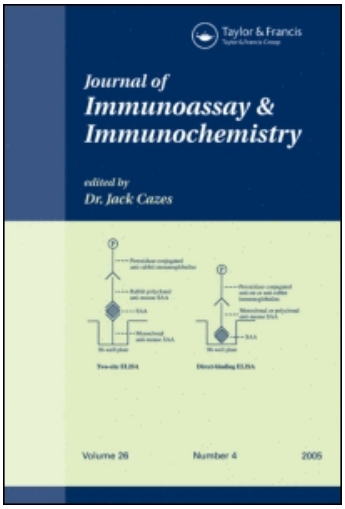

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INFLUENCE OF THE ANTIBODY-PEROXIDASE COUPLING METHODS ON THE CONJUGATES STABILITY AND ON THE METHODOLOGIES FOR THE PRESERVATION OF THE ACTIVITY IN TIME

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ABSTRACT

We studied the influence of conjugation methods and storage conditions on the stability of immunoconjugates with peroxidase. We demonstrate here that conjugates formed by the maleimide-sulfhydryl method and by the periodate oxidation method lose activity when mantained in diluted solutions. However, while the loss of activity of MSM conjugates is due exclusively to hydrolysis of the thioether bond, the loss of activity of periodate complexes is caused by a reduction of both the enzymatic and antibody immunochemical activities. Based on these observations, we developed a buffer that stabilizes the thioether bond, thus permitting long time storage of these immunoconjugates at low concentration and at above freezing temperatures.
(KEY WORDS: Horseradish peroxidase; immunoglobulin; conjugate stability; ELISA)

INTRODUCTION

Immunoconjugates are the components of immunochemical analytical devices that provide a measurable signal in the presence of a specific analyte. A conjugate must meet certain criteria of sensitivity, specificity and stability. In the case of conjugates of antibodies and enzymes, it is evident that specificity is

strictly a function of the antibody, while sensitivity depends both on the affinity of the antibody and on the kinetic properties of the enzyme. The overall stability of the complex is therefore the result of the stability of the immunochemical and enzymatic activities and of the stability of the bond that links the component molecules. Stability of the bond in turn may be influenced by the buffer in which it is stored. This influence is particularly evident when the complex is kept at low concentration.

Conditions favouring stability in dilute solutions of immunoconjugates with horseradish peroxidase (HRP) have not been thoroughly investigated although data are available on the stability of conjugates stored frozen (1), at -20°C in 50% glycerol (2,3), at 4°C with proteins as stabilizers (4,5), or as ammonium sulphate precipitates (6). Few reports have dealt with the relationship between conjugate concentration and stability (5) while in general, the influence of the type of linkage on the conjugate stability has been neglected.

In this study, we investigated the rule of each of these factors in the overall activity of the complex. The results obtained confirm that the activity of the conjugate may be reduced or lost because of inactivation of the enzyme, and loss of immunochemical actitvity. These results also showed that loss of activity may be due to hydrolysis of the bond, thus providing evidence that the two most common coupling procedures, periodate (POM) (7) and maleimide-sulfhydryl methods (MSM) (4) have a direct bearing on the stability of immunoconjugates.

Based on these observations, we developed a stabilizing buffer that allows preservation of immunoconjugate activity in which the reagent is mantained at low concentration and at above freezing temperatures.

MATERIALS AND METHODS

Antibody Purification

The IgG fraction of anti-human IgG goat hyperimmune sera (heavy and light chain specific) (goat anti-hIgG) was purified by ammoniun sulfate precipitation and DEAE-Sephadex chromatography (8).

Coupling Procedures

Periodate Oxidation Method (POM): Purified goat anti-hIgG was coupled to HRP (Boehringer Mannheim GmbH Biochemia, Mannheim, Germany) according to the Nakane and Kawaoi method (7), omitting the enzyme amino group blocking.

Maleimide-Sulfhydryl Method (MSM): HRP was activated by reaction with sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC; Pierce, Rockford IL 61105, U.S.A.) reagent. Goat anti-hIgG was reacted with the N-succinimidyl-S-acetylthioacetate (SATA; Pierce) reagent and thiol groups deprotected. Reactions were carried out according to the manufacturer's directions. The activated proteins were purified

from the reaction mixtures by gel permeation chromatography, and mixed in a 4:1 (HRP:antibody) molar ratio, fluxed with nitrogen, and left overnight at room temperature.

The unreacted proteins were separated from the complexes by gel permeation chromatography on a 1.5 X 100 cm Spectra/Gel AcA44 (Spectrum Medical Industries Inc., Los Angeles CA 90060, U.S.A.) column, eluting with borate buffer (0.1 M sodium borate buffer pH 7.8, 0.15 M sodium chloride, 20 mg/l merthiolate) and collecting 1.5 ml fractions. The column void volume (Vo) was determined as the elution volume of Blue-Dextran (Pharmacia LKB Biotecnology, Uppsala, Sweden). Fractions corresponding to the column Vo, which showed absorbance value ratios (A_{403nm}/A_{280nm}) equal or higher than 0.5, were collected and stored at 4°C until use.

Determination of the Antibody Concentration and of the Enzyme-Antibody Ratio in the Complex

The HRP and goat anti-hIgG immunoglobulins and their ratio in the conjugate were determined by spectrophometric analysis according to the following relationships :

$HRP\ (mg/ml) = A_{403nm}/E_{HRP}$

$Free\ IgG\ (mg/ml) = A_{280nm}/E_{IgG}$

$Conjugated\ IgG\ (mg/ml) = [A_{280nm}-(A_{403nm}/C)]/E_{IgG}$

$HRP/IgG\ (M/M) = [HRP\ (mg/ml)/IgG\ (mg/ml)]x(MW_{IgG}/MW_{HRP})$

Where: $E_{HRP}=2.25$ is the extinction coefficient of a 0.1% solution of HRP at 403_{nm} (1 cm path length); $E_{IgG}=1.4$ is the extinction

coefficient of a 0.1% solution of immunoglobulins at 280_{nm} (1 cm path length); C=3.1 is the ratio between the absorbance at 403_{nm} and 280_{nm} of HRP; the value of the ratio MW_{IgG}/MW_{HRP} is 3.5.

Determination of Antibody Immunochemical Activity

The capacity of the antibodies to recognize specific antigens (immunochemical activity) was determined by solid phase radioimmuno assay using human IgG as a specific antigen.

Microvil^{R} microplates (Dynatech Laboratories, Alexandria, VA 22314, U.S.A.) were coated with 100 µl/well of a 1 µg/ml solution of purified human IgG (Cappel Organon Teknika Corporation, West Chester PA 19380, U.S.A.) in 0.1 M sodium bicarbonate buffer pH 9.6, with overnight incubation at 4°C. Plates were rinsed with 0.01 M sodium phosphate buffer pH 7.2, 0.15 M sodium chloride, 0.125 ml/L Tween-20 (PBS-Tween) and then blocked by incubation with 200 µl/well of 3% bovine serum albumin (BSA; Miles Scientific, Naperville, IL 60566, U.S.A.) solution in PBS, for 2-3 hours at 37°C. After extensive washing with PBS-Tween, plates were treated with 100 µl/well of two-fold serial diluted solutions, starting at 10 µg/ml, of native goat anti-hIgG and goat anti-hIgG conjugated to HRP, in PBS containing 3 % BSA. After overnight incubation at 4°C, plates were washed as reported above, treated with 100 µl/well of ^{125}I-labeled rabbit IgG anti-goat immonoglobulins (100,000 cpm), and incubated for 2 h at 37°C. After washing in PBS-Tween the wells were cut and counted in a gamma counter. The immuochemically active goat anti-hIgG fraction present 1n the total immunoglobulins

conjugate was assayed by comparing the specific immunochemical activity of labeled and native immunoglobulins.

Determination of Peroxidase Enzymatic Activity

The enzymatic activity of native and conjugated HRP was determined using 2mM 2-2´-azino-di-[3-ethylbenzthiazoline sulfonate (6)] (ABTS; Boehringer) in 0.1 M potassium phosphate buffer pH 7.0 as a substrate, in the presence of 0.35 mM hydrogen peroxyde at 25°C, following the reaction kinetics at 405_{nm}. One unit of enzymatic activity was defined as the enzyme activity which oxidizes one µmole of ABTS per minute. The HRP enzymatic activity found in the conjugate was measured and expressed as the percentage of native HRP activity.

Determination of Conjugate Activity

For the sake of clarity we use the term "conjugate activity" to mean the overall activity of a conjugate which is the result of the combined antibody immunochemical and HRP enzymatic activities. The overall conjugate activities were tested in the recognition of anti-toxoplasma IgG antibodies. Briefly *Toxoplasma Gondii* coated wells taken from the ELISA TOXO-IgG Kit (cat. no. 90183, Sclavo Diagnostics Srl, Siena, Italy) were treated, according to the manufacturer´s istructions, with 100 µl/well of anti-*Toxoplasma Gondii* human IgG positive serum and incubated for 30 minutes at 37°C. Plates were rinsed three times with washing buffer by an automated washer (Sclavo Washer 2). 100 µl/well of goat anti-hIgG-

HRP conjugates were added after which, plates were incubated at 37°C, for 30 minutes. The plates were then washed as previously described and color developped by adding 100 µl/well of 3,3´,5,5´-tetramethylbenzidine (TMB; Boehringer) (9). After exactly 30 minutes incubation at room temperature, the reaction was stopped by adding 100 µl/well of 1 M sulfuric acid. Optical density readings at 450 nm were determined with a Microtiter Reader SR-400-AT (Sclavo). We defined as "conjugate activity" the conjugate concentration giving an optical density reading of 1, in the experimental conditions described. In this manner, it is possible to determine the difference in activities between different immunoconjugates.

Determination of the Conjugate Integrity

To determine the extent of dissociation of the conjugate components, aged immunoconjugates were chromatographed and enzymatic activities were determined in each fraction. A sample of conjugate (about 0.5 µg of conjugate in 100 µl) was loaded on to a 1x50 cm Spectra/Gel AcA44 column, and eluted in 0.1 M sodium borate pH 7.8 buffer, 0.15 M sodium chloride, 20 mg/l merthiolate. Five µl of each 0.4 ml fraction was transferred to untreated flat-bottom wells of standard microplates, and 0.1 ml of TMB substrate simultaneously added. The reaction was stopped after 5 minutes and O.D. reading taken at 450 nm. The amount of enzymatic activity found as free enzyme was expressed as the percentage of the total enzymatic activity recovered in the chromatogram.

Determination of Conjugate Stabilty

Stability of the conjugate was determined by assaying the conjugate activity of the complexes obtained by the two methods under study with respect to time. To determine the influence of storage buffers on stability, different buffers were tested.

Goat anti-hIgG-HRP MSM and POM conjugates were diluted at 1.3 and 10.6 μg/ml respectively in the following buffers:

1) 0.1 M sodium borate buffer pH 7.8, 5% BSA (enzyme-free grade; Miles), 0.5 mg/ml cytochrome-C (Sigma Chemical Company, St. Louis MO 63178, U.S.A.), 0.05 μg/ml gentamicin sulfate (Sigma), 0.1% merthiolate (Sigma);

2) buffer (1) supplemented with 30% glycerol (Sigma);

3) buffer (1) in which borate buffer was replaced with 0.1 M sodium phosphate buffer pH 6.5, 0.15 M sodium chloride;

4) buffer (3) supplemented with 10% glycerol; and

5) buffer (3) supplemented with 30% glycerol.

Solutions were sterilized by filtration on 0.22 μm membranes, (Millipore, Bedford, MA 01730, U.S.A.) dispensed in 0.5 ml aliquots in sterile vials and stored at 4°C and 37°C in the dark. At various times samples were diluted 20 fold and analyzed for their capacity to detect human anti *toxoplasma gondii*-IgG in sera, as described above. Conjugate stability was expressed as the percentage of the activity with respect to time zero.

RESULTS AND DISCUSSION

To investigate the effect of the conjugation method and of the buffer components on the stability of the immunoconjugates, we used the complexes between anti-hIgG immunoglobulins and HRP obtained by the POM and MSM methods. The overall activity of the complexes was assessed by using the test for the determination of anti-*Toxoplasma Gondii* antibodies in human sera as reported in the Materials and Methods section.

The data reported in Table 1 show the efficiency of the conjugation methods and the antibody immunochemical and HRP enzymatic activity recovered after conjugation. The efficiency of the two methods is comparable, since the POM procedures gives 2 moles of HRP per mole of IgG while the MSM method gives a ratio close to 3 (table 1). The recovery of antibody immunochemical and HRP enzymatic activity is low for both methods. In particular, the POM complex retained 3% of the original antibody immunochemical activity, while the residual immunochemical activity in the MSM conjugate was 11%. In both cases, the complexes retained 40% of the original HRP enzymatic activity. Thus, the conjugation procedures affect differently the final conjugate activity of the complex obtained, so that the MSM conjugate was found as a tracer in ELISA, about 10 time more efficient than the POM conjugate, as reported in table 1.

TABLE 1

Characterization of Goat anti Human IgG-HRP Conjugates

Conjug. Method	HRP/IgG (M/M)	Percentage of Biochemical activity recovered (1)		Conjugates Activity ng/ml(2)
		IgG	HRP	
POM	2.0	3	40	530
SMS	2.8	11	43	65

1) The immunochemical activities of goat anti-hIgG immunoglobulins and the HRP enzymatic activity in the conjugates were expressed as percentages of the IgG and HRP activities before conjugation;
2) The Conjugate activity was expressed as the complex concentration giving an absorbance = 1 at 450 nm in the experimental conditions reported in the Materials and Methods section.

As regards the stability over time of these conjugates, we made tests to find the conditions that can allow storing of this type of immunoreagent at diluted solutions, similar to those usually used in commercial kits. We studied the ageing of these complexes, kept at 4 and 37°C in solutions twenty fold higher than those used in the assay, to conform, by an adequate dilution in the assay buffer, to the ELISA test conditions. As shown in fig. 1, the conjugates prepared with both methods show an initial accentuated loss of activity in the first week of the storage test, followed by a less accentuated decay with time. The residual activity of the POM conjugate was 47% after two weeks and 32% after one month at 37°C, while that of the MSM conjugate was 52% and 43% at the same time points. At 4°C, the reduction of conjugate activity presented

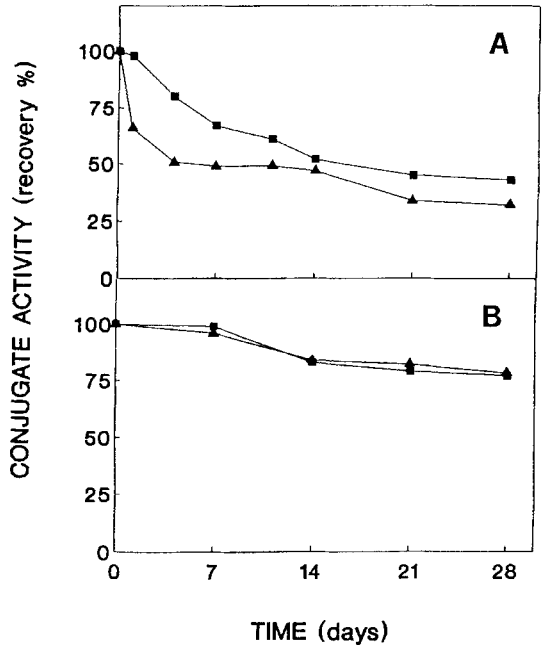

FIGURE 1: Stability curves of MSM (■—■—■) and POM (▲—▲—▲) goat anti-hIgG-HRP conjugates, 10.6 µg/ml and 1.3 µg/ml in the storage buffer (see Materials and Methods section) at 37°C (A) and 4°C (B), respectively. The conjugate activity was tested in the determination of human anti-*toxoplasma gondii* IgG in ELISA, measured in triplicate analysis of each sample. Standard deviations were calculated, found less than 3% and then omitted in the figure. Conjugate stability was expressed as a percentage of the value measured at the beginning of the storage.

similar results to those at 37°C, although quantitively smaller: the residual activity in this case was 84% after two weeks and 78% after one month for both types of conjugates. These ageing profiles for both the conjugates, do not seem to obey the Arrhenius equation regarding the relation between temperature and biological activity dacay (10).

TABLE 2

Conjugate Analysis after Storing 15 Days at 37°C in Storage Buffer

Conjugation Method	Percentage of Biochem. Activities Recovered(1)			Residual Enzymatic Activity Associated to the Complex (2)
	Conjugate	IgG	HRP	
POM	48	34	47	100
MSM	53	100	100	30

1) The immunochemical and enzymatic activities of goat anti-hIgG immunoglobulins and HRP in the complex and the overall conjugate activity, after storage at 37°C for 2 weeks, were expressed as percentage of starting values.
2) Enzymatic activity recovered associated with the chromatographic peak corresponding to the non hydrolyzed complex and determined by gel-permeation chromatography was expressed as a percentage of the total enzymatic activity recovered in the chromatogram.

Although the reduction of conjugate activity was similar for the complexes obtained by the two procedures, the data presented above do not give any information as to the causes of this reduction. Consequently, we compared the separate antibody immunochemical and HRP enzymatic activities at various times as reported in table 2. These data show that after two weeks at 37°C, the POM conjugate retained 34% of antibody activity and 47% of the HRP enzymatic activity. The MSM conjugate showed no loss of antibody or enzymatic activity after two weeks at 37°C. These results explain the observed reduction (50%) of the POM conjugate activity, but do not account for the reduction observed for the MSM conjugate (table 2).

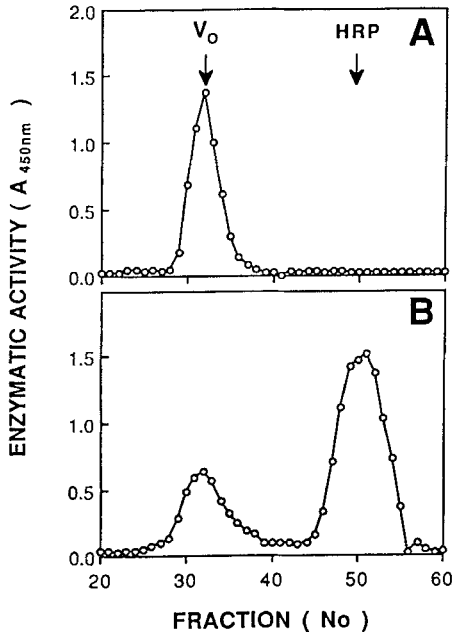

FIGURE 2: Elution profile from a Spectra/Gel AcA 44 column 1x50 cm of POM (A) and MSM (B) goat anti-hIgG-HRP conjugates after storage for 15 days at 37°C.

To ascertain whether the loss of activity of the MSM complex may be caused by breakdown of the complex, this was analysed by column chromatography as described in Materials and Methods. As shown in the elution profiles in Fig. 2, two peaks of enzymatic activity were obtained, corresponding to the retention volumes of the conjugate and of the free enzyme respectively. Only 30% of the total enzymatic activity in the chromatogram was found to be associated with the peak corresponding to the complex. The enzymatic activity of the POM preparation analyzed in a similar column was found to be associated entirely with the peak of the conjugate (Fig. 2).

TABLE 3

Stability of the MSM Conjugate Stored in Different Buffers for 15 Days at 37°C in Diluted Solutions.

Buffer	Percentage of Conjugate Activity Recovered (1)
Borate pH 7.8 (2)	53
" " " + Glycerol 30%	68
Phosphate pH 6.5(3)	72
" " " + Glycerol 10%	88
" " " + " 30%	98

1) Expressed as percentage of the difference between the conjugate activity of the sample stored for 15 days at 37°C, and compared to the same sample stored at 4°C.
2) 0.1 M sodium borate buffer pH 7.8, 5% BSA enzyme-free grade, 0.5 mg/ml cytocrome-C, 0.05 µg/ml gentamicin sulfate and 0.1% merthiolate.
3) 0.1 M sodium phosphate buffer pH 6.5, 0.15 M sodium chloride, containing the same protein composition as (2).

These results show that loss of conjugate activity is due to a reduction of both antibody and enzymatic activity in the POM complex, with no dissociation of the complex. The loss of activity of the MSM conjugate is caused exclusively by dissocation of the complex.

We have shown it to be possible to stabilize MSM complexes under conditions that stabilize the thiother bond. Since it is known that the thioether bond is more stable at a low pH, we tried two different approaches to stabilize the MSM conjugate. The first was to acidify the storage buffer to pH 6.5, as reported (4). The second was to add glycerol which works both as a stabilizer of

proteins in solution and has been reported to be relatively acidic in aqueous solution (11).

As the data in table 3 demonstrates, lowering the pH to 6.5 increased the residual conjugate activity from 53 to 72%. Likewise, the addition of 30% glycerol to the inital buffer, increased the residual activity to a similar extent. The effect of glycerol was dose dependent (table 3). If both modifications are introduced the effect on stabilization reaches almost 100%, as reported in table 3. Thus the effect of pH and glycerol are synergistic rather than additive. This modified buffer has little effect on the stability of the conjugate activity of the POM complex (not shown).

These conditions have been successful in preserving the activity of conjugates with purified immunoglobulins from antisera, but have not yet been tested with monoclonal antibodies.

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REFERENCES

1. Kofler, H., Kofler, R., Wolf, H., Müller, P.U. and Wick, G. Development of an Enzyme-Linked Immuno-assorbent Assay for the Detection of Autoantibodies against Thyroglobulin in Chickens. J. Immunol. Methods 1984; 69: 243-52.

2. Suter, M., Pike, B.L. and Nossal, G.J.V. An ELISA Assay Efficiently Detects Clonal Antibody Formation by Single, Apten-Specific B Lymphocytes. J. Immunol. Methods 1985; 84: 327-41.

3. Ferrua, B., Becker, P., Schaffar, L., Shaw, A. and Fehlmann, M. Detection of Human IL-1α and IL-1β at the Subpicomolar Level by Colorimetric Sandwich Enzyme Immunoassay. J. Immunol. Methods 1988; 114: 41-8.

4. Ishikawa, E., Masayoshi, I., Hashida, S., Yoshitake, S., Hamaguchi, Y. and Ueno, T. Enzyme-Labeling of Antibodies and their Fragments for Enzyme Immunoassay and Immunohistochemical Staining. J. Immunoassay 1983; 4: 209-327.

5. Coll, J.M. Heme Increases Peroxidase-Antibody Activity in Aged Conjugates. J. Immunol. Meth. 1987; 104: 259-63.

6. Montoya, A. and Castell, J.V. Long-Term Storage of Peroxidase-Labelled Immunoglobulins for Use in Enzyme Immunoassay. J. Immunol. Meth. 1987; 99: 13-20.

7. Nakane, P.K. and Kawaoi, A. Peroxidase-Labeled Antibody a New Method of Conjugation. J. Histochem. Cytochem. 1974; 22: 1084-91.

8. Harlow, E. and Lane, D. Antibodies: a Laboratory Manual. Cold Spring Harbor ed. 1988.

9. Goka, A.K. and Farthing, M.J.K. The Use of 3,3′,5,5′ Tetramethylbenzidine as a Peroxidase Substrate in Microplate Enzyme-Linked Immunoassorbent Assay. J. Immunoassay 1987; 8: 29-41.

10. Anderson, G., and Scott, M. Determination of Product Shelf Life and Action Energy for Five Drugs of Abuse. Clin. Chem. 1991; 37/3:398-402.

11. Ballinger, P. and Long, F.A. Acid Ionization Constants of alcohols. II. Acidities of some Substituted Methanols and Related Compounds. J. Am. Chem. Soc. 1960; 82: 795-8.