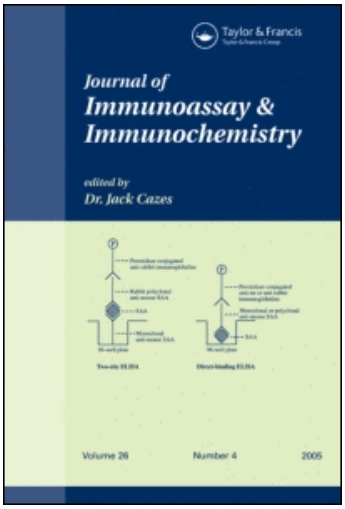

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3,3',5,5'-TETRAMETHYLBENZIDINE AS AN AMES TEST NEGATIVE CHROMOGEN FOR HORSE-RADISH PEROXIDASE IN ENZYME-IMMUNOASSAY

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ABSTRACT

The use of 3,3',5,5'-tetramethylbenzidine as non-mutagenic chromogen for the end point determination in enzyme-immunoassay (EIA) is described. In sandwich EIAs for HCG and HBsAg and in a competitive EIA for testosterone, the colour yield with TMB was superior to that obtained with o-phenylene diamine (OPD), which was by far the best chromogen for horse-radish peroxidase until now. This led to an improvement of sensitivity and precision of the assays and makes EIA even more competitive with other types of immunoassays.

INTRODUCTION

Enzyme-immunoassay (EIA) has become a generally accepted and applied technique for identification and quantitation of antigens, haptens and antibodies. One of the various enzymes suitable for use in EIA is horse-radish peroxidase (HRP) (1). In colorimetric determination of HRP, o-phenylene diamine (OPD), 2,2'-azino-di[3-ethyl-benzthiazoline-sulphonate] (ABTS) and 5-aminosalicylic acid (5-ASA) are the most commonly used hydrogen donors (2 - 4). EIAs with ABTS and OPD showed excellent sensitivities. However, both compounds

were found to be mutagenic in the Ames test (5,6). 5-ASA showed no mutagenic activity (6), but the poor colour yield obtained with this compound does not allow a high sensitivity (7). Therefore, we looked for a non-mutagenic chromogen that yields a colour intensity at least comparable with that of OPD.

Benzidine has long been used as a sensitive and specific reagent for the detection of haemoglobin, which has been found to exhibit a peroxidase-like activity (8). However, it is carcinogenic in animals and in man (9). Because it was shown that o-hydroxylation of aromatic amines is the main cause of the carcinogenicity (10), benzidine was replaced by a derivative in which o-hydroxylation was impossible, viz. 3,3',5,5'-tetramethylbenzidine (TMB) (11). This compound has no mutagenic activity, neither alone nor in conjunction with liver enzyme preparations (12). Prolonged subcutaneous administration to rats gave a negligible yield of tumours contrary to benzidine (11).

In the present paper, the use of TMB as a highly sensitive chromogen for HRP in enzyme-immunoassays is described.

MATERIALS AND METHODS

Horse-radish peroxidase (RZ 0,6 or 3,0) was from Boehringer, Mannheim, G.F.R., bovine serum albumin (BSA) from Armour, Eastbourne, U.K.; 3,3',5,5'-tetramethylbenzidine and o-phenylene diamine dihydrochloride from Fluka AG, Buchs, Switzerland; Tablets containing urea hydrogen peroxide from Organon Teknika, Turnhout, Belgium and flatbottom polystyrene microelisa ^{R} plates from Greiner, Nürtingen, G.F.R. All other reagents were of analytical grade.

Determination of Optimal Assay Conditions

Solutions of HRP in a concentration range of 0,1 µg/l to 2,5 µg/l were prepared in 0,03 mol/l citric acid-sodium phosphate (McIlvaine) buffer or 0,1 mol/l sodium acetate-citric acid buffer, each containing 1 g/l BSA. TMB was dissolved into the organic solvent of choice and this solution was added dropwise to the McIlvaine or acetate-citric acid buffer. Just before use, (urea) hydrogen peroxide was added.

To 0,5 ml of HRP solution, 1,5 ml of substrate solution was added and incubation was performed for 60 min at room temperature. The reaction was stopped with 0,5 ml of acid (see below).

Absorption spectra of the oxidation products, before and after the addition of acid, were recorded with a Gilford 250 spectrophotometer equipped with a Gilford 2530 wavelength scanner and a Kipp BD9 recorder. The absorbance was read in a Gilford 300 N spectrophotometer.

For assays in microtitration plates, 50 µl of the HRP solution was pipetted into the wells. 150 µl of substrate solution was added and incubation was performed for 60 min at room temperature. The enzyme reaction was stopped by the addition of 50 µl of acid (see below). Absorbances were read in a through the plate measuring photometer (OT Reader, Organon Teknika, Turnhout, Belgium).

Detailed reaction conditions in the individual experiments are described in the legends of the figures or in the text.

Antisera

Antisera against HCG were raised in rabbits or sheep (13). Antisera against testosterone were raised in female rabbits using

11-α-hydroxytestosterone-11-succinyl-BSA as immunogen (14).

IgG was isolated from these antisera by sodium sulphate precipitation (final concentration 1,07 mol/l) as described by Keckwick (15).

$F(ab')_2$-fragments of IgG were prepared according to the procedure of Davies et al. (16).

Coating of Microtitration Plates

Wells of microtitration plates were coated with 0,1 ml of a 20 mg/l (anti-HCG)-IgG solution according to Hamaguchi et al. (17) or with 0,1 ml of a 10 mg/l (anti-testosterone)-IgG solution (18).

HRP Conjugates

$F(ab')_2$-fragments of sheep (anti-HCG)-IgG were coupled to HRP (RZ 3,0) using N-succinimidyl-2-pyridyldithiopropionate (SPDP) as cross-linking agent (19). The conjugates were stored in 0,3 mol/l NaCl; 1 g/l BSA in small portions at -20°C.

Testosterone-3-carboxymethyloxime was bound to HRP using a modified mixed anhydride method (14). The testosterone-HRP conjugate was stored in lyophilised form at 4°C until use.

Sandwich EIA for HCG

A 0,1 ml sample of HCG solutions in 0,1 mol/l NaCl; 1

g/l BSA; 0,04 mol/l sodium phosphate buffer pH 7,4 (Buffer A) in a concentration range of 1,0 to 100 IU/l was pipetted into a well of an (anti-HCG)-coated microtitration plate and incubated for 60 min at room temperature while gently shaking. The contents of the wells were discarded and the plates washed twice with a 0,2 mol/l Tris-buffer pH 7,4, containing 0,5 g/l Tween 20 (Buffer B) and twice with bidistilled water. Subsequently, 0,1 ml of (anti-HCG)-F(ab')$_2$-HRP conjugate in buffer A was added and allowed to react for 60 min at room temperature in the dark. After discarding the contents of the wells, the plates were washed twice with buffer B and with bidistilled water. The substrate solution was added and after 60 min of incubation at room temperature in the dark, the enzyme reaction was stopped (see: End point determination).

EIA for Testosterone

A 0,08 ml sample of testosterone solutions (0,6 - 100 nmol/l) in buffer A was pipetted into each well of an (anti-testosterone)-coated microtitration plate and was allowed to react for 2 h at room temperature while shaking. 0,02 ml of a 10 mg/l testosterone-HRP solution in buffer A was added and the incubation continued for 1 h. The contents of the wells were discarded and the plates washed twice with buffer B and twice with bidistilled water. Substrate solution was added to each well and after 60 min of incubation at room temperature in the dark, the reaction was stopped and absorbances were read in a photometer (see: End point determination).

EIA for HBsAg

The enzyme-immunoassay for HBsAg was carried out according to the procedure of Wolters et al. (20).

End Point Determination

OPD

OPD.2HCl was dissolved in 0,03 mol/l McIlvaine buffer pH 5,0 to a final concentration of 4 mmol/l. Just before use, hydrogen peroxide or urea hydrogen peroxide was added (final concentration 1,3 mmol/l or 0,75 mmol/l respectively) and 100 µl of the substrate solution was pipetted into each well of the microtitration plate. After 60 min of incubation in the dark, the enzyme reaction was stopped by the addition of 100 µl of 2 mol/l H_2SO_4. The absorbance at 492 nm was read in the OT Reader.

TMB

TMB was dissolved in DMSO (final concentration 42 mmol/l) and 10 ml of this TMB solution were added dropwise under gentle shaking to 1 l of 0,1 mol/l sodium acetate/citric acid buffer pH 6,0. Just before use, hydrogen or urea hydrogen peroxide was added (final concentration 1,3 mmol/l or 0,5 mmol/l respectively) and 0,2 ml of this substrate solution were pipetted into the wells of the microtitration plates. After 60 min of incubation at room temperature, the enzyme reaction was stopped with 0,05 ml of 2 mol/l H_2SO_4. The absorbance was read at 450 nm in the OT Reader.

RESULTS AND DISCUSSION

Since TMB is poorly soluble in aqueous solutions (21) the possibility of preparing stable solutions containing a suitable amount of TMB with the aid of organic solvents was investigated. TMB was dissolved to a final concentration of 10 g/l in various organic solvents and these solutions were added to a 0,03 mol/l McIlvaine or to 0,1 mol/l sodium acetate buffer pH 6,0. TMB was insoluble in lower alcohols, diethylether and aromatic solvents like benzene, toluene and xylene. Phase separation between buffer and solvent occurred with solutions of TMB in chloroform and dichloroethane. Only with water-mixable, aprotic solvents like dioxan, dimethylformamide (DMF) and dimethylsulphoxide (DMSO) a stable solution of 0,1 g/l TMB could be prepared. Because dioxan may contain peroxides and both dioxan and DMF show toxic effects (22) subsequent experiments were carried out with DMSO as a primary solvent which does not inhibit the enzyme activity at the concentrations applied.

When a substrate solution consisting of 0,1 g/l TMB and 1,3 mmol/l H_2O_2 in McIlvaine or acetate buffer pH 6,0 was added to dilution series of HRP, a blue colour developed during incubation. The absorption spectrum of the oxidation product showed three peaks at 370 nm, 450 nm and 655 nm (Fig. 1). This is in agreement with literature data (23, 24). The small peak at 450 nm was absent at low enzyme concentrations. As can be seen in Fig. 2, a linear relationship exists between the absorbance at 655 nm and the peroxidase concentration.

After the addition of 2 mol/l sulphuric acid, the colour of the reaction mixture changed from blue to bright yellow. The absorption

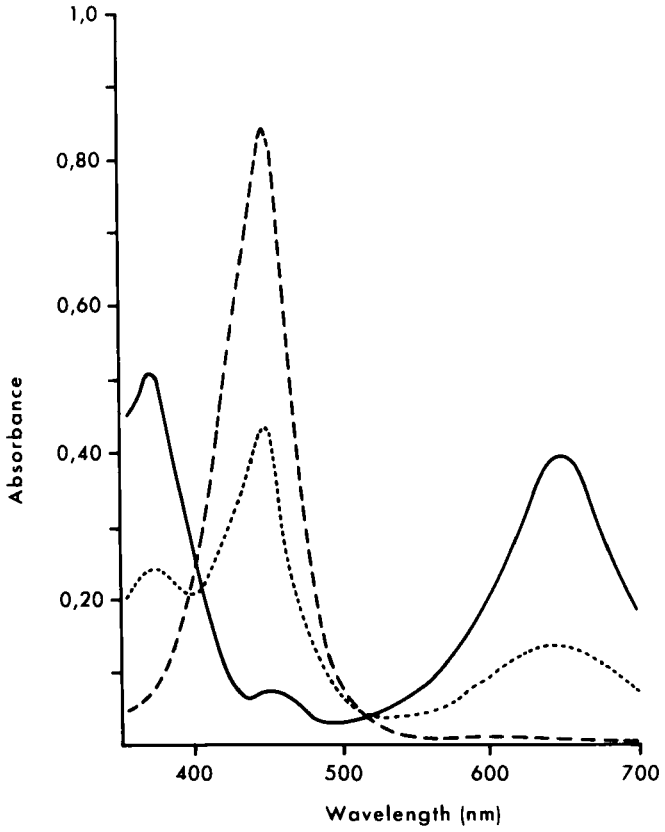

FIGURE 1

Absorbance spectra of the oxidation product of TMB before and after addition of stop reagents.

———————— : unstopped, 4 mol/l acetic acid

..... : 4 mol/l formic acid, 1,3 mol/l citric acid

– – – – – – – : 2 mol/H_2SO_4, 4 mol/l HCl, 4 mol/l TFA

peaks at 370 nm and 655 nm disappeared completely and only one sharp peak at 450 nm remained (Fig. 1). As is shown in Fig. 2, the colour intensity increased more than two-fold. The same result was obtained when 4 mol/l HCl or 4 mol/l trifluoroacetic acid (TFA) were added to the reaction mixture. 4 mol/l Acetic acid caused no change of absorption spectrum. Addition of 4 mol/l perchloric acid and nitric acid caused a turbidity. Stopping of the enzyme reaction with 4 mol/l formic acid or 1,3 mol/l citric acid resulted in a partial spectrum shift and a pale green colour of the reaction mixture (Fig. 1).

From these experiments, it is concluded that a complete spectrum shift and the concomitant hyperchromic effect could only be achieved by the addition of strong, non-oxidising acids (pKa $\leq$ 2,0). Since the absorbance at 450 nm after the addition of sulphuric acid was stable for at least 90 min and a rapid non-specific oxidation occurred in the HCl- and TFA-stopped reaction mixture, 2 mol/l H_2SO_4 was chosen as stop reagent in further experiments.

The reaction of HRP with TMB as hydrogen donor is optimal at pH 5,5 in McIlvaine buffer and between 5,0 and 6,0 in acetate buffer (Fig. 3). Because the intensity of colour developed in acetate buffer was substantially higher (Figs. 2 and 3 A) and a blue precipitate of oxidation products formed at elevated absorbance values in McIlvaine buffer, the experiments were carried out in acetate buffer. pH 6,0 was chosen as a compromise between colour intensity and blank values.

The optimal concentration of the chromogen was 0,1 g/l (Fig. 3B). At higher concentrations, part of the TMB precipitated during the incubation, giving a turbid reaction mixture. The optimal peroxide concentration was found to be 1,3 mmol/l for hydrogen peroxide and 0,5 mmol/l for urea peroxide (Fig. 3C).

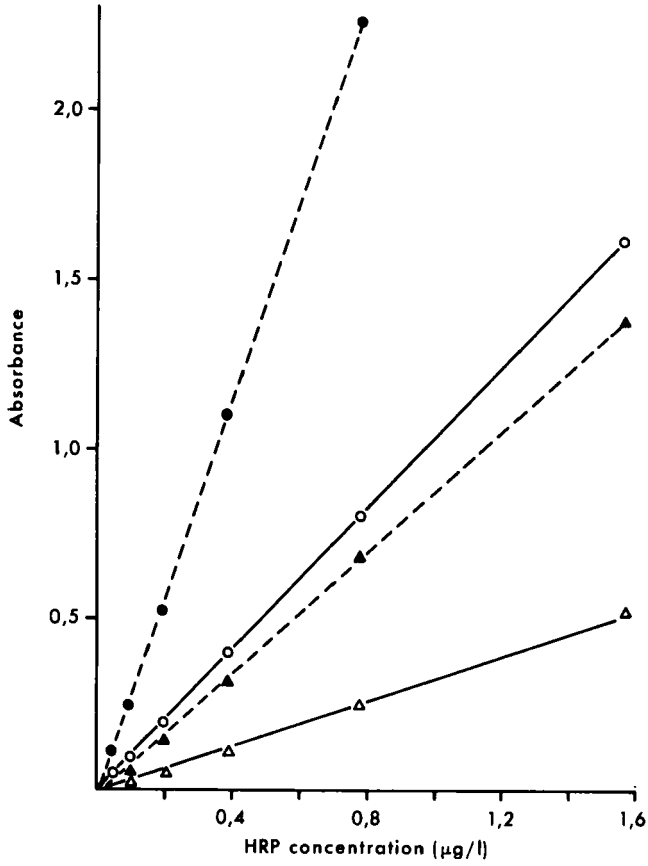

FIGURE 2

Correlation between peroxidase concentration and optical density. Incubation was performed for 60 min at room temperature in the dark.

▲– – – –▲ : TMB in 0,1 mol/l acetate-citric acid pH 6,0, unstopped, 655 nm

●– – – –● : TMB in 0,1 mol/l acetate-citric acid pH 6,0, stopped with 2 mol/l H_2SO_4, 450 nm

△————△ : TMB in 0,03 mol/l citric-acid-phosphate pH 6,0, unstopped, 655 nm

○————○ : TMB in 0,03 mol/l citric acid-phosphate pH 6,0, stopped with 2 mol/l H_2SO_4, 450 nm

The peroxidase reaction with TMB is complete after 60 min of incubation at room temperature (Fig. 3D). In contrast to the reaction with OPD, it is not susceptible to light and needs no special precautions (Fig. 3E).

The within-assay precision of the peroxidase reaction with TMB in a macro- and microassay was determined and compared with the values found for OPD. The coefficients of variation were virtually identical for both chromogens. In the macroassay CVs of 0,8% were found at absorbances over 1,0 and of 1,5 - 1,8% at absorbances below 1,0, whereas in the microversion these values were 1,8% and 2,2 - 2,9% respectively. This difference may be due to the difference of photometer applied. In the macro- as well as in the microassay the blank values were strikingly low, viz. an A_{450} value of 0,002 in both assay types. The values clearly demonstrate that end point determination of an enzyme label does not affect the precision of the immunoassay when compared with measuring radioactivity in a RIA. The coefficient of variation in the counting of radioactive samples is of the same order of magnitude (25).

When TMB was used for the end point determination of a sandwich assay for HCG and a competitive EIA for testosterone, the colour yield was clearly superior to that obtained with OPD (Fig. 4). In the sandwich EIA for HBsAg the difference is less pronounced since the concentration of OPD applied was twice as high as in the other assays. The within-assay CVs were the same for both chromogens in the HCG assay and testosterone assay i.e. around 4% and 7% respectively. In the assay for HBsAg a slight difference was found in favour of TMB, viz. 5% and 7% for OPD. In all experiments with TMB the slope of the standard curves was increased, which implies a higher precision of the assay and the detec-

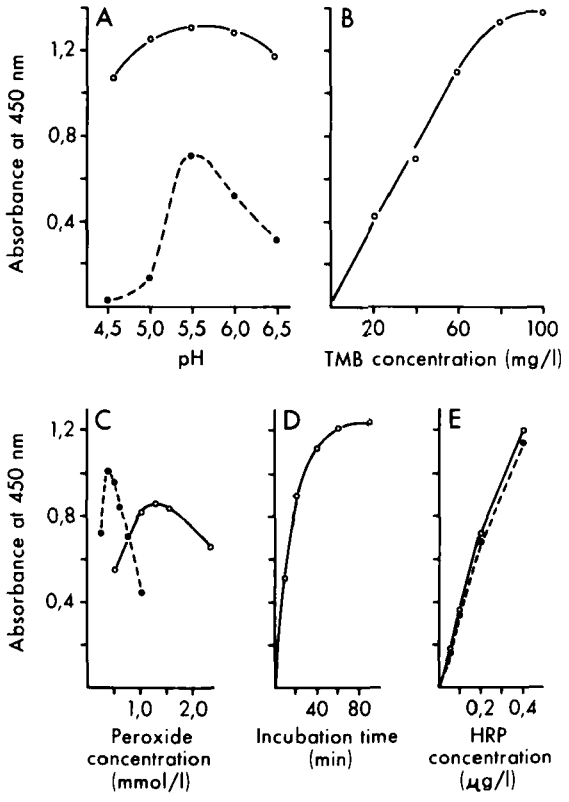

FIGURE 3

Characteristis of the peroxidase reaction with TMB

The incubations were carried out with 0,39 µg/l HRP in a macro-assay.

A. pH-Optima. Incubation was performed for 60 min at room temperature at a urea hydrogen peroxide concentration of 0,4 mmol/l.
O———O : 0,1 mol/l acetate buffer
●– – – –● : 0,03 mol/l McIlvaine buffer

B. Optimal chromogen concentration. Incubation was performed for 60 min at room temperature at a concentration of 0,4 mmol/l urea hydrogen peroxide in 0,1 mol/l acetate buffer pH 6,0.

tion limit was reduced from 5 IU/l to 2 IU/l for the HCG assay and from 0,25 µg/l to 0,15 µg/l ad-antigen in the assay for HBsAg. In the testosterone assay the measurable concentration range was at least two-fold extended by the use of TMB and the detection limit was reduced from 2,8 nmol/l to 1,4 nmol/l testosterone which corresponds with 70 pmol/assay.

So, precision and sensitivity of the EIA are significantly improved by the use of TMB as chromogen.

CONCLUSION

The aim of the study was to find a substitute for the current chromogens for the peroxidase label in enzyme-immunoassays. The alternative chromogen should be Ames-negative and since the

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C. Optimal peroxide concentration. Incubation was performed for 60 min at room temperature at a concentration of 100 mg/l TMB in 0,1 mol/l acetate buffer pH 6,0.
O———O : hydrogen peroxide
●– – – –● : urea hydrogen peroxide

D. Time course. Incubation was performed at room temperature, the TMB concentration being 100 mg/l and the urea hydrogen peroxide concentration being 0,4 mmol/l in 0,1 mol/l acetate buffer pH 6,0.

E. Light-susceptibility of the peroxidase reaction with TMB. Incubation was performed for 60 min at room temperature at a concentration of 0,4 mmol/l urea hydrogen peroxide and 100 mg/l TMB in 0,1 mol/l acetate buffer pH 6,0.
O———O : absorbance at 450 nm after incubation in the dark
●– – – –● : absorbance at 450 nm after incubation in the light

Blank values increased from 0,003 to 0,012.

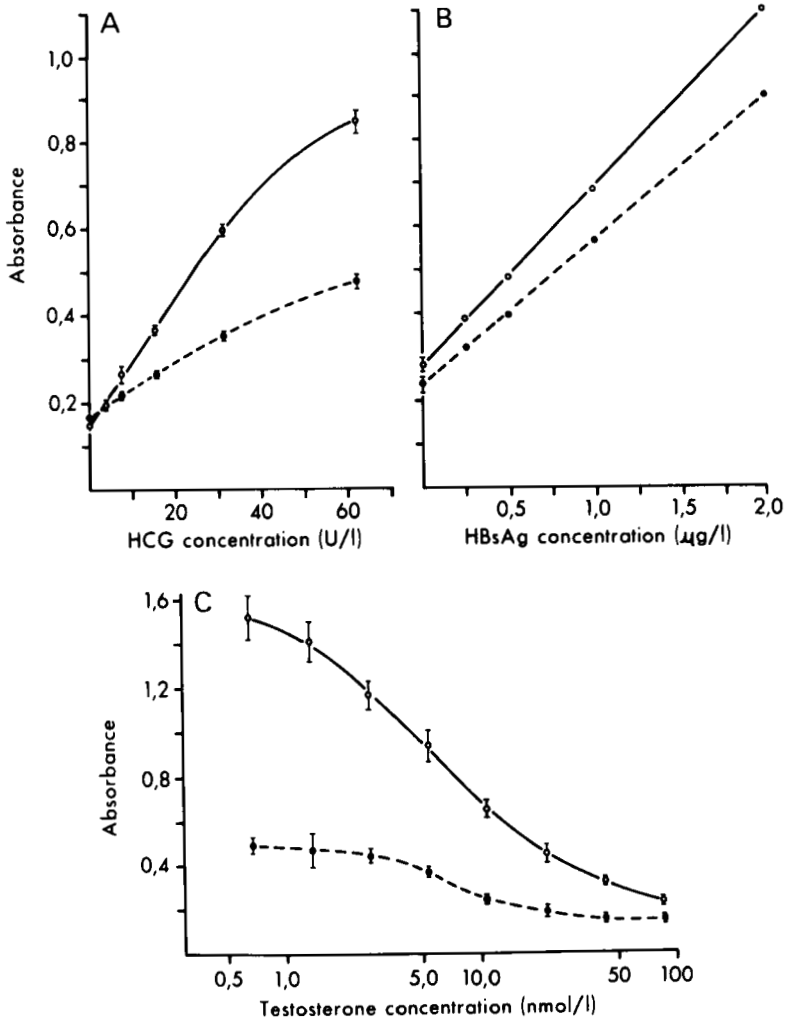

FIGURE 4

Standard curves of the enzyme-immunoassay for HCG, HBsAg and testosterone.

The assays were carried out as described under Methods.

O———O : TMB, 450 nm

●- - - - -● : OPD, 492 nm

A. Sandwich EIA for HCG
B. Sandwich EIA for HBsAg
C. Competitive EIA for testosterone.

sensitivity of the eventual assay should not be impaired, it had to be at least equivalent in terms of colour yield to OPD, which is by far the best chromogen available at this moment.

TMB and its metabolites are Ames-negative and therefore probably non-mutagenic, as was demonstrated by Garner at al. (12) and confirmed by experiments in our laboratories (I. Waalkens, J. Joosten, unpublished results). Furthermore, TMB is virtually, if not completely, non-carcinogenic (11). So, as for safety aspects of the chromogen, TMB appears to be adequate. As is shown in this paper, the intensity of colour development in the end point determination of EIAs is clearly superior to that obtained with OPD. In combination with an equal variation, this results in a significant improvement of sensitivity and precision. Thus, TMB also meets the second requirement for an alternative chromogen for OPD.

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