 2.0 prior to conjugate preparation
A solution of rabbit anti-HCG IgG (4–5 mg/ml in 5 mmol NaCl/l pH 7.0) was adjusted to pH 2.0 and incubated for 1 h at 4°C; the pH was readjusted to 7.4 and the IgG solution was used for conjugate preparation. The concentration of “pH 2 treated IgG” during the conjugate synthesis was varied, and the various conjugates were screened as described in 12.3.2.

A concentration of 0.033 mg “pH 2 treated IgG”/ml reaction mixture, at a sol concentration corresponding with \( A_{532}^{1%} = 5 \), appeared to be the optimal value.

Comparison of conjugates based on native IgG and “pH 2 treated” IgG clearly demonstrated the advantage of the latter with respect to reactivity in the agglutination test:
12.3.4. Effect of additives on the reactivity of conjugates

Addition of a destabilizing agent (e.g. MgSO₄) to the conjugate prior to addition of the sample may yield a decrease in reaction time and/or detection limit of the agglutination assay, particularly in case of conjugates highly stable per se. Anti-HCG/Palanil® Red BF conjugates, based on the dye batches 5228513 (powder) and 4742893 (wet dispersion), were incubated with a dilution series of MgSO₄ yielding a final concentration range of 0—20 mmol MgSO₄/l total test mixture, and Aₘₙ₅₀ was determined at regular time intervals. Concentrations of 1.2 and 9.5 mmol MgSO₄/l total test mixture, respectively, were found to be compatible with a still suitable stability of the conjugates (decrease of Aₘ₅₀ less than 0.1—0.2 after 2 h).

A dilution series of HCG in FFB (see section 1.4.; 0—5 IU/ml sample) was incubated with anti-HCG/Palanil® Red BF (4742893) conjugate with an without 9.5 mmol MgSO₄/l total test mixture. The conjugate without MgSO₄ gave only a slight decrease in A₁₅₀, as compared to the blank whereas in the presence of MgSO₄, a decrease in absorbance, significantly differing from the blank, was observed:

<table>
<thead>
<tr>
<th>IgG in conjugate</th>
<th>(HCG) (IU/ml)</th>
<th>Decrease in A₁₅₀ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>native IgG</td>
<td>2.5</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td>pH-2 treated IgG</td>
<td>2.5</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>28</td>
</tr>
</tbody>
</table>

12.3.5. Reactivity of anti-HCG/Palanil® Red BF conjugate, based on "pH 2 treated" IgG, and in the presence of MgSO₄

The combined effects, described in 12.3.3/12.3.4, were investigated with Palanil® Red BF conjugates (based on the wet and dry commercial preparation), and a HCG dilution series in FFB (0—4 IU/ml sample). The obtained detection limits (defined as the concentration of HCG yielding a response equal to (BL—2×SDI)) are summarized below:

<table>
<thead>
<tr>
<th>[MgSO₄] (µmol/ml total test mixture)</th>
<th>(HCG) (IU/ml)</th>
<th>decrease in A₁₅₀ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.5</td>
<td>3</td>
</tr>
<tr>
<td>9.5</td>
<td>2.5</td>
<td>13</td>
</tr>
<tr>
<td>0</td>
<td>5.0</td>
<td>4</td>
</tr>
<tr>
<td>9.5</td>
<td>5.0</td>
<td>21</td>
</tr>
</tbody>
</table>

12.3.6. Effect of particle size on conjugate reactivity

All experiments up to now were performed with only one fraction of the total dye dispersion, viz. the material remaining in the supernatant at 1000—1100 g (9800—10 800 N/kg), which corresponds roughly with a particle size ≤0.2 μm diameter. The effect of particle size was further investigated by preparing anti-HCG conjugates of different "g-fractions" of Palanil® Red BF, and testing them in an HCG agglutination assay. Significant effects were observed (cf. Figure 4), yielding optimal results for the dye fraction isolated between 1500 and 2500 g.

Example 13

Lyophilization of disperse dye/immunoglobulin conjugates; stability.
A Palanili® Red BF/rabbit anti-HCG conjugate was lyophilized from an aqueous dispersion ($A_{230}^{\text{em}}$=5) containing the following constituents:

5 mmol NaCl
5 g BSA
1 g sodium methiolate per litre;
2.5 g dextran (Mw 40 000 Dalton)
5.0 g lactose

The reconstituted dry conjugate showed no loss in reactivity as compared to the original wet preparation. The conjugate retains its immunoreactivity for at least 2$\frac{1}{2}$ months at -20°C, 4°C and room temperature. Some loss in activity was found after 2$\frac{1}{2}$ months/37°C, a considerable loss was found after 2$\frac{1}{2}$ months/45°C.

Claims

1. Process for the qualitative and/or quantitative determination of an immunochemically reactive component such as a hapten, antigen or antibody in an aqueous test medium using the immunochemical reactivity of such components with respect to each other, whereby one or more labelled immunochemically reactive components are employed and whereby during or after a certain reaction period for the immunochemical reaction, optionally after separation of the free and bound labelled component(s), in the test medium or in one of the fractions obtained after separation, the nature and/or the quantity of the label is determined using methods known for this purpose, characterized in that the or each labelled immunochemically reactive component is obtained by direct or indirect attachment of such a component or components to particles of an aqueous sol of a hydrophobic organic dye or pigment.

2. Process according to claim 1, characterised in that the detection takes place by establishing, either visually or by measurement, the physico-chemical changes of the dye sol.

3. Process according to claim 1 and/or 2, characterised in that two or more immunochemically reactive components are determined simultaneously in that for each component to be indicated or determined a corresponding immunochemically reactive component is used, which is labelled with an organic dye sol particle characteristic for that component by using chromophores which can clearly be distinguished from each other spectrally and/or visually.

4. Process according to claim 1, characterised in that the or each labelled component is obtained by adding to the organic dye or pigment sol a certain quantity of the immunochemically reactive component to be labelled, the latter enveloping the dispersed particles wholly or partially, after which if required supplementary coating can take place with a polar macromolecule which is immunochemically inert in the corresponding determination.

5. Process according to claim 1, characterised in that the or each labelled component is obtained by adding to the sol of the organic dye or pigment one or more macromolecules which are immunochemically inert in the corresponding determination, and which coat the sol particles, after which by means of adsorption, possibly bio-specific, or via covalent attachment, the immunochemically reactive component is attached to the coating material.

6. Process according to claim 1, characterised in that the or each labelled component is obtained by the organic dye and/or organic pigment sol being placed in an environment of monomers, by causing the latter to be polymerised or co-polymerised in situ, resulting in envelopment of the sol particles, and subsequently adsorbing or covalently attaching the immunochemically reactive component to the polymer material.

7. Process according to claim 1, characterised in that the or each labelled component is obtained by covalently attaching the immunochemically reactive component to colloidal organic dye particles, either by prior chemical activation of suitable functional groups in the dye and/or the immunochemically reactive component, or by the use of conjugates of organic hydrophobic chromophores and reactive groups, known as reactive (dispersion) dyes.

8. Process according to claim 5, characterised in that the organic dye and/or the organic pigment sol particles are first protected by a hydrophilic macromolecule, after which (co-)polymerisation takes place under the influence of an inorganic initiator.

9. Immunochemical reagent, to be used for the determination of one or more immunochemically reactive components in an aqueous medium, comprising a labelled immunochemically reactive component obtained by the direct or indirect attachment of such a component to particles of an aqueous sol of a hydrophobic organic dye or pigment.

10. Freeze-dried or spray-dried immunochemical reagent obtained by freeze-drying or spray-drying the immunochemical reagent according to claim 9.
11. Test kit to be used for the qualitative and/or quantitative determination of one or more immunochromically reactive components in an aqueous test medium, containing in addition to other reagents one or more immunochromic reagents according to claim 9 or 10.

5 Patentansprüche

1. Verfahren zur qualitativen und/oder quantitativen Bestimmung einer immunochromischen reaktionsfähigen Komponente, wie ein Hapten, Antigen oder Antikörper, in einem wässerigen Testmedium unter Verwendung der immunochromischen Reaktionsfähigkeit von solchen Komponenten in bezug aufeinander, wobei eine oder mehrere markierte immunochromische reaktionsfähige Komponenten verwendet werden und wobei während oder nach einer gewissen Reaktionszeit für die immunochromische Reaktion, gegebenenfalls nach Trennung der freien und gebundenen markierten Komponenten(n), im Testmedium oder in einer der nach der Trennung erhaltenen Fraktionen die Natur und/oder die Menge der Markierung bestimmt wird unter Verwendung bekannter Methoden zu diesem Zweck, dadurch gekennzeichnet, dass die oder jede markierte immunochromische reaktionsfähige Komponente durch direkte oder indirekte Bindung einer solchen Komponente oder Komponenten an Teilchen eines wässerigen Sols eines hydropohen organischen Farbstoffes oder Pigmentes erhalten wird.

2. Verfahren nach Patentanspruch 1, dadurch gekennzeichnet, dass der Nachweis durch Feststellung, entweder visuell oder durch Messung, der physikalisch-chemischen Veränderungen des Farbstoffsol gelingt.

3. Verfahren nach Patentanspruch 1 und/oder 2, dadurch gekennzeichnet, dass zwei oder mehr immunochromisch reaktionsfähige Komponenten gleichzeitig bestimmt werden, indem für jede nachzuweisende oder zu bestimmende Komponente eine entsprechende immunochromische reaktionsfähige Komponente verwendet wird, welche mit einem organischen Farbstoffsolpartikel markiert ist, welches für jene Komponente charakteristisch ist, durch Verwendung von Chromophoren, welche klar voneinander spektral und/oder visuell unterschieden werden können.

4. Verfahren nach Patentanspruch 1, dadurch gekennzeichnet, dass eine oder jede markierte Komponente erhalten wird durch Zusatz zum organischen Farbstoff- oder PigmentsoI einer gewissen Menge der immunochromisch reaktionsfähigen, zu markierenden Komponente, wobei die letztere die dispergierten Partikel ganz oder teilweise umhüllt, worauf, falls erforderlich, ein zusätzliches Ueberziehen stattfinden kann mit einem polaren Macromolekül, welches immunochromisch inert ist in der entsprechenden Bestimmung.

5. Verfahren nach Patentanspruch 1, dadurch gekennzeichnet, dass die oder jede markierte Komponente erhalten wird durch Zusatz zu dem Sol des organischen Farbstoffes oder Pigmentes eines oder mehrerer Macromoleküle, welche immunochromisch inert sind in der entsprechenden Bestimmung, und welche die Solpartikel überziehen, worauf mit Hilfe von Adsorption, eventuell biospezifisch, oder über covalente Bindung die immunochromisch reaktionsfähige Komponente an der Ueberzugsmaterial gebunden wird.

6. Verfahren nach Patentanspruch 1, dadurch gekennzeichnet, dass die oder jede markierte Komponente erhalten wird, indem das organische Farbstoff- und/oder organische Pigmentsol in eine Umgebung von Monomeren verbracht wird, die letzteren zum Polymerisieren oder Copolymerisieren in situ gebracht werden, wodurch ein Uehmüllen der Solpartikel erzielt wird, und anschliessend die immunochromisch reaktionsfähige Komponente an das polymere Material adsorbiert oder covalent gebunden wird.

7. Verfahren nach Patentanspruch 1, dadurch gekennzeichnet, dass die oder jede markierte Komponente erhalten wird, indem die immunochromisch reaktionsfähige Komponente an dispergierte organische Farbstoffpartikel covalent gebunden wird, entweder durch vorgängige chemische Aktivierung von geeigneten funktionellen Gruppen im Farbstoff und/oder der immunochromisch reaktionsfähigen Komponente, oder durch die Verwendung von Konjugaten von organische hydrophoben Chromophoren und reaktionsfähigen Gruppen, bekannt als reaktionsfähige (Dispersion)-Farbstoffe.

8. Verfahren nach Patentanspruch 5, dadurch gekennzeichnet, dass die organische Farbstoff- und/oder die organischen Pigmentdoppartikel zuerst durch ein hydrophil geschrägt wird, worauf (Co)-Polymersisation stattfindet unter dem Einfluss eines anorganischen Initiators.

9. Immunochromisches Reagens zur Verwendung für die Bestimmung von einer oder mehreren immunochromisch reaktionsfähigen Komponenten in einem wässerigen Medium, enthaltend eine markierte immunochromisch reaktionsfähige Komponente, welche durch direkte oder indirekte Bindung einer solchen Komponente an Partikel eines wässerigen Sols eines hydropohen organischen Farbstoffes oder Pigmentes erhalten wurden.


0 032 270

Reven
dications

1. Procédé de dosage qualitatif et/ou quantitatif d’un composant à réactivité immuno
chemique tel qu’un hapten, un antigène ou un anticorps dans un milieu d’essai aqueux en utilisant la réactivité
immuno
chemique mutuelle de tels composants de manière à utiliser un ou plusieurs composants à
réactivité immuno
chemique marqués et de manière que pendant ou après une certaine période de réaction,
ev
te
tuellement après séparation du ou des composants marqués libres et liés, dans le milieu d’essai ou
dans l’une des fractions obtenues après séparation, la nature et/ou la quantité du marqueur soient
déterminées en utilisant des méthodes connues dans ce but, caractérisé par le fait que le ou chaque
composant à réactivité immuno
chemique marqué est obtenu par fixation directe ou indirecte d’un tel ou de
tels composants aux particules d’un sol aqueux d’un colorant ou pigment organique hydrophobe.

2. Procédé selon la revendication 1, caractérisé par le fait que la détection a lieu en établissant,
visuellement ou par mesure, les variations physicochimiques du sol de colorant.

3. Procédé selon la revendication 1 et/ou 2, caractérisé par le fait que deux composants à réactivité
immuno
chemique ou plus sont déterminés simultanément, en ce que pour chaque composant à indiquer
ou déter

minir, ou utiliser un composant à réactivité immuno
chemique correspondant, qui est marqué avec
une particule de sol de colorant organique caractéristique de ce composant en utilisant des chromophores
qui peuvent être nettement distingués les uns des autres spectralement et/ou visuellement.

4. Procédé selon la revendication 1, caractérisé par le fait que le ou chaque composant marqué est
obtenu par l’addition au sol de colorant ou de pigment organique d’une certaine quantité du composant à
réactivité immuno
chemique à marquer, ce dernier enveloppant les particules dispersées entièrement ou
partiellement, après quoi, si nécessaire, un revêtement supplémentaire peut avoir lieu avec une
macromolécule polaire qui est immuno
chemiquement inactive dans la détermination correspondante.

5. Procédé selon la revendication 1, caractérisé par le fait que le ou chaque composant marqué est
obtenu par l’addition au sol de colorant ou pigment organique d’une ou plusieurs macromolécules qui sont
immuno
chemiquement inertes dans la détermination correspondante, et qui revêtent les particules de sol,
après quoi, par adsorption, éventuellement bio-spécifique, ou par fixation covalente, le composant à réactivité immuno
chemique est fixé à la matière de revêtement.

6. Procédé selon la revendication 1, caractérisé par le fait que le ou chaque composant marqué est
obtenu par la mise en place du sol de colorant organique et/ou de pigment organique dans un milieu de
monomères, en provoquant la polymérisation de ceux-ci ou leur copolymérisation in situ, ce qui entraîne
l’enveloppement des particules de sol, puis adsorption ultérieure ou fixation par covalence du composant à
réactivité immuno
chemique à la matière polymère.

7. Procédé selon la revendication 1, caractérisé par le fait que le ou chaque composant marqué est
obtenu par fixation par covalence du composant à réactivité immuno
chemique aux particules de colorant
organique colloïdales, soit par activation chimique préalable de groupes fonctionnels appropriés dans le
colorant et/ou le composant à réactivité immuno
chemique, soit par l’utilisation de conjugués de
chromophores hydrophobes organiques et de groupes réactifs, connus en tant que colorants réactifs
dispersion.

8. Procédé selon la revendication 5, caractérisé par le fait que les particules de sol de colorant
organique et de pigment organique sont tout d’abord protégées par une macromolécule hydrophile,
après quoi la (copolymérisation) a lieu sous l’influence d’un initiateur minéral.

9. Réactif immuno
chemique à utiliser pour la détermination d’un ou plusieurs composants à réactivité
immuno
chemique dans un milieu aqueux, comprenant un composant à réactivité immuno
chemique marqué obtenu par la fixation directe ou indirecte de ce composant aux particules d’un sol aqueux d’un
colorant ou pigment organique hydrophobe.

10. Réactif immuno
chemique lyophilisé ou séché par atomisation obtenu par lyophilisation ou séchage
par atomisation du réactif immuno
chemique selon la revendication 9.

11. Trousse d’essai à utiliser pour le dosage qualitatif et/ou quantitatif d’un ou plusieurs composants à
réactivité immuno
chemique dans un milieu d’essai aqueux, contenant, en plus d’autres réactifs, un ou
plusieurs réactifs immuno
chemiques selon la revendication 9 ou 10.
Figure 1. Standard curves for HCG in phosphate buffer (●) and urine (▲), according to a "RIA-sandwich" (cf. p. 8).
Figure 3  Spectra of a Palanil\textsuperscript{R} Red BF sol/rabbit anti-HCG immunoglobulin conjugate, in the presence of 0, 1, and 5 IU HCG/ml, respectively, after incubation during 20 h at room temperature (cf. p. 9).
Photograph 1.
Scanning electron microscope photo (enlargement: 15,000 X) of a sol of the transfer dye Lurafix Red BF prepared according to the method as described in Example 9.2.2.a.
Photograph 2

Scanning electron microscope photo (enlargement: 15,000 X) of a sol of the transfer dye Lurafix Red BF prepared according to a method as described in Example 9.2.2.b.