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BIOTINYL-ESTRADIOL DERIVATIVES IN ENZYME IMMUNOASSAYS: STRUCTURAL REQUIREMENTS FOR OPTIMAL ANTIBODY BINDING

L. X. TIEFENAUER* and R. Y. ANDRES
Medical Bioanalytics Project, Paul Scherrer Institute, CH-5232 Villigen PSI, Switzerland

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Summary—The use of the avidin/biotin complex in immunoassays is well documented. No comprehensive studies, however, are available on the structural requirements of the linkage between biotin and small molecules to get an optimal antigen–antibody interaction.

We have synthesized seven different biotinylated estradiol derivatives. They were evaluated in an antibody- and in an antigen-immobilized enzyme immunoassay system. All three derivatives lacking a spacer group were useless for use in immunoassays, demonstrating the importance of a long distance between the biotin- and estradiol-moiety. In addition, the chemical structure of the linkage at the site of attachment to the steroid skeleton is very important for the antibody recognition: it may either be rigid but identical to that one used in the immunogen (6-carboxymethyloxime), or must be structurally flexible as exemplified by a 6-amido-linkage. A rigid structure (hydrazone) different from that of the immunogen absolutely prevents antibody binding.

INTRODUCTION

The quantification of steroids by ELISA techniques has been described in many reports in the past [1–8]. The preparation [9, 10] and the stability [11] of the conjugates and the immobilization of the antibody [12] or antigen [13] on a solid surface are crucial steps which have a direct impact on the quality of the assay.

Recently we have presented a comparison of two ELISA systems using avidin and a biotinyl-estradiol derivative as the key components [14]. In the first system, the antibody is immobilized on a microtiter plate coated with protein A and the biotinyl-estradiol molecules bound to the antibody are detected with the use of a streptavidin-peroxidase conjugate. In the second assay system, the antigen biotinyl-estradiol is bound by the avidin coated to the plates and the antibody bound to the immobilized antigen is subsequently measured using a second antibody fragment labeled with peroxidase. Both ELISA systems display a high sensivity, good precision and reproducibility which make them attractive for a commercial application.

Previously we have found that an amino-estradiol derivative is the most suitable starting material for an iodinated tracer applied to a radioimmunoassay. Bridge binding of antibodies can be prevented using this altered tracer structure [15], resulting in a more sensitive assay. This observation demonstrates the need of a careful design of the estradiol derivative to get an optimal antigen–antibody interaction.

Therefore we have compared in this study the biotinyl-amino-estradiol with a biotinyl-estradiol derivative having a bridge identical to that present in the immunogen. In addition, a hydrazone structure has been introduced as an alternative bridge. Three further derivatives were synthetized by introducing a spacer group (6-amidohexanoic acid) into the different bridge structures mentioned above.

The antibody recognition of these biotinyl-estradiol derivatives has been assessed in both the antigen- and the antibody-immobilized assay.

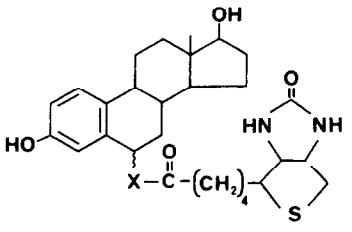
EXPERIMENTAL

Purification of E_2-Bio derivatives by HPLC

The E_2-Bio derivatives (structures see Fig. 1) were purified on a reverse phase column (Spheri-RP-8, 5 μm, 0.46 × 22 cm, Brownlee Labs, Santa Clara, U.S.A.) using a gradient system gased with helium (acetonitrile/50 mmol/l ammonium acetate, pH 5) with a flow rate of 1.5 ml/min [except for product (**5**)]. The optimal gradient profile is described for each synthesis separately.

*To whom correspondence should be addressed.

Abbreviations: Ab, antibody; Ag, antigen; Bio, biotin; CMO, *O*-(carboxymethyloxime); DMF, dimethylformamide; E_2, estradiol; ELISA, enzyme linked immunosorbent assay; HPLC, high-pressure liquid chromatography; Hy, hydrazone; LC, "long-chain"; NH, amino; O.D., optical density; RIA, radioimmunoassay.



Compound	−X−	Abbreviations
(1)	····NH−C(=O)−$(CH_2)_5$−NH−	E_2 - NH - LC - Bio
(2), (3)	~NH−	E_2 - NH - Bio
(4)	=N−NH−C(=O)−$(CH_2)_5$−NH−	E_2 - Hy - LC - Bio
(5)	=N−NH−	E_2 - Hy - Bio
(6), (7)	=N−O−CH_2−C(=O)−NH−NH−C(=O)−$(CH_2)_5$−NH−	E_2 - CMO - LC - Bio

Fig. 1. Structures of E_2-Bio derivatives used in this study. Compound (1) represents the 6α-form; compounds (2) and (3) represents two chromatographically separated forms, presumably the 6α- and the 6β-form; compounds (6) and (7) represents two chromatographically and immunologically different forms, presumably the *Z*/*E*-isomers.

Synthesis and purification of 3,17β-dihydroxy-1,3,5(10)estratrien-6α-N-(ε-biotinyl)-aminocaproamide (E_2-NH-LC-Bio, (1)). The synthesis of this substance has been described earlier [14].

Synthesis and purification of 3,17β-dihydroxy-1,3,5(10)estratrien-6α-N-ε-biotinylamid (E_2-NH-Bio, (2) and (3)). 1 mg (3.5 μmol) of 6α-amino-estradiol [15] was dissolved in 100 μl DMF containing 10.5 μmol of triethylamine. 3.1 mg (7 μmol) of *N*-hydroxysuccinimide-biotin ester (Pierce Chemical Company, Rockford, U.S.A.) in 31 μl DMF was added and the mixture was incubated at 4°C overnight. The solvent was evaporated with nitrogen and the product extracted with ethyl acetate. The dried extract was redissolved in 1 ml ethanol.

HPLC-gradient: 0–15% acetonitrile over 10 min, and 15–25% over 30 min. The two relevant compounds eluted after 31 min (2) (0.11 mg) and after 32.5 min (3) (0.32 mg), respectively.

(3) u.v. λ_{max}(ethanol): 283 nm (ϵ = 1750).

Synthesis and purification of 3,17β-dihydroxy-1,3,5(10)estratrien-6-hydrazido-(ε-biotinyl)-aminocaproamide (E_2-Hy-LC-Bio, (4)). 20.4 mg (70 μmol) of 6-keto-estradiol (Sigma, St Louis, U.S.A.) and 74.2 mg (200 μmol) of biotin-LC hydrazide (Calbiochem, La Jolla, U.S.A.) were dissolved in 20 ml methanol containing 62 mg of sodium acetate and 1.24 ml of acetic acid. The mixture was refluxed for 20 h. After the evaporation of the solvent, 20 ml of H_2O was added to precipitate the biotin-LC-hydrazide. The aqueous phase was extracted three times with 10 ml ether, the solvent evaporated and 9 mg of the crude material was redissolved in 2 ml ethanol.

HPLC-gradient: 0–27% acetonitrile over 10 min and 27–37% over 20 min. The product (4) eluted 11.5 min after injection. After lyophilization white amorphous material was obtained.

u.v. λ_{max}(ethanol): 265 nm (ϵ = 575). m.s. *m/e*: 553 (4%), 413 (16%), 329 (27%), 227 (10%), 176 (100%). m.p.: 126°C (decomp.).

Synthesis and purification of 3,17β-dihydroxy-1,3,5(10)estratrien-6-hydrazido-ε-biotinylamide (E_2-Hy-Bio, (5)). 100 mg (350 μmol) of 6-keto-estradiol and 252 mg (977 μmol) of biotinyl-hydrazide (Calbiochem) were dissolved in 80 ml methanol containing 250 mg sodium of acetate and 5 ml of acetic acid. The mixture was refluxed for 20 h. The solvent was evaporated and 60 ml of H_2O was added to precipitate the biotin-hydrazide. The crude product was extracted with ethyl acetate and dried (yield: 44%).

A crude purification was achieved by thin-layer chromatography on preparative silica plates (Merck, Darmstadt, F.R.G.) with benzene–ethanol–acetic acid (70:29:1, by vol) as the mobile phase. The partially purified product (R_f = 0.39) was extracted with ethanol and dried. The residue was redissolved in methanol. A part of it was further purified by HPLC (Biosil HP-10, BioRad Lab., Richmond, U.S.A.)

using a gradient of methanol in chloroform: 2% methanol over 10 min and 2–10% methanol over 10 min. The product (**5**) (6.7 mg) eluted after 12.5 min.

u.v. λ_{max} (ethanol): 283 nm ($\epsilon = 900$). m.s. *m/e*: 414 (6%), 281 (7%), 97 (32%), 43 (100%). i.r. ν_{max}: 3430 (hydrazone), 3250, 2929, 1685, 1565, 1413 cm^{-1}.

Synthesis and purification of 3,17β-dihydroxy-1,3,5(10)estratrien-6-one-6-O-(carboxymethyloxime)-hydrazido-(ε-biotinyl)-aminocaproamide (E_2-CMO-LC-Bio, (6) and (7)). 5 µl of tributylamine and 5 µl of isobutylchloroformiate (both diluted 1:10 in dry dioxane) were added to a solution containing 1.2 mg (3.2 µmol) of 6-CMO-estradiol (Steraloids, Wilton, U.S.A.) in 35 µl dioxane. After an incubation at 8°C for 30 min, 100 µl of dioxane was added. 2 mg (5 µmol) of biotin-LC-hydrazide was dissolved in 500 µl DMF, mixed with the activated steroid and kept overnight at 4°C before purification

HPLC-gradient: 0–40% acetonitrile over 24 min and 40–47% over 16 min. The two compounds eluting after 22.5 min (**6**) (0.6 mg) and 31.5 min (**7**) (0.5 mg) were identified as E_2-Bio derivatives. The solvent was evaporated by nitrogen and the solid residue redissolved in 1 ml ethanol and stored at 4°C.

u.v. λ_{max} (ethanol): (**6**): 260 nm ($\epsilon = 4560$), 300 nm sh (**7**): 260 nm, 310 nm. m.s. *m/e*: (**7**): 551 (16%), 395 (18%), 372 (69%), 177 (100%).

Antibodies

Unless mentioned otherwise, an antiserum raised in our laboratory against estradiol-6-CMO bovine serum albumin was used without purification. This antiserum shows no bridge binding and has a high affinity constant of 3.8×10^{11} l/mol. For comparative reasons, a second antibody exhibiting bridge binding [15] was occasionally used (e02/2/83 from I.F.C.I., Casalecchio, Italy) with an affinity constant of 2.9×10^{10} l/mol.

Enzyme immunoassays

The preparation of avidin- and protein A-coated plates and the respective assay formats has been described in details [14]. Briefly the procedures are as follows:

For the antigen-immobilized system, avidin is first coated on microtiter plates (Immunoplates I, Nunc, Roskilde, Denmark). After an incubation of the respective E_2-Bio (1 h, room temperature), the plates were washed and the suitably diluted antiserum added simultaneously with the calibrator or cross-reactant. The subsequent incubation was performed at 37°C for 1 h; after washing, a second antibody-peroxidase conjugate was incubated for 2 h at room temperature. Unbound enzyme conjugate was removed by washing and the binding of the conjugate was vizualized by the addition of the substrate tetramethylbenzidine.

For the antibody-immobilized system, protein A in acetate buffer was first coated on plates. After the antibody incubation, the respective E_2-Bio derivative was incubated simultaneously with the competing substance (calibrator or crossreactant). E_2-Bio bound to the immobilized antibody was detected by the enzymatic reaction after a previous incubation with avidin-peroxidase. All data are corrected for non-specific binding, which was usually about 5%.

Each antigen (E_2-Bio derivative) was titrated on avidin- and protein A-antibody coated plates, respectively, to determine the concentration required to give a signal of 0.5 O.D. (see Table 1).

Radioimmunoassays

The general procedure for this assay is described in Ref. [15]. The crossreactivity of biotinylated estradiol derivatives towards estradiol was determined in polystyrene tubes following this procedure. Additionally, the influence of immobilized E_2-Bio derivatives onto the antibody-[^{125}I]estradiol tracer binding was determined as follows: increasing concentrations of E_2-Bio was added to avidin coated plates for 150 min. The plates were washed five times to remove free molecules. 50 µl of tracer (estradiol-CMO-[^{125}I]iodohistamide [15]), and 100 µl of anti-estradiol antibody was added to each well. After an incubation for 3 h at room temperature, an aliquot of 150 µl was withdrawn from each well and transferred to a test tube. Bound and free tracer was separated by charcoal as described [15]. Competition of the immobilized E_2-Bio with the soluble estradiol tracer for antibody

Table 1. Performance of various E_2-Bio derivatives in antibody- and antigen-immobilized systems

Compound	Structure	Required E_2-Bio concentration (pmol/well)	
		Ab-coated ELISA	Ag-coated ELISA
(1)	E_2-NH-LC-Bio	0.024	3.2
(2)	E_2-NH-Bio	n.s.	n.s.
(3)	E_2-NH-Bio	n.s.	n.s.
(4)	E_2-Hy-LC-Bio	63	n.s.
(5)	E_2-Hy-Bio	n.s.	n.s.
(6)	E_2-CMO-LC-Bio	0.009	0.87
(7)	E_2-CMO-LC-Bio	0.007	2.4

Different concentrations of estradiol derivatives were used in both ELISA systems under standardized conditions [14]. The data displayed represent the amount of E_2-Bio required to give an optical signal of 0.5 O.D. n.s.: no signal, using up to 500 pmol/well.

binding is expressed as the mass of plate bound estradiol derivative required to reduce tracer binding to 50%.

Crossreactivities

Crossreactivity is calculated as the ratio of 50% of the maximal tracer binding observed for the calibrator and the crossreactant, respectively. Crossreactants (estrogens, E_2-Bio derivatives) were used in concentrations from 10 μg/ml to 10 pg/ml.

Biotin assay

In microtiter plates coated with avidin (see above), biotin as a calibrator (1–18 ng/ml) or E_2-Bio derivatives in various concentrations were incubated for 1 h. After a washing step, 200 μl (0.3 ng) of a biotin-peroxidase conjugate (Sigma, St Louis, U.S.A.) was incubated for 1 h. The unbound biotin-peroxidase was washed away and the bound enzyme activity measured by addition of the substrate as described for ELISA. The presence of biotin in the E_2-Bio derivatives was assessed assuming an affinity for avidin identical to that of free biotin.

RESULTS

Preparation and purification of E_2-Bio derivatives

An effective purification by HPLC turned out to be a prerequisite for a successful application of the E_2-Bio derivatives in the two ELISA systems. A full chemical analysis was not possible in all cases due to lack of sufficient amounts of material. Even if NMR or mass spectroscopy results were available, only tentative conclusions could be drawn, because molecules of such complexity give rise to very complicated data sets. For instance, the molecule ion was never found in the mass spectrometry data, but only fragments of E_2-Bio derivatives after loss of the ethyl-urea group from the biotin moiety. The u.v. spectroscopy indicates the presence of the phenolic ring of estradiol.

All substances were biochemically investigated and found to contain both estradiol and biotin. In summary, theoretical studies of the reaction mechanisms, the chemical analytical data and the biochemical results confirm the proposed structures of the various E_2-Bio derivatives to a degree sufficient for the context of this study.

Surprisingly two immunologically active E_2-Bio derivatives were found in both the synthesis of the short-chain amino and the long-chain CMO derivatives. Since the short-chain amino derivatives did not yield useful results (see below) only the two long-chain CMO derivatives were carefully examined. They are clearly resolved in HPLC and their u.v. spectra differ: Compound (**6**) shows a shoulder at 300 nm, whereas compound (**7**) has a second peak at 310 nm. Already the starting material (E_2-CMO) consists of two compounds (see Fig. 2). We interpret compound (**6**) and (**7**) as the biotin derivatives of the *Z*/*E*-isomers present in the starting material. The impact of this structural difference onto the antibody recognition is discussed below.

Binding of E_2-Bio derivatives by antibody and avidin

The ability of E_2-Bio derivatives (Fig. 1) to sequentially bind the respective protein was investigated in both the antibody- and the antigen-immobilized system (Table 1). All derivatives having a spacer group [(**1**), (**4**), (**6**) and (**7**)] were found to bind to the

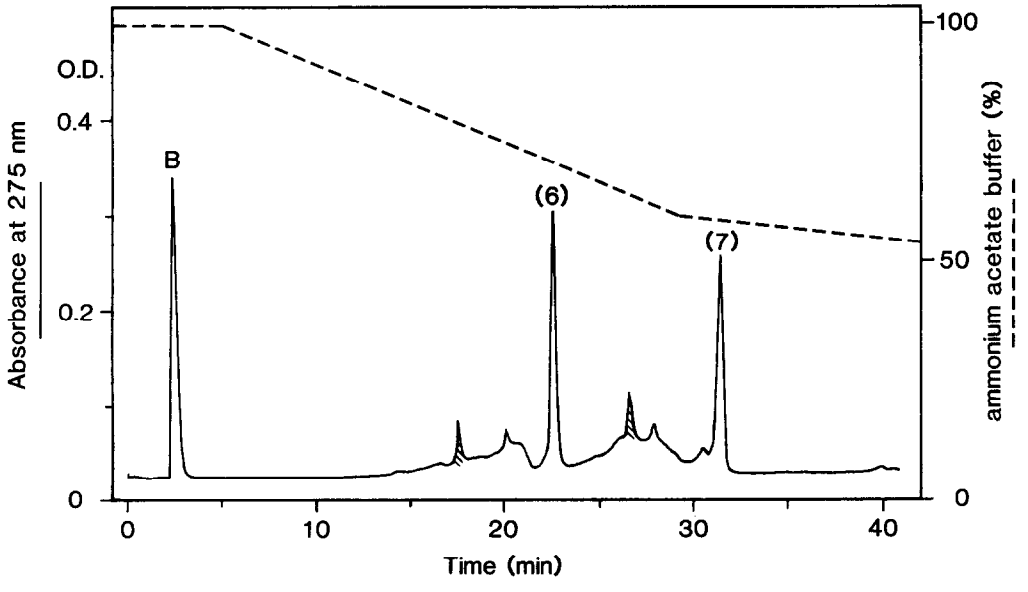


Fig. 2. Purification of E_2-CMO-LC-Bio by HPLC. Two isomers, compounds (**6**) and (**7**), are eluted from the column after injection of the reaction mixture. The hatched peaks represent the starting material (E_2-CMO). Peak "B" is the Bio-LC-hydrazide. The gradient of acetonitrile in ammonium acetate buffer is drawn as a dashed line.

Table 2. Recognition of soluble and immobilized E_2-Bio derivatives by the antibody

Compound	Structure	Crossreactivity (%)	Competition (pg/well)
(1)	E_2-NH-LC-Bio	39	22
(2)	E_2-NH-Bio	5	25
(3)	E_2-NH-Bio	6	9
(4)	E_2-Hy-LC-Bio	0.002	$\geqslant$630
(5)	E_2-Hy-Bio	4	72
(6)	E_2-CMO-LC-Bio	13	22
(7)	E_2-CMO-LC-Bio	0.002	140

The crossreactivity of each derivative was determined in a liquid phase RIA system (estradiol = 100%). In addition, the ability of immobilized E_2-Bio to compete with a liquid phase iodinated estradiol tracer for antibody binding has been assessed. The concentration of the respective E_2-Bio derivative needed to displace 50% of the estradiol tracer is indicated.

immobilized antibody and they can be detected by avidin labeled with peroxidase. However, sequential binding of compounds (1), (6) and (7) to immobilized avidin and to the liquid phase antibody required approximately a 100-fold higher concentration to reach the same optical signal. The suggested structural difference between compound (6) and (7) (see above) is manifested by an almost 3-fold higher concentration required of compound (7) in the antigen-immobilized system, while the same concentration of both E_2-CMO-LC-Bio derivatives is needed in the case of antibody-immobilization.

Competition of E_2-Bio with [^{125}I]estradiol for antibody binding

The abilities of various surface bound E_2-Bio derivatives to compete with liquid phase [^{125}I]estradiol permits to assess their relative avidity for anti-estradiol (Table 2). With the exception of compound (4) all E_2-Bio derivatives are able to compete with the iodinated estradiol for antibody binding. The relative potencies vary by a factor of approximately 20.

The structural differences of the E_2-Bio derivatives are better revealed, when they are in solution (Table 2): the two compounds (1) and (6) are very well recognized, as it was expected from the previous data (Table 1), whereas the crossreactivity of compound (7) is remarkably low.

Specificity of ELISA systems using selected E_2-Bio derivatives

In addition to the competition experiments, three of the long chain E_2-Bio derivatives have been selected and used in both the antigen- [compounds (1) and (6)] and the antibody-immobilized ELISA system [compounds (1), (4) and (6)]. The crossreactivities of five estrogens were determined using an antibody without bridge effect (Table 3) and an antibody which has previously demonstrated bridge binding [15] (Table 4). In the antibody-immobilized system the crossreactivity for a given estrogen seems generally to be constant, regardless of the type of the E_2-Bio derivative and the antibody used. Some unsystematical exceptions remain unexplained. In an antigen-immobilized system, the overall specificity is improved when using E_2-CMO-LC-Bio [compound (6)] as opposed to E_2-NH-LC-Bio [compound (1)].

DISCUSSION AND CONCLUSION

In an earlier report [14], we have indicated the usefulness of a biotinylated derivative of 6α-amino-

Table 4. Specificity of antibody-immobilized ELISA systems

Crossreacting estrogen	Crossreactivity (%) Ab-coated ELISA compound used (1)	(4)	(6)
Estrone	4.5	0.78	3.4
Estriol	0.24	0.24	0.41
Estradiol-3-glucuronide	1.4	1.65	0.96
Estradiol-17-glucuronide	0.12	0.17	0.12
17-Ethinyl-estradiol	0.01	0.02	0.02

Crossreactivitiy of five estrogens have been determined in an antibody-immobilized ELISA system using three different E_2-Bio derivatives and antibody e02/2/83 with a marked bridge effect. The crossreactivity is compared to estradiol (100%) and calculated at 50% tracer displacement.

Table 3. Specificity of antigen- and antibody-immobilized ELISA systems

Crossreacting estrogen	Crossreactivity (%) Ab-coated ELISA compound used (1)	(4)	(6)	Ag-coated ELISA compound used (1)	(6)
Estrone	3.6	4.2	5.3	17	24
Estriol	0.6	0.8	5.7	4	1.9
Estradiol-3-glucuronide	0.1	0.9	0.23	3.6	0.48
Estradiol-17-glucuronide	0.09	0.05	0.06	0.12	0.008
17-Ethinyl-estradiol	0.003	0.005	0.02	0.07	0.04

The crossreactivities of five estrogens have been determined in the two ELISA systems using three different E_2-Bio derivatives. The crossreactivity is compared to estradiol (100%) and calculated at 50% tracer displacement. The antibody used displays no bridge effect.

estradiol [compound (**1**)] in immunoassay systems. In this study, some structural aspects of the moiety linking biotin to estradiol have been investigated.

The biotin moiety of the E_2-Bio derivative is entirely embedded into the polypeptide loops of the streptavidin molecule [16]. Therefore the binding of the estradiol moiety to the antibody seems to be strongly impeded in derivatives lacking a spacer group. However, the binding of a radioactive tracer to the antibody in a solution is still affected by immobilized compound (**2**) or (**3**), two derivatives lacking a spacer group (Table 2). Thus we assume, that the antibody can recognize the estradiol-moiety even in these cases, but the binding is not stable enough.

Our data indicate that the binding of biotin to avidin is not affected by the presence of a bulky rigid structure close to the carboxyl group present in the side chain. This finding is in agreement with observations of unimpaired binding to avidin of a great variety of biotinylated substances.

The chemical structure at C–6 of the steroid is of great importance as exemplified for iodinated tracer structures [15]. Both, the CMO- [compounds (**6**) and (**7**)] and the amido-structure [compound (**1**)] in the E_2-Bio derivative fit well into the binding pocket of the antibody raised against the immunogen estradiol-CMO-bovine serum albumin. These results are in full agreement with our previous findings.

From the two isomers of E_2-CMO-LC-Bio, compound (**6**) is well recognized when present in solution, whereas the crossreactivity of compound (**7**) is about 10,000 times lower under the same conditions (Table 2). This difference almost disappears, when these two compounds are immobilized. We assume, that the ureido-group present in the biotin moiety is able to form a hydrogen bond to the hydroxyl-group present in position 3 of the estradiol ring A, but only in the diastereomer derivating from the Z-isomer. The recognition by an antibody is therefore seriously impeded and the ultraviolet spectrum is slightly different compared with the spectrum of 6-CMO-estradiol. In an ELISA using immobilized antigen only three times more of compound (**6**) than of compound (**7**) is required. This is in contrast to the great difference found, when these compounds are present in solution. The assumed formation of an intramolecular hydrogen bond is obviously prevented by the immobilization of compound (**7**).

The stereochemical structure of the hydrazone-bridge (Hy) is very similar to that of the CMO-hydrazido-bridge. We therefore expected only slightly altered antibody binding. However, when immobilized on a surface, E_2-Hy-LC-Bio is absolutely not accessible to the antibody. The rigid structure seems to prevent the antibody binding. This assumption is supported by the result obtained by the RIA, where this immobilized antigen does not compete with the iodinated tracer. However, in the case of immobilized antibody, subsequently bound E_2-Hy-LC-Bio molecules can be detected using a streptavidin-peroxidase conjugate. Steric hindrance seems not to prevent antibody binding absolutely.

The crossreactivity data show that the specificity is only slightly and not systematically dependent on the E_2-Bio derivative used (Table 3). The same unsystematical results were found using an antibody with bridge binding (Table 4). Thus, the competition between the estrogens and E_2-Bio derivatives seems to be hardly predictable. A multitude of sterical and thermodynamical factors seems to determine the kinetics and the stability of the antigen binding [17].

The sum of our results confirms that the design of the bridge in steroid immunoassay is the central problem [18]. Three main factors seem to determine antibody binding:

Firstly: the chemical structure linking the bridge to the steroid skeleton is very important. This is in agreement with the findings known from iodinated tracer structures [19–21]. A slight modification is sufficient to prevent antibody binding, as demonstrated in this report for the hydrazone structure. On the other hand, a more drastic change as represented by the amido-structure has only little influence on the recognition by the antibody. The tetrahedral C-6 structure may give more freedom for motion and molecular adaptation than a rigid planar structure. The structural requirements identified here might be exceptionally severe, because the steroid itself is a very rigid molecule. Direct extrapolation to other more flexible haptens might therefore not be useful.

Secondly: the introduction of a spacer is a necessity. However, even when E_2-Bio derivatives lacking a spacer group are immobilized, the estradiol moiety is still recognized by the antibody. This binding seems to be unstable and apparently does not survive the multiple washing steps of an ELISA.

Thirdly: the immobilization of reactants drastically influences the immunochemical reaction. The diffusion of free molecules to the plastic surface and the spacial arrangement of the immobilized molecules are decisive factors [22]. Preferably the bulky antibody molecule should be immobilized and not the relatively small antigen as suggested from the data on E_2-Hy-LC-Bio (Table 1).

In summary, it seems that from the compounds tested here both the homologous (E_2-CMO-LC-Bio) and the heterologous bridge derivative (E_2-NH-LC-Bio) are suitable reagents for an avidin/biotin based ELISA. These substances may potentially be used in other advanced immunoanalytical systems.

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