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ELISA inibition technique for the demonstration of sulphones in body fluids

Comparison of two ELISA methods*

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Summary A new enzyme-linked immunosorbent assay (ELISA) for sulphones is described. The main tool is a dapsone-enzyme conjugate (E-DDS). The technique is compared with the one described earlier, in which the main tool is a specific antibody-enzyme conjugate (E-Ig). The E-DDS-based ELISA is 50% inhibited by as little as 4 ng DDS/ml, i.e. it is 7.5 times more sensitive for DDS than the E-Ig-based ELISA. In both ELISA's other sulphones cross-react with DDS, although the patterns are different. Cross-reactions with sulphone analogues, such as sulphonamides, do not occur. The sensitivity of the new ELISA is not reduced when E-DDS is lyophilized. A possible explanation for the difference in sensitivity of the two ELISA's is given, and the practical applicability of the new technique is discussed.

Introduction

In two previous papers[1, 2] we described the development of a simple enzyme-linked immunosorbent assay (ELISA) for the demonstration of sulphones in body fluids. The main tool of this inhibition technique is a sulphones-specific antibody-enzyme conjugate (E-Ig). Because of its high sensitivity it can be applied to detect substantial failure in dapsone (DDS) self-administration by leprosy patients (unpublished data).

Recently, basic alterations in the method led to a second ELISA for sulphones, even more sensitive than the first one. The main tool of this new inhibition technique is a DDS-enzyme conjugate (E-DDS). The present paper describes the new ELISA and compares it with the one reported earlier.

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Materials and Methods

CONJUGATION OF DDS AND PEROXIDASE

Horseradish peroxidase (10 mg) was conjugated to DDS according to the method of Nakane and Kawaoi.[3] In the second step a saturated solution of DDS in PBS was used. Unconjugated DDS was removed by repeated ultrafiltration, until no DDS could be detected[4] in the PBS filtrate. The concentrated conjugate (E-DDS) was stored at 4°C in a final volume of 2.5 ml.

E-DDS-BASED ELISA INHIBITION TECHNIQUE

Each well of a microtitre tray was incubated with a solution of 2.4 μg sulphones specific Ig[1] in 100 μl carbonate buffer of pH 9.6 (2 h, 56°C). The tray was washed with PBS/Tween as described.[1] Then 50 μl aliquots of specified solutions of sulphones or analogues in normal horse serum containing 0.05% Tween 20 were added to the wells, followed by 50 μl aliquots of a 4×10^{-3} dilution of the E-DDS concentrate in PBS/Tween containing 5% normal horse serum (PBS/Tween/Serum). After incubation (30 min, 56°C) the tray was washed and a $5AS/H_2O_2$ solution was added as described.[1] However, the reaction was not stopped by addition of NaOH, and readings were done after 2 h using a Titertek Multiskan (Flow Laboratories) at 492 nm (O.D. 492).

E-Ig-BASED ELISA INHIBITION TECHNIQUE

Starting from a coating with BSA-DDS as described,[1] this ELISA was set up in 3 modifications:

(a) using unconjugated sulphones specific Ig,[1] followed by horseradish-peroxidase-conjugated anti-rabbit IgG antiserum in an additional incubation step;[5]
(b) using freshly prepared sulphones specific E-Ig;[1]
(c) using lyophilized sulphones specific E-Ig.[2]

In all modifications 50 μl-aliquots of serial dilutions of a DDS solution were added to the wells as described,[1] using PBS/Tween/Serum as diluent. (For this ELISA 5% serum addition to PBS/Tween gave optimal inhibitions, whereas the sensitivity of the other ELISA appeared to be optimal when 100% serum was used as a solvent for the inhibiting compounds.) Readings were made after 2 h at 492 nm as above.

PRINCIPLES

Figure 1 shows the principles of the 2 ELISA inhibition techniques.

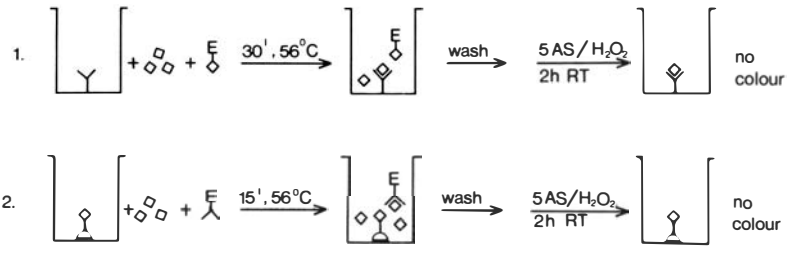

Figure 1. Two ELISA inhibition techniques. In both the attachment of the enzyme-conjugate is inhibited by free molecules of sulphones. Symbols: ⋎ = sulphones specific Ig; ◇ = sulphones; ⋎◇ = E-DDS; ⋎⌓ = DDS conjugated to BSA (⌓); ⋎⋎ = E-Ig.

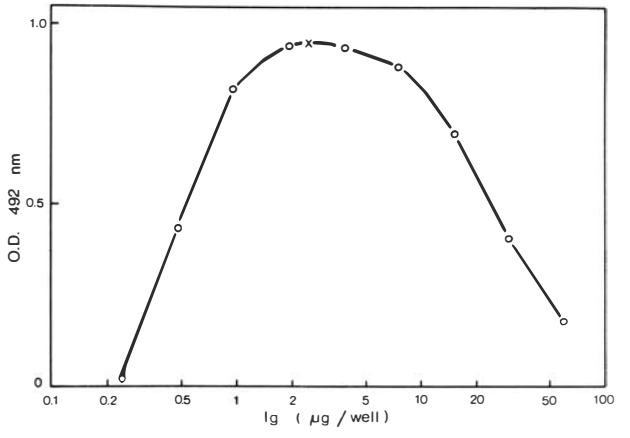

Figure 2 Dose-response curve for sulphones specific Ig coating in ELISA. From the curve an Ig quantity of 2.4 µg per well (x) was chosen for coating in this ELISA.

Results

In preliminary experiments the sulphones specific Ig coating gave an optimal ELISA response, if Ig quantities of 1–10 µg/well were used (Fig. 2). Coating in all further experiments with the new ELISA was done using 2.4 µg/well. Figure 3 is a dose-response curve of E-DDS in ELISA without inhibition. From this curve an E-DDS dilution of 4×10^{-3} was chosen for the inhibition tests with sulphones and analogues.

The inhibition of the E-DDS ELISA by DDS is illustrated in Fig. 4, together with similar inhibition curves for the E-Ig ELISA. Based on these curves, Table 1 lists the sensitivities of the ELISA's for DDS, expressed in ng DDS/ml and ng DDS/well used at 50% response. The E-DDS ELISA appears to be about 7.5 times more sensitive for DDS than the E-Ig ELISA when fresh conjugate is used. The new ELISA maintains its high sensitivity after lyophilization of the conjugate, whilst the sensitivity of the old one is 1.7 times reduced by a similar manipulation.

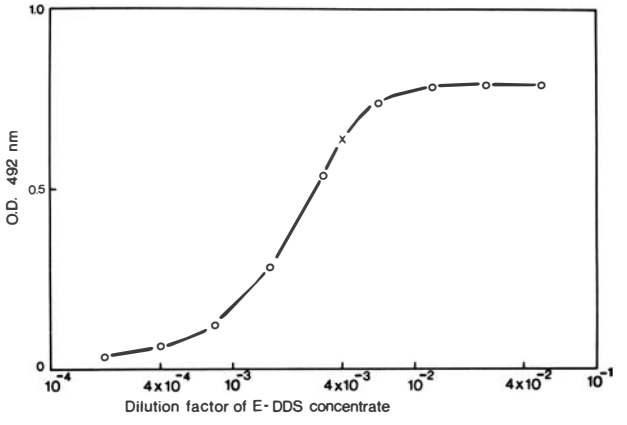

Figure 3 Dose-response curve for E-DDS in ELISA. From the curve an E-DDS dilution factor of 4×10^{-3} (×) was chosen for the inhibition tests.

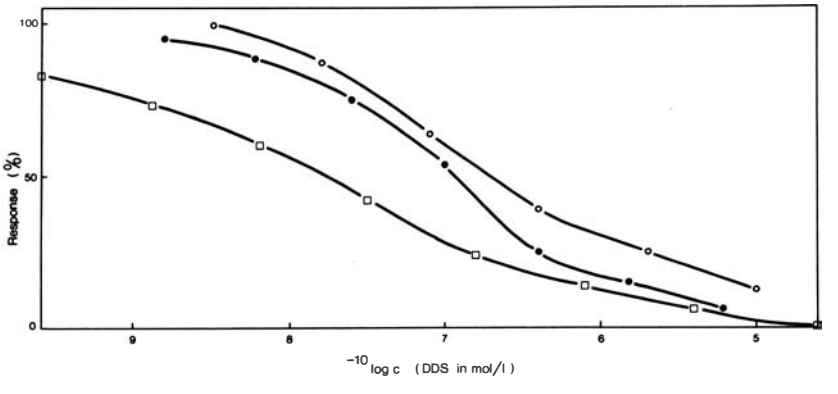

Figure 4 ELISA inhibition by DDS. □, E-DDS-based ELISA (fresh and lyophilized E-DDS gave identical curves); ●, E-Ig-based ELISA using fresh conjugate (the double antibody modification gave an identical curve); ○, E-Ig-based ELISA using lyophilized conjugate.

Table 1. Sensitivities of ELISA's for DDS

	50% response	
ELISA	ng DDS/ml	ng DDS/well
E-DDS fresh	4	0.2
E-DDS lyophilized	4	0.2
Double antibody	30	1.5
E-Ig fresh	30	1.5
E-Ig lyophilized	50	2.5

Results are taken from the inhibition curves illustrated in Figure 4.

Table 2. Relative sensitivities of ELISA's for sulphones and analogues

Compound	cross-reaction (%) E-DDS	E-Ig
1. $H_2N-C_6H_4-SO_2-C_6H_4-NH_2$ (DDS)	100	100
2. $CH_3CONH-C_6H_4-SO_2-C_6H_4-NHCOCH_3$ (DADDS)	24	275
3. $H_2N-C_6H_4-SO_2-C_6H_4-NHCOCH_3$ (MADDS)	7	183
4. $H_2N-C_6H_4-SO_2-C_6H_4-NHSO_3K$	3	183
5. $H_2N-C_6H_4-SO_2-C_6H_4-NO_2$	121	157
6. $H_2N-C_6H_4-SO_2-C_6H_4-NHOH$	43	110
7. $H_2N-C_6H_4-SO_2-C_6H_5$	31	110
8. $C_6H_5-SO_2-C_6H_5$ (Diphenylsulphone)	0.8	<0.1
9. $H_2N-C_6H_4-S-C_6H_4-NH_2$	0.9	<0.1
10. $H_2N-C_6H_4-SO_2NH-C_4H_3N_2$ (Sulphadiazine)	≪0.1	≪0.1
11. $H_2N-C_6H_4-COOH$	≪0.1	≪0.1

The figures indicate the amounts (molarity) needed to reduce the ELISA response to 50% relative to these figures for DDS (= 100% cross-reaction). The figures for the E-Ig-based ELISA are taken from a previous paper.[1]

Table 2 gives the sensitivities of the 2 ELISA's for other sulphones and analogues, relative to the sensitivity of the respective ELISA for DDS. There is a significant difference between the 2 ELISA's as regards the sensitivity for DDS itself relative to that for the other sulphones. Whereas the E-Ig conjugate has a higher affinity for most other sulphones tested, only one other sulphone inhibits the E-DDS-based ELISA more than DDS. An important characteristic of both systems is that there is no cross-reaction with sulphonamides.

Discussion

From the few reports about ELISA's on drugs both inhibition techniques might be expected to have equally high sensitivities.[6, 7] It is only partly understood why the modified ELISA for sulphones is more sensitive than the initial one. An explanation for this may be that in the modified method the competition for the antibody is between free sulphones and a conjugate (E-DDS) in which the bridge attaching DDS is different from the one used in the conjugate against which the antibodies were raised. In the initial method the bridges are

homologous. In 1975 Van Weemen and Schuurs[8] reported on the advantage of heterologous combinations in enzyme-immunoassays. However, introduction of a heterologous bridge to BSA-DDS, with which the free sulphones have to compete in the initial method, has not yet been shown to increase the sensitivity.

Differences in patterns of cross-reactions (Table 2) might be explained in a similar way. What is important is that for the qualitative demonstration of sulphones in body fluids both patterns of cross-reactions are equally acceptable.

The E-DDS ELISA is 7.5 times more sensitive for DDS than the E-Ig ELISA, and 12.5 times more sensitive if lyophilized materials are used. Although there is no need to improve the sensitivity of the initial ELISA urine test (unpublished data),[2] higher sensitivity means a greater flexibility and a wider applicability to other body fluids.

The only apparent disadvantage of the modified ELISA is that the incubation times are longer. Coating and E-DDS incubation at room temperature require respectively 24 and 4 h. Production costs and shelf life of the reagents of both methods are about the same.

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