Reactivity of an immobilized anti-progesterone antiserum with homologous and heterologous progesterone–horseradish peroxidase conjugates

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The binding and selectivity features of an immobilised anti-progesterone antiserum were studied by the use of four different enzyme tracers: progesterone 11α-hemisuccinate–horseradish peroxidase (P-11α-HS–HRP), progesterone 11α-carboxymethyl ether–horseradish peroxidase (P-11α-CME–HRP), progesterone 11β-carboxymethyl ether–horseradish peroxidase (P-11β-CME–HRP) and progesterone 3-(O-carboxymethyl)oxime–horseradish peroxidase (P-3-CMO–HRP). The antiserum–tracer affinities generally showed a remarkable reduction in respect to the affinity of the analyte because of the steric hindrance of the enzyme and, among the four tracers, the higher affinity value was evaluated for the P-11α-HS–HRP (homologous to the immunogen molecule). The concentration of antibody binding sites interacting with the tracers showed the presence of different classes of antibodies able to react with variable affinity with tracers and analyte, as confirmed by the cross-reactivity values measured towards different progesterone derivatives. The assays performed with the tracers showed that an increase of sensitivity can be obtained using enzyme tracers provided with heterologous structure features with respect to the immunogen molecule.

Introduction

In an immunometric assay based on the competition between the analyte and the tracer (an analyte derivative coupled to an enzyme) for the antibody binding sites immobilised on a solid phase, the tracer structure can affect the selectivity and the sensitivity features in a determinant way.[1–4]

Studies previously carried out for the determination of plasmatic steroids in the human antiserum by heterogeneous competitive radioimmunoassays showed that iodinated tracers,[2,5–7] provided with a homologous linking bridge (having the same chemical structure and stereochemical configuration like that of the immunogen), bind the antibody binding sites more than the analyte does. Such a feature depends on the antiserum ability to recognise in a selective and specific way both the steroid molecule and the linking bridge, a property known as 'bridge effect'.

Since former studies on a rabbit anti-progesterone antiserum[7,8] showed that it effectively manages to bind the homologous linking bridge, the binding properties of such an antiserum towards several enzyme tracers (using the horseradish peroxidase as enzyme) provided with different linking bridges were studied in order to evaluate the effect of the structural features of the tracer on the sensitivity and selectivity of the enzyme immunoassay.

Experimental

Materials

Progesterone 11α-hemisuccinate was obtained from Steraloids (Wilton, NH, USA). Progesterone 3-(O-carboxymethyl)oxime, 11-ketoprogesterone, horseradish peroxidase (HRP), type VI-A, were obtained from Sigma (St. Louis, MO, USA). The Coomassie Protein Assay Reagent was obtained from Pierce (Rockford, IL, USA).

Infrared spectra were recorded on a FTIR Nicolet Impact 410 (Madison, WI, USA) using potassium bromide pastilles. Mass spectra were obtained by desorption chemical ionisation, with a Finnigan-MAT 95Q (San José, CA, USA). Sephadex G-25 Superfine and low pressure chromatographic apparatus (peristaltic pump P-1, columns 10 × 100 mm, fraction collector FRAC-100, monitor UV-M and chart recorder REC-482) were supplied by Pharmacia (Uppsala, Sweden). All UV measurements were recorded on a Varian Cary 1E double beam spectrophotometer (Sunnyvale, CA, USA). Microplates were obtained from Nunc (Roskilde, Denmark), Microplate washer (Novapath Washer), microplate incubator and microplate reader (Microplate Reader 3550) were supplied by Bio-Rad (Hercules, CA, USA).

All the antiserum and the enzyme tracer dilutions for the binding studies on microtiter plates were made by means of the diluent buffer (sodium phosphate buffer 0.02 mol dm^{-3}, sodium chloride 0.13 mol dm^{-3}, potassium chloride 0.05 mol dm^{-3}, bovine serum albumin 0.1% w/v, Tween 20 0.05% v/v, pH 7.4).

Immunoreagents

The rabbit polyclonal antiserum raised against progesterone 11α-hemisuccinate, conjugated to bovine serum albumin, was kindly supplied by G. Bolelli, Servizio di Fisiopatologia della Riproduzione, Ospedale S. Orsola, Bologna, Italy. The antibodies against bovine serum albumin were precipitated by addition of the protein to the crude antiserum.

The goat polyclonal anti-rabbit γ-globulin antiserum was also kindly supplied by G. Bolelli. The IgG fractions were purified from the crude antiserum by immunoaffinity chromatography on a 10 × 13 mm column of rabbit IgG–Affiprep 10 (Bio-Rad). The antiserum was diluted 1 + 2 v/v with phosphate buffer 0.02 mol dm^{-3}, sodium chloride 0.13 mol dm^{-3}, EDTA 0.001 mol dm^{-3}, pH 7.4 and adsorbed on the column. The column was washed with the same buffer and the eluate absorbance was

recorded at 280 nm. The anti-rabbit IgG fractions were desorbed with glycine buffer 0.05 mol dm^{-3}, sodium chloride 0.1 mol dm^{-3}, pH 2.5. Protein fractions (1 cm^3) were collected and immediately diluted with 2 cm^3 of phosphate buffer, then stored at 4 °C.

The purified antibody concentration was determined by the Coomassie Protein Assay Reagent.[9,10] The immunoreactive solid phase was obtained by coating the polystyrene microplate wells with 0.3 cm^3 of 0.01 mg dm^{-3} anti-rabbit goat IgG solution and then stored at 4 °C, according to the literature.[11]

Synthesis of progesterone 11α- and 11β-carboxymethyl ethers

Both the steroid molecules with a carboxymethyl ether side chain at the position 11, α and β orientated, were synthesised starting from the 11-ketoprogesterone. In order to avoid the reduction, the selective protection of the ketonic structures at the positions 3 and 20 were performed by exchange dioxolanation with 2-ethyl-2-methyl-1,3-dioxolane as reported in literature.[12] The stereoselective reduction of 11-ketoprogesterone-3,20-diethylenketal to 11β-hydroxy derivative was performed by sodium borohydride in basic methanol,[13] while the stereoselective reduction to 11α-hydroxy derivative was performed by metallic sodium with propan-1-ol as solvent.[13] The functionalization of the two hydroxyl derivatives was made by ethyldiazo acetate according to literature.[14,15] The ethyl ester of carboxymethoxyl derivatives obtained were hydrolysed under acid and basic conditions. The crystalline products were characterised by MS and IR techniques.

Relevant mass spectrum signals (for progesterone 11α-CME) are: 205, 265, 311, 403, 417, 479, 531, 775 *m/z* and the molecular ion is identified as the signal with *m/z* ratio equal to 387. Relevant mass spectrum signals (for progesterone 11β-CME) are: 123, 187, 311, 403, 447, 778 *m/z* and the molecular ion is identified as the signal with *m/z* ratio equal to 387. Infrared data (ν_{max}) are: α-ether C_{11} (1057 cm^{-1}) and β-ether C_{11} (1134 cm^{-1}), α-unsaturated carbonyl C_3 (1653 cm^{-1}), carbonyl C_{20} (1701 cm^{-1}), carboxyl group (1733 cm^{-1}).

Synthesis of the enzyme tracers

The progesterone derivative –enzyme conjugates (progesterone 11-α-hemisuccinate–horseradish peroxidase, P-11α-HS–HRP; the progesterone 11-α-carboxymethyl ether–horseradish peroxidase, P-11α-CME–HRP; the progesterone 11-β-carboxymethyl ether–horseradish peroxidase, P-11β-CME–HRP and the progesterone 3-(O-carboxymethyl)oxime–horseradish peroxidase, P-3-CMO–HRP) were synthesised, purified by size exclusion chromatography and characterised according to the literature.[10] The average number of progesterone derivative molecules for each peroxidase molecule, determined by spectrophotometric analysis[10] were: 0.9 (P-11α-HS–HRP), 1.9 (P-11α-CME–HRP), 1.2 (P-11β-CME–HRP) and 1.3 (P-3-CMO–HRP).

Solid phase coating procedure of the anti-progesterone antiserum

Portions (0.2 cm^3) of the anti-progesterone antiserum, properly diluted with the diluent buffer, were dispensed in the microplate wells (coated with the anti-rabbit goat IgG), incubated 24 h at room temperature and then washed 3 times with a 0.05% v/v solution of Tween 20 (washing solution). The measurement of the non specific binding (NSB) of the enzyme tracer was made replacing the anti-progesterone antiserum with the buffer.

Colorimetric detection

Portions (0.2 cm^3) of chromogen–substrate solution were dispensed in each well. After incubation in the dark at 37 °C (1 h for P-11α-CME–HRP, P-11β-CME–HRP and P-3CMO–HRP, 10 min for P-11α-HS–HRP and unconjugated HRP), the enzyme reaction was stopped with 0.1 cm^3 of 1 mol dm^{-3} sulfuric acid and the absorbance (A) was read at 450 nm (λ_{max}) or, when the absorbance values were over 2, at 405 nm.

Optimal antiserum dilution on the solid phase

As the heterologies of the enzyme tracers used can affect their binding behaviour on the solid phase (increasing or decreasing the resulting analytical signal), it is necessary to define both the anti-progesterone antiserum working titer for each enzyme tracer and the more appropriate concentrations of each tracer to have an analytical signal (absorbance) on the solid phase of about 1. As a matter of fact an absorbance of about 1 represents the minimum value useful to have a substantial difference between the minimun and the maximum of the experimental curves.

The coating with the anti-progesterone antiserum was made by dilution at 1 : 4000, 1 : 8000, 1 : 16 000, 1 : 32 000, 1 : 64 000, 1 : 128 000 and 1 : 256 000 (v/v) with the diluent buffer according to the procedure. Measurements were taken in duplicate. Portions (0.2 cm^3) of enzyme tracer properly diluted with the diluent buffer (45.5, 11.4, and 2.27 nmol dm^{-3} for P-11α-CME–HRP and P-11β-CME–HRP, 22.7, 6.82, and 2.27 nmol dm^{-3} for P-11α-HS–HRP and P-3CMO–HRP) were dispensed in each well and incubated 24 h at room temperature. The microplates were washed three times with the washing solution, then the bound enzyme tracer was measured according to the colorimetric detection procedure.

Tracer specific activity

Measurements were taken in duplicate on immunoreactive microplates (coated with the anti-rabbit goat IgG) and all the dilutions were made with the diluent buffer. The non specific colour development was measured by replacing the properly diluted tracer with the buffer. Portions (0.025 cm^3) of each tracer and unconjugated horseradish peroxidase were dispensed at concentrations of 0.68, 1.14, 1.59, 2.27, 6.82, 15.9, 22.7 pmol dm^{-3}, then the colorimetric detection was carried out.

The tracer specific activity (SA) was calculated by the slope of the plot absorbance *vs.* enzyme concentration and it represents the absorbance of the enzyme tracer per concentration unit in the condition fixed for the colorimetric detection.

Tracer binding constant for the solid phase

The anti-progesterone antiserum was immobilised on the immunoreactive solid phase at the dilution of 1 : 32 000 (v/v) as described above. Afterwards, 0.2 cm^3 of each tracer at concentrations of 0.068, 0.114, 0.159, 0.227, 0.341, 0.455, 0.682, 0.909, 1.70, 2.27, 3.41, 4.54, 9.09, 18.2 and 22.7 nmol dm^{-3} were dispensed in each well and, after a 24 h incubation at room temperature, microplates were washed with the washing solution and the colorimetric detection was carried out.

The equilibrium binding constant was calculated by fitting the experimental absorbance *vs.* tracer concentration by means of the mathematical expression (eqn. 4) shown in the Appendix. The non-linear curve fitting was done by means of the Marquardt–Levenberg algorithm, using the FigP Software (Biosoft, Cambridge, UK). This software allowed the error

estimates of the equation parameters, based on the scattering of the experimental points around the fitted curve. The binding site concentration (B_{max}) on the solid phase was calculated by the eqn. 3.

The cross reactivity measurement

A mixture of 0.1 cm^3 of the buffered solution of competitor and 0.1 cm^3 of the enzyme tracer properly diluted (2.3 nmol dm^{-3} of the P-11α-HS–HRP, 10 nmol dm^{-3} of the P-3-CMO–HRP and 15 nmol dm^{-3} of the P-11α-CME–HRP and the P-11β-CME–HRP) were dispensed in each well (coated with the anti-progesterone antiserum), and were incubated overnight at room temperature. In order to obtain similar absorbance values for all the enzyme tracers, the anti-progesterone antiserum had been previously immobilized at the dilutions of 1 : 32 000 for P-11α-HS–HRP, 1 : 16 000 for P-3-CMO–HRP, and 1 : 6000 for P-11α-CME–HRP and P-11β-CME–HRP. The zero concentration of the competitor was measured replacing the competitor with the diluent buffer. Then the microplates were washed with the washing solution and the colorimetric reaction was carried out.

The competition curves were obtained by plotting the absorbance at 450 nm *versus* the competitor concentration and were fitted by using the four parameter logistic equation.[16] To allow the direct comparison of the curves, the absorbance values were converted into their corresponding test-inhibition value (B/B_0) as follows:

$$B/B_0 = (A - A_{sat})/(A_0 - A_{sat})$$

where A represents the absorbance value of calibrators, A_{sat} represents the absorbance value corresponding to a saturating analyte concentration (as evaluated by the four parameter logistic function). The detection limit was significantly evaluated as the concentration of progesterone that gives an absorbance equal to the absorbance of the zero standard (A_0) minus 3 standard deviations, evaluated with ten repeated measurements. The mid-point value of the competition curves was evaluated as the concentration of competitor that gives a test inhibition value of 50%. The cross reactivity was calculated according to the literature.[17]

Results and discussion

The experimental results to define the antiserum optimal dilution showed that the proper working dilutions used for the affinity measurements have to be 1 : 32 000 (v/v) for all the tracers considered.

The analytical signal of the tracer bound to the antiserum immobilised on the solid phase as a function of the total tracer concentration in solution (binding isotherm) is shown in Fig.1 and Fig. 2 for the different tracers studied.

The equilibrium binding constant (K) and the antibody site concentration (B_{max}) on the solid phase, obtained from the curve fit with the eqn. 2, are reported in Table 1. The reliability of the estimates of the binding site concentrations and equilibrium binding constants strongly depends on the validity of the assumption regarding the ligand excess, *i.e.*, $L_o >> [PL_s]$. The concentration of the bound ligand $[PL_s]$ can be easily calculated, by means of the eqn. 1 (see Appendix), from the data of K and B_{max} reported in Table 1 for all the enzyme tracer concentrations used to determine the binding isotherm. Even in the case of the lowest concentration used, the fraction of tracer bound to antibody binding sites are 3.8% for P-11α-HS–HRP, 0.024% for P-11α-CME–HRP, 0.017% for P-11β-CME–HRP and 0.14% for P-3CMO–HRP. Thus the assumptions underlying the analysis of the binding data are widely satisfied. The experimental data show that the affinity of the homologous tracer (P-11α-HS–HRP) is ten-fold lower than that measured for the tritiated progesterone[8] with the same antiserum, even if the studied antiserum shows the bridge effect. Such an affinity drop suggests that, on the contrary to what happens with the radiolabelled tracers, the bridge effect is not present with the enzyme tracer, in spite of the presence of a linking bridge equal to that of the immunogen. The lack of the bridge effect can be explained by the following suppositions: (1) assuming that the steroid molecule cannot deeply enter into the binding sites for

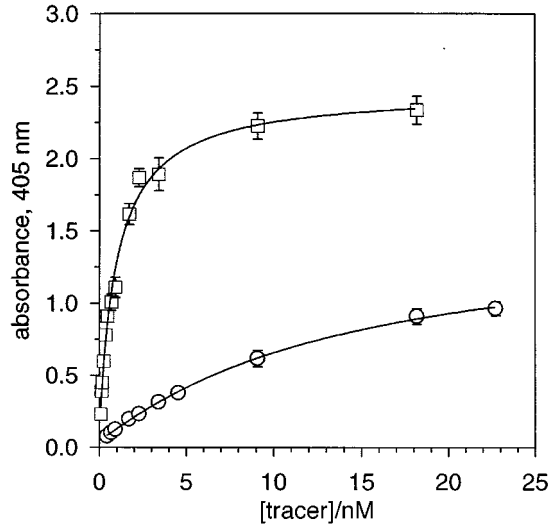

Fig. 1 Binding isotherms for the interaction of the anti-progesterone antiserum with the tracers P-11α-HS–HRP (□) and P-3-CMO–HRP (○). Each point represents the mean ± SD of double experimental data.

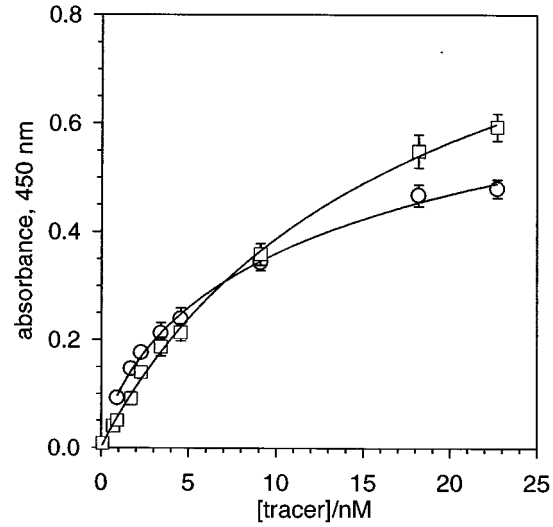

Fig. 2 Binding isotherms for the interaction of the anti-progesterone antiserum with the tracers P-11α-CME–HRP (○) and P-11β-CME–HRP (□). Each point represents the mean ± SD of double experimental data.

Table 1 Specific activity (SA) of the enzyme tracers, tracer binding constants (K) and binding site concentrations (B_{max}) measured for the interaction of the tracers with the anti-progesterone antiserum. The specific activity of the native HRP is 3.8 × 10^{11} A mol^{-1} dm^3

Tracers	SA × 10^{-11}/ A mol^{-1} dm^3	K × 10^{-8}/ mol^{-1} dm^3	B_{max}/ pmol dm^{-3}
P-11α-HS–HRP	1.7 ± 0.20	10 ± 2.7	41 ± 2.5
P-11α-CME–HRP	2.6 ± 0.21	1.0 ± 0.44	2.4 ± 0.26
P-11β-CME–HRP	3.5 ± 0.37	0.60 ± 0.15	2.8 ± 0.30
P-3-CMO–HRP	2.1 ± 0.22	0.61 ± 0.12	23 ± 1.9

the steric hindrance; (2) otherwise, the lower affinity can be caused by the additional free energy variation requested to alter partially the enzyme structure around the steroid and to allow the complete access to the antibody site.

The affinities of the heterologous tracers P-11α-CME–HRP, P-11β-CME–HRP and P-3-CMO–HRP show that, whatever the nature of the heterology, an affinity decrease is always observed in comparison with the homologous tracer, related again to the steric hindrance. In fact, if such an effect is so important with a linking bridge like the hemisuccinate, we can think all the more that such a steric hindrance is important on the affinity of tracers with a shorter linking bridge.

If the linking bridge heterology can change the access to the binding sites, we can not exclude that the tracers P-11α-CME–HRP, P-11β-CME–HRP and P-3-CMO–HRP occupy different antibody sites. Such hypothesis is confirmed by the site concentrations occupied by the heterologous tracers that are about 2–20 fold lower than that of the homologous tracer. In particular, for the tracer P-3-CMO–HRP, the lateral position of the linking bridge (in comparison with the linking bridge of P-11α-HS–HRP) causes the occupied sites to be structurally different in respect to that of the homologous tracer.

The low binding site concentrations of the tracers with linking bridge 11α- and 11β-CME confirm the hypothesis that these do not enter into the high affinity sites occupied by the P-11α-HS–HRP because of the very pronounced steric hindrance. These tracers preferably interact with binding sites which are more superficial and accessible, but able to recognise, even in a less specific way, the steroidal component.

In order to evaluate the selectivity of the immobilised antiserum for each enzyme tracer considered in this work, the cross-reactivity values of several progesterone derivatives (P-11α-HS, P-11α-CME, P-11β-CME, P-3-CMO, P-11α-OH, P-17α-OH) were determined by the corresponding competition curves. In order to compare the competition curves and to have quite similar absorbance values (ranging from 1.3 to 1.6) for all the enzyme tracers considered, the anti-progesterone antiserum dilutions were changed with respect to those used to determine the equilibrium binding constants. Examples of competition curves used to calculate the cross-reactivities are reported in Fig. 3 and Fig. 4. The relative potency does not represent a constant value with respect to the changing of B/B_0. However, the choice of expressing the cross-reactions in terms of 50% of B/B_0 values was made in order to show the selectivity differences of assays performed with different enzyme tracers. The complete list of the competitors, the enzyme tracer concentrations used as well as the measured mid-points, the calculated cross-reactivity values and the detection limit of the calibration curves with progesterone obtained with the different enzyme tracers are reported in Table 2. Whichever tracer one

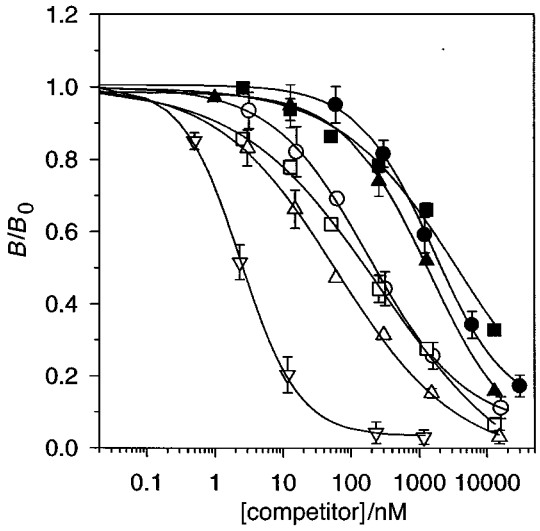

Fig. 3 Competition curves of the tracer P-11α-HS–HRP with the progesterone (○) and different progesterone derivatives: P-17α-OH (●), P-11α-CME (□), P-11β-CME (■), P-11α-OH (△), P-3-CMO (▲) and P-11α-HS (▽). Each point represents the mean ± SD of double experimental data.

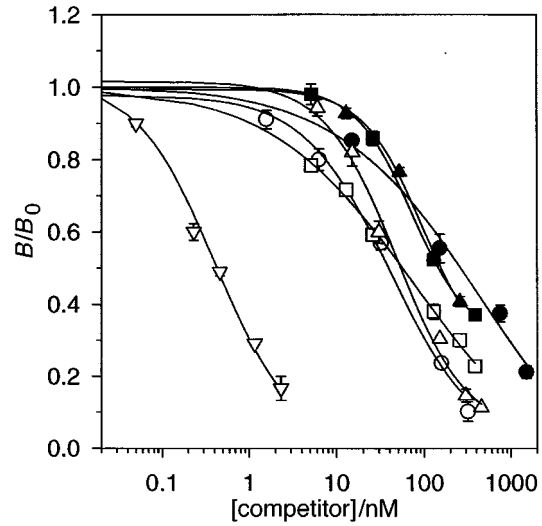

Fig. 4 Competition curves of the tracer P-11α-CME–HRP with the progesterone (○) and different progesterone derivatives: P-17α-OH (●), P-11α-CME (□), P-11β-CME (■), P-11α-OH (Δ), P-3-CMO (▲) and P-11α-HS (▽). Each point represents the mean ± SD of double experimental data.

Table 2 Tracers and concentrations used, mid-point values, cross-reactivities (CR) calculated for each progesterone derivative by the competition curves and detection limits (DL) measured on the progesterone calibration curves

Tracer/concentration nmol dm^{-3}	Competitor	Mid-point/ nmol dm^{-3}	CR/ nmol dm^{-3}	DL/ nmol dm^{-3}
P-11α-HS–HRP (2.3)	P	185	100	1.8
	P-11α-HS	2.3	8.0×10^3	
	P-11α-CME	140	130	
	P-11β-CME	3600	5	
	P-3-CMO	1336	14	
	P-11α-OH	55	335	
	P-17α-OH	2300	8	
P-3-CMO–HRP (10)	P	6	100	0.5
	P-11α-HS	0.1	5.8×10^3	
	P-11α-CME	38	16	
	P-11β-CME	580	1	
	P-3-CMO	15	40	
	P-11α-OH	20	30	
	P-17α-OH	200	3	
P-11α-CME–HRP (15)	P	45	100	0.6
	P-11α-HS	0.3	14×10^3	
	P-11α-CME	54	80	
	P-11β-CME	140	30	
	P-3-CMO	160	28	
	P-11α-OH	54	80	
	P-17α-OH	230	20	
P-11β-CME–HRP (15)	P	46	100	0.9
	P-11α-HS	0.24	20×10^3	
	P-11α-CME	90	50	
	P-11β-CME	46	100	
	P-3-CMO	160	28	
	P-11α-OH	130	35	
	P-17α-OH	250	18	

used in the assay, the cross-reaction of the P-11α-HS appears to be very high because of the recognition of the linking bridge from the antibody binding sites. Moreover, the lower the tracer affinity is, the more pronounced the effect appears to be. This high cross-reaction and its dependence on the tracer affinity can be explained by the presence of different classes of antibody binding sites able to react with variable affinity with the tracer and the analyte, as previously observed for an analogous progesterone immunoassay based on P–HRP tracers.[18] This explanation is also confirmed by the binding site concentrations reported in Table 1, which shows that the different tracers studied have to react with different antibody binding sites as discussed above.

Apart from the progesterone and the progesterone derivative homologous to the immunogen molecule, it can be observed that the derivative homologous to the tracer used in the assay always shows the highest cross-reactivity among the different derivatives considered. This behaviour is due to the ability of such a homologous derivative to compete efficiently for the occupation of antibody binding sites, which are able to bind that defined steroid-linking bridge structure more strongly than other antibody sites.

Another feature common to the assays performed with the four tracers examined is the more pronounced cross-reactivity of those progesterone derivatives provided with chemical substituents at the same position and/or with the same steric orientation as the immunogen structure. In fact, the derivative P-11α-CME always shows a cross-reactivity higher than the derivative P-11β-CME, except in the assay performed with the P-11β-CME–HRP as tracer, and in the same way P-11α-OH interacts more strongly than P-17α-OH. The differences of the cross-reactivity values appear again more pronounced when the tracer affinity is high, as a high affinity implies a tight interaction between the steroid molecule and the binding site, that enhances the structural differences between the interacting molecules. More generally, the cross-reactivities observed in the assays performed with low affinity tracers (P-11α-CME–HRP and P-11β-CME–HRP) show that the differences of selectivity between P-11α-CME and P-11β-CME, or between P-11α-OH and P-17α-OH are lower if compared to those observed with high affinity tracers. This confirms that the antibody binding sites involved in the reaction with low affinity tracers also interact in a less specific way with the steroid molecule.

The experimental data reported in the Table 2 show that the assay performed with P-3-CMO–HRP as tracer shows the maximum of sensitivity (minimum mid-point value and minimum detection limit). This result can be related to the presence of at least two classes of different antibody binding sites, as it may be deduced by the mid-point values for the progesterone and the P-11α-HS. As the native progesterone can be considered able to bind a number of antibody binding sites higher than that bound by the enzyme tracers, the explanation of the different sensitivities observed with different tracers has to be based on the competition between the progesterone and the progesterone–enzyme tracer for different binding sites. As different antiserum dilutions were used in the assays performed with the different enzyme tracers considered, the concentration of the antibody sites able to bind the progesterone clearly increases by lowering the working dilution of the anti-progesterone antiserum immobilized on the solid phase.

The discussion has to be planned in a quite different way as regards the antibody sites able to bind the enzyme tracers considered. In the case of the tracers P-11α-CME–HRP and P-11β-CME–HRP, high antibody binding site concentrations can not be reached even with low dilutions of anti-progesterone antiserum, as it can be seen by the data reported in Table 1. The comparison between the tracers P-3-CMO–HRP (antiserum working dilution 1 : 16 000) and P-11α-CME–HRP (antiserum working dilution 1 : 6000) shows that the higher binding site concentration observed in the case of P-3-CMO–HRP leads to a competition for the progesterone less effective than that which happens with the tracer P-11α-CME–HRP, which has quite similar affinity. On the other side, the higher concentration of antibody sites able to bind the native progesterone in the enzyme assay with the P-11α-CME–HRP leads to a less effective competition for the tracer itself, if compared with what happens in the assay with the P-3-CMO–HRP. These two effects, affecting the analyte–tracer competition in diametrically opposed ways, lead to similar sensitivities in both cases.

In the case of the assay performed with the P-11β-CME–HRP (provided with an affinity lower than that of the P-11α-CME–HRP), quite similar absorbance values can be effectively obtained through a binding site concentration slightly higher than P-11α-CME–HRP, with a consequent loss of sensitivity.

The situation can be considered completely different for the assay performed with the tracer P-11α-HS–HRP. In this case, there are less sites able to bind the native progesterone both with the same antibody binding site concentration used in the affinity measurements (antiserum dilution 1 : 32 000) and with a lower tracer concentration with respect to the experimental conditions adopted for the other competition curves. These features, though able to make easier the competition with the analyte, do not manage to balance the loss of sensitivity linked to the sharp increase of the tracer affinity, yielding as a result the decrease of the sensitivity of this assay in comparison with the previous ones considered.

Finally, the same considerations explain the increase of the mid-point value, but not of the detection limit, observed in the assay performed with the P-11α-CME–HRP as tracer with respect to the assay with the P-3-CMO–HRP. In fact, this behaviour is due to the different slopes of the competition curves, also shown in Figs. 3 and 4, that can be just related to the existence of different antibody binding sites able to bind the competitor with different affinity values.

Conclusions

The steric hindrance of the enzyme appears to have an important effect on the affinities of the enzyme tracers provided with short linking bridges. This aspect affects the selectivity too, because it can move the competition towards potential interferents. Nevertheless, the heterology of the linking bridge allows assays to be performed with a sensitivity higher than that obtainable with the homologous linking bridge. Thus, it can be thought that only a proper reduction of the steric hindrance, achievable by the use of a linking bridge longer than the hemisuccinate (the homologous tracer), together with the introduction of structure heterologies, can show an advantage in terms of sensitivity and selectivity in respect to the use of the homologous tracer.

Appendix

Calculation of binding constants from experimental saturation curves

The strength of the interaction between a binding protein P and a monovalent ligand L is numerically expressed by the equilibrium binding constant, *K*, related to the complex formation between the protein and the ligand:

$$P + L \leftrightarrows PL$$

and defined as

$$K = [PL]/([P][L])$$

where [PL], [P] and [L] represent the molar concentrations of bound ligand (or occupied binding sites), unoccupied binding sites and unbound ligand, respectively, at equilibrium.

This simple equilibrium implies a monovalent binding protein (or a multivalent binding protein with equivalent and independent binding sites). When the binding protein is immobilised on the surface of the reaction vessel, and the concentration of the bound ligand, [PL], is measured after the unbound ligand is removed by rinsing, a reliable equilibrium association binding constant measurement can be obtained but only if, during the rinsing step, all the unbound ligand is removed and all the bound ligand dissociates negligibly. Moreover, if the binding protein is immobilised on the solid phase by interaction with another binding protein (*e.g.*, an antibody to γ-globulins in this work), the reliable measurement of K also requires a negligible dissociation of the binding protein P (with or without bound ligand) from the surface.

If these conditions are satisfied, the equilibrium association binding constant for the interaction of an immobilised binding protein P to a ligand L can be written as

$$K = [PL_s]/[P_s]\,[L]$$

where the subscript *s* denotes the concentration of species immobilised on the solid phase. These concentrations are expressed as the moles of reagent immobilised on the surface (*i.e.*, the surface density of binding sites or bound ligand times the surface area) divided by the volume of the reaction mixture.

Taking into account the mass balance for binding protein and ligand, at total concentration P_0 and L_0, respectively, the equilibrium binding constant can be written as:

$$K = (P_0 - [P_s])/[P_s]\,[L] \text{ and } [P_s] = P_0/(1 + K\,[L])$$

When $L_0 >> [PL_s]$, the mass action law can be written as:

$$[PL_s] = (K\,P_0\,L_0)/(1 + K\,L_0) \quad (1)$$

The analytical signal S is proportional to the molar concentration of the immunocomplex, so:

$$S = a\,[PL_s] \text{ or } [PL_s] = S/a \quad (2)$$

where the proportionality constant, a, is the specific activity of the tracer, expressed in absorbance units per molar concentration.

At very high tracer concentrations, the analytical signal reaches a maximum value corresponding to antibody binding site saturation:

$$S_{max} = a\,P_0 = a\,B_{max} \quad (3)$$

Then, substituting the eqn. (1) with eqns. (2) and (3), an expression for the analytical signal is obtained:

$$S = (K\,S_{max}\,L_0)/(1 + K\,L_0) \text{ or } S = (S_{max}\,L_0)/(1/K + L_0) \quad (4)$$

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