J. Steroid Biochem. Molec. Biol. Vol. 43, No. 6, pp. 523–527, 1992
Printed in Great Britain.

0960-0760/92 $5.00 + 0.00

BIOTIN-HYDRAZIDE DERIVATIVES FOR THE DEVELOPMENT OF STEROID ENZYME-LINKED IMMUNOASSAYS

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(Received 6 April 1992)

Summary—We are describing the synthesis and use of biotinamidocaproyl-derivatives of 18-oxocortisol-3-carboxymethoxylamine for the development of enzyme labels using the avidin–peroxidase system for the design of enzyme-linked immunoassays (ELISA). An ELISA for 18-oxocortisol and -hydroxycorticosterone was devised which showed improved sensitivity and specificity in comparison to an RIA using a tritiated tracer. The system is easy to prepare and offers the possibility to design immunoassays when no tritiated tracer is available.

INTRODUCTION

The quantification of steroids by immunoassay have traditionally used tritium labeled steroids or iodinated derivatives of the steroids conjugated to histamine, tyramine or tyrosine methyl ester [1–4]. In the last few years, enzyme linked immunoassays (ELISA) have been gaining popularity [5–7] due in part to the ever increasing regulatory difficulties with the disposal of radioactive materials. Most corticosteroid immunoassays are performed using antibodies raised with steroid derivatives having either a hemisuccinate or a carboxymethoxylamine bridge for the conjugation to an immunogenic protein. Steroid iodinated derivatives or steroid derivatives linked to an enzyme usually use the same bridge to conjugate the steroid to the iodinatable component or to the enzyme. Unfortunately, the recognition of this bridge by the antibody is a common problem frequently resulting in assays with decreased sensitivity despite the higher specific activity of the iodinated tracer or the greater signal generated by the enzyme compared to that of tritiated steroids. There have been multiple approaches toward solving the problem of bridge recognition such as including the use of different steroid derivatives to generate the antibody and to conjugate to the iodinatable moiety [1–3, 8].

There are many cases (usually not reported) in which antibodies perform well using tritiated steroids but do not work when using iodinated tracers or ELISAs [1, 3, 8]. The biotin–avidin system has been extensively used for the detection of antibodies, RNA, DNA, etc. and recently has been reported to be useful in the ELISA for estradiol [9]. We are reporting our studies using biotinylated derivatives of 18-oxocortisol (18-oxoF) and 18-hydroxycorticosterone (18-OH-B) as labels for ELISA. We are also reporting the effect of conjugation and length of the bridge on the performance of the ELISAs.

EXPERIMENTAL

Reagents for buffers, avidin-peroxidase and streptavidin-peroxidase were purchased from Sigma Chemical Co. (St Louis, MO). The steroids 18-oxoF, 18-hydroxycortisol, 18-hydroxydeoxycorticosterone and 18-OH-B were synthesized according to established procedures [10–12]. The tritiated steroid [1,2,^{3}H]18-oxoF (sp. act. 55 Ci/mmol) was prepared by catalytic tritiation using Wilkinson catalyst from the 1,2-diene precursor and purified by HPLC [11]. Biotin-ϵ-aminocaproylhydrazide (biotin-X-H) and biotin-(ϵ-aminocaproyl)$_2$hydrazide (biotin-X-X-H) were purchased from Clontech Corporation (Palo Alto, CA). Antibodies against 18-oxoF-3-carboxymethoxylamine (CMO) conjugated to urease and 18-OH-B-3-CMO-bovine serum albumin were raised in New Zealand white rabbits.

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The 3-carboxymethyloxime derivatives of 18-oxocortisol (18-oxoF-3-CMO) and 18-OH-B were prepared as described [2, 11]. 18-OxoF-3-biotin-ϵ-aminocaproylhydrazone was prepared by a procedure as described previously [2]. The conjugates of 18-oxoF-3-CMO and 18-OH-B-3-CMO with biotin-X-H and 18-oxoF-CMO with biotin-X-X-H were prepared by the mixed anhydride technique [2] and purified by TLC using the system chloroform–acetonitrile–methanol–*N*-methylmorpholine (76:20:15:0.1 by vol) on a 2 mm preparative Silica Gel G254 plate. The peaks corresponding to the product were eluted with methanol–acetonitrile (1:1). In the case of the 18-OH-B derivative, the solution contained 0.05% *N*-methylmorpholine to prevent dimerization or ether formation. 18-OxoF-3-CMO was also conjugated directly to per-oxidase or to cationized bovine serum albumin.

ELISA procedure

Microtiter plates were coated overnight at 4°C with an affinity purified goat anti-rabbit IgG-Fc antibody (Jackson ImmunoResearch) at a concentration of 1 μg/200 μl per well in a carbonate buffer 0.1 M, pH 9.4. The plates were washed 3 times with phosphate buffered saline (PBS) containing 0.1% Tween-80 and were either used immediately or stored at 4°C with plain PBS. The assay was performed by adding the standard or samples in 100 μl followed by 100 μl of the steroid antibody. These were mixed well and then 100 μl of the biotin derivative was added. After mixing, the plate was incubated overnight, then washed 5 times with the washing buffer. Avidin-peroxidase (300 μl) was then added and the plate incubated for 45 min at room temperature followed by washing and incubating with substrate solution [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS) 0.4 mM plus urea peroxide 1 mM in citrate buffer 0.1 M, pH 4.0] for 1 h. The plates were then read in a Dynatech plate reader using a 410 μ filter. Thorough washing was found to be of crucial importance in improving reproducibility, especially after the addition of avidin-peroxidase. This required filling the plate with buffer after the third washing, taking it out of the automatic washer, and shaking it in a mixer (Fisher) for 1 min before continuing 1 additional washing.

Additional studies using 18-oxoF-3-CMO-peroxidase conjugate were done essentially as above, as well as by a method involving the immobilization of the antigen, 18-oxoF-3-CMO-cationized bovine serum albumin, to the plates as described previously [6, 7]. The bound antibody was detected using a goat anti-rabbit IgG (Fc + FAB) affinity isolated peroxidase conjugate.

Crossreactivities

To assess crossreactivity, increasing concentrations of competing steroids were incubated. The concentration which produced an o.d. which was 50% of the zero in the standard curve was used for the calculation of the relative crossreactivity. The results were expressed as percentage crossreactivity.

RESULTS

The indirect ELISA procedure used immobilized 18-oxoF-3-CMO-cationized bovine serum albumin worked very poorly. Since large quantities of 18-oxoF were required to produce a displacement of the antibody from the immobilized hapten, it was evaluated no further. Direct ELISA using 18-oxoF-3-CMO-peroxidase required a similar concentration of antibody to that needed for the biotinylated tracers, but large quantities of 18-oxoF were also required to displace the peroxidase label. Thus, this type of assay was evaluated no further. The titers of the antibody for both assays were 10 times greater than for assays using the tritiated tracers.

The biotinylated hydrazide labels (Fig. 1) were used in an attempt to mitigate the side-chain recognition problem. Displacement of the bound tracer 18-oxoF demonstrated that the 3-CMO-X- and -X-X- tracers were similar to that in which the direct hydrazone of 18-oxoF was used, but the crossreactivities were lower with the 3-CMO-biotin derivatives. There were no significant differences between the two tracers differing in the length of the bridge between steroid and biotin (X vs XX). All subsequent studies were done with the 3-CMO-X-biotin hydrazide derivatives.

The sensitivity of the standard dose–response curve for 18-oxoF, defined as 3 standard deviations from the signal given by the zero blank, was better for the ELISA than for the radioimmunoassay (RIA) (Table 1). The sensitivities using the various biotin derivatives were very similar. The crossreactivities using the biotin derivatives were less than by RIA (Table 1) and all the steroids tested showed striking less crossreactivity. Antibodies from 2 bleedings from 2

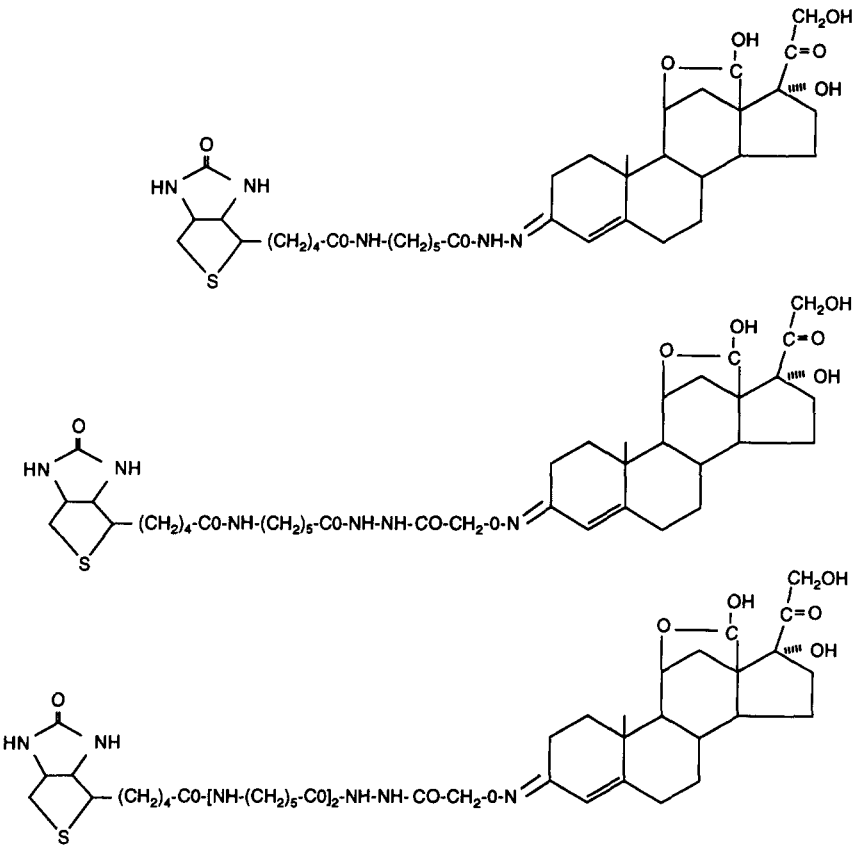

Fig. 1. The structure of 18-oxoF-3-biotinamidecaproylhydrazone, 18-oxoF-3-CMO-biotinamidecaproylhydrazide and 18-oxoF-3-CMO-(biotinamidecaproyl)$_2$hydrazide are shown.

rabbits immunized against 18-OH-B were also evaluated since the commercial availability of tritiated tracer has recently ceased. There were wide differences between the 2 animals and between the 2 bleedings. The sensitivity of these antibodies was 2 pg/tube (5.5 fmol/tube) for both the ELISA and RIA.

Two technical details were found to be very important. First, was the source of avidin-peroxidase. Several batches exhibited a very high nonspecific binding of the avidin-peroxidase to the 96 well plates. This was never solved even by using a variety of proteins and detergents in the buffer. Second, as mentioned in the Experimental section, we found that it was crucial to shake the plates at least once during the wash cycle after the addition of the avidin-peroxidase. This step significantly reduced the nonspecific binding and the variability between wells.

DISCUSSION

Tritium labeled steroids were initially used for the determination of steroids in RIA. The most common specific activities of the various tritiated derivatives for steroids have been between 40 to 150 Ci/mmol. The synthesis of less

Table 1. Characteristics and crossreactivities of an antibody against 18-oxoF

Steroid	RIA	18-OxoF-B	18-OxoF-3-CMO-X-B	18-OxoF-3-CMO-X-X-B
18-OxoF	100%	100%	100%	100%
Aldosterone	34.8	0.33	0.3	1.08
Cortisol	11.4	0.33	0.56	0.42
18-Hydroxycortisol	0.42	0.013	0.02	0.07
Corticosterone	2.86	0.005	0.04	0.06
18-OH-B	0.3	0.003	<0.001	<0.001
11-Deoxycortisol	4.4		0.35	
Deoxycorticosterone	0.44		0.02	
Progesterone	0.8		0.04	
19-Hydroxydeoxycorticosterone	0.09		0.03	
18-Hydroxydeoxycorticosterone	0.09		0.01	
Sensitivity	67 fmol	9 fmol	17 fmol	13 fmol

Table 2. Crossreactivities for antibodies against 18-OH-B-3-CMO

	Antibody number (rabbit code)			
Steroid	1001 (21)	1002 (22)	1023 (21)	1024 (22)
18-OH-B	100%	100	100	100
18-Hydroxydeoxycorticosterone	3.6	30	3.46	20
18-Hydroxycortisol	42.3	0.24	5.63	0.17
Aldosterone	0.036	0.033	0.01	0.016
Corticosterone	3.6	0.029	0.21	0.02
Cortisol	0.66	<0.001	0.038	<0.001
Deoxycorticosterone	0.013	<0.001	<0.001	<0.001
11-Deoxycortisol	<0.001	<0.001	<0.001	<0.001
Progesterone	<0.001	<0.001	<0.001	<0.001
18-OxoF	<0.001	<0.001	<0.001	<0.001
19-Hydroxydeoxycorticosterone	<0.001	<0.001	<0.001	<0.001

common tritiated steroids not available commercially has been either beyond the technical expertise of most laboratories and/or prohibitively expensive. Iodinated derivatives of steroids used herein were prepared in a way similar to the immunogens. The steroids were conjugated to the phenolic amine such as tyramine or tyrosine methyl ester, or an imidazole such as histamine, then iodinated with ^{125}I. They became popular in the early 70s because of the ease of their preparation and because ^{125}I has a specific activity 88 times that of tritium. The addition of an iodine yielded a practical specific activity which was approx. 40 times greater than the most common tritiated tracers [1–3, 8]. The expected increase in sensitivity with a tracer of higher specific activity occurred only in very rare instances [3]. In many cases, unusable conjugates were generated for antibodies which recognized the bridge between the steroid and carrier protein or iodinatable compound [1–3]. Different chemical bridges or iodinated derivatives conjugated at a site on the steroid different from that used to generate the antibody were tried as partial solutions. This yielded good results in some cases [2, 3, 8], but no uniform solution was available for all antibodies.

Direct ELISA (enzyme-linked immunoassays in which the label was the steroid conjugated to an enzyme) [5] or indirect ELISA (the steroid is conjugated to a protein different from the one used for antibody generation and then immobilized to a solid support) were similarly used with good results [6, 7]. In the indirect ELISA, the antibody binding was detected by using an anti-first antibody enzyme label. However, in a significant number of cases, this approach to the development of ELISAs failed (as in the case of 18-oxoF using an 18-oxoF-3-CMO-peroxidase label or an 18-oxoF-3-CMO-peroxidase label or an 18-oxoF-cationized serum albumin derivative). In the case presented here, the antibody binded to the tracer, but the displacement by the native steroid was so poor that the assay was unusable. Our difficulties with the production of a few other antibodies is probably not unique; most failures to produce usable antibodies never get published. An alternative approach has been to use the biotin–avidin technology. An assay for estradiol has been published which claimed good results [9]. We have reported here that the use of a biotin-spacer hydrazide derivative (ϵ-aminocaproyl or 2 ϵ-aminocaproyl chains) worked well in one case, that of 18-oxoF, where a more traditional enzyme labels did not work. The sensitivity of the assay as well as the crossreactivities were shown to be clearly superior to that of an RIA with tritium labeled 18-oxoF. It is possible that the difference between the amide conjugation to the immunizing protein and the hydrazide conjugation to the biotin were sufficiently different that the recognition of the bridge was decreased and adequate sensitivity was obtained. The use of a 3-hydrazone derivative iodinated tracer synthesized in a similar fashion as those described herein did not offer any advantages and the sensitivities were worse than tritium [2]. In the present case with the biotin-hydrazone, the sensitivities were similar to the CMO-biotin derivatives, but the crossreactivities were worse suggesting that only some clones within the polyclonal antibody were recognized. No significant differences were found between the use of a derivative with a single ϵ-aminocaproyl or 2 ϵ-aminocaproyl long chains, however the single chain derivative is significantly less expensive than the 2 ϵ-aminocaproyl derivatives. Some avidin-peroxidase derivatives were found to give unacceptable levels of nonspecific binding with no clear explanation. Streptavidin derivative may also be used to minimize nonspecific binding, but the derivative is significantly more expensive than avidin. We have now used this biotin–avidin system with 6 other steroids with uniform success (unpublished observations).

Acknowledgements—These studies were supported by Medical Research Funds from the Department of Veterans Affairs and NIH Grants HL 27737 and HL 27525.

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