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RAPID PURIFICATION OF ANTISTEROID ANTIBODIES BY HIGH-PERFORMANCE LIQUID AFFINITY CHROMATOGRAPHY

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SUMMARY

An adsorbent for the high-performance affinity chromatography of antisteroid antibodies was prepared, based on a commercial pre-packed column. The column contained activated microparticulate silica beads bearing epoxide functions, on which the steroid dexamethasone was covalently linked. The column was used successfully for the rapid and complete isolation of several hundred microgram amounts of specific antidexamethasone antibodies from rabbit antisera. The practical aspects of the purification procedure, especially the optimization of the washing and of the elution steps, are detailed. Despite non-biospecific elution with 20% acetonitrile in an acidic buffer, the purification yield was very satisfactory and the biological activity of the purified immunoglobulins appeared excellent.

INTRODUCTION

Since the initial report of Ohlson *et al.*[1], who were the first to use HPLC-grade silica beads for preparing bioaffinity matrixes and thus to initiate high-performance liquid affinity chromatography (HPLAC), increasing attention has been paid to this new technique, which combines the high-speed characteristics of high-performance liquid chromatography (HPLC) with the selectivity of biospecific interactions. Numerous papers have appeared in the last few years on improving the derivatization of silica[2], discussing both the practical[3–7] and theoretical[7–9] aspects of HPLAC and describing many separations[3–27]. However, most of the work involved analytical applications and only a few dealt with the particular problems encountered during preparative work[15,24,25]. Moreover, owing to the lack of ready-to-use activated silica beads, the spread of HPLAC was restricted to laboratories with expertise in silica chemistry. Thus, the relative scarcity of reports on preparative HPLAC applications from biochemical laboratories with the need to solve specific purification problems was not unexpected. However, the very recent appearance of various commercial pre-activated HPLAC supports will probably change the situation. In this paper we report the use of a commercial macroporous silica matrix bearing activated epoxide groups for the covalent immobilization of the steroid dexamethasone and the appli-

cation of the stationary phase obtained to the complete purification of rabbit poly-
clonal antidexamethasone antibodies.

EXPERIMENTAL

Materials

[1,2(n)-^{3}H]Dexamethasone, 40 Ci mmol^{-1}, was obtained from Amersham In-
ternational (Amersham, U.K.) and unlabelled dexamethasone from Roussel-Uclaf
(Romainville, France). The Ultraffinity-EP column (50 × 4.6 mm I.D.) and the Ul-
traffinity-EP column capacity kit were obtained from Beckman (Berkeley, CA,
U.S.A.). Ultrogel ACA 202 was purchased from Industrie Biologique Francaise
(Villeneuve-la-Garenne, France). All others chemicals were of analytical-reagent
grade.

Antisera

Antisera to dexamethasone were raised in New Zealand rabbits by immuni-
zation with dexamethasone-21 hemisuccinate–bovine serum albumin conjugates us-
ing conventional techniques[28,29]. Immunoglobulins were precipitated by ammonium
sulphate, redissolved in phosphate-buffered saline (10 mM sodium phosphate, 150
mM sodium chloride, pH 7.0) and submitted to gel filtration on an Ultrogel ACA
202 column equilibrated in the same buffer. The excluded fractions containing the
immunoglobulins were pooled and used for further purification by HPLAC.

Derivatization of the column

A preliminary derivatization experiment was performed using the Ultraffini-
ty-EP column capacity kit. Eight micromoles of [^{3}H]dexamethasone (specific activity
2 mCi mmol^{-1}) were dissolved in 1.6 ml of acetonitrile–water (96:4) and added to
the vial containing the activated silica (106 mg), which was then placed on a rotating
shaker for 40 h at 37°C. After careful washing with acetonitrile and tetrahydrofuran,
the amount of silica-bound steroid was assayed by scintillation counting of an aliquot
of the derivatized gel. Deactivation of the remaining epoxy functions was obtained
by incubation of the gel with 10% glycerol in 1 M phosphate buffer (pH 7.0) as
recommended by the manufacturer.

The Ultraffinity-EP column was connected to a Beckman Model 1108 sol-
vent-delivery system. An 11.5-μmol amount of [^{3}H]dexamethasone (specific activity
1.75 mCi mmol^{-1}) was dissolved in 15 ml of acetonitrile–water (98:2), the solution
was degassed by sonication and pumped through the column at a 0.2 ml min^{-1}
(closed circuit) as recommended by the manufacturer. The column was immersed in
a water-bath thermostated at 45°C and the derivatization was carried on for 40 h.
The column was then washed with 70 ml of acetonitrile at room temperature. The
amount of steroid bound to the column was obtained from differential assay of the
radioactivity in the coupling solution before and after derivatization. The column
was then equilibrated in an aqueous solvent after several intermediary washing steps
with acetonitrile–water mixtures of increasing water content. The remaining epoxy
functions were deactivated by pumping 20 ml of 0.5 M mercaptoethanol in 0.1 M
potassium phosphate (pH 6.5) overnight (closed circuit) at 0.23 ml min^{-1}. The col-
umn was then washed with water containing 0.02% sodium azide.

Affinity chromatography procedure

Apparatus. The HPLAC system consisted of a Beckman 1108 solvent-delivery system, a Waters U6K injector equipped with a 10-ml sample loop, an Altex 210A sample injection valve used as a column-switching valve and fitted with the Ultraffinity-EP column in place of the sample loop, a Beckman Model 160 absorbance detector connected to a recorder and a programmable Gilson Model 201 fraction collector. The detection wavelength was 280 nm. The Altex 210A valve was used to direct the eluent either to the column (in the "inject" position) or to a column by-pass (in the "load" position).

Adsorption. The γ-globulin samples were filtered on a 0.22 μm Millex GS filter (Millipore) before use and injected at room temperatrure and at a definite flow-rate (0.2–1.0 ml) in the column previously equilibrated in phosphate-buffered saline. The absorbance at 280 nm was recorded and 0.8-ml fractions were collected and assayed for [³H]dexamethasone specific binding activity. As soon as the absorbance began to decrease, the flow-rate was increased to 2 ml min^{-1} and washing with phosphate-buffered saline was continued until a complete return to the baseline was achieved.

Elution. The column was rapidly isolated from the solvent line by switching the Altex valve, and was immersed in a tank filled with ice. The solvent line and the detector were then rapidly equilibrated at 0°C with the eluting buffer, 6.7 mM sodium citrate–6.7 mM sodium phosphate–11.4 mM sodium borate–20% acetonitrile (pH 3.1). Once the new baseline had been obtained the flow-rate was reduced to 0.2 ml min^{-1} and the column was switched on the line. Fractions of 0.4 ml were collected in tubes already containing 0.1 ml of 1 M potassium phosphate buffer (pH 8.5). In some instances a stopped-flow elution was performed, which consisted in diverting the flow around the column as soon as the phosphate-buffered saline was replaced in the column by the acidic strong eluting buffer. Then stopped-flow elution was continued in the column and after a few minutes the column was switched on the solvent line again and the eluate was collected.

Antibody detection assay

Rabbit antisera, ammonium sulphate fractionated immunoglobulins and affinity chromatography effluents were submited to the appropriate dilution with phosphate-buffered saline and 0.4-ml aliquots were incubated in duplicate at 0–4°C with 30 nM [³H]dexamethasone. After 17 h the bound radioactivity was determined in duplicate by charcoal adsorption assay[30]. The non-specific binding was measured by incubating parallel samples in the presence of a 1000-fold excess of non-radioactive dexamethasone.

Miscellaneous

The Coomassie blue assay of Bradford[31] modified according to Read and Northcote[32] was used for protein determination, using purified rabbit immunoglobulins G as standard. Radioactivity was measured in an Intertechnique SL 4000 liquid spectrometer, using Aqualyte (Baker Chemicals, Deventer, The Netherlands) as scintillation cocktail (35% tritium efficiency).
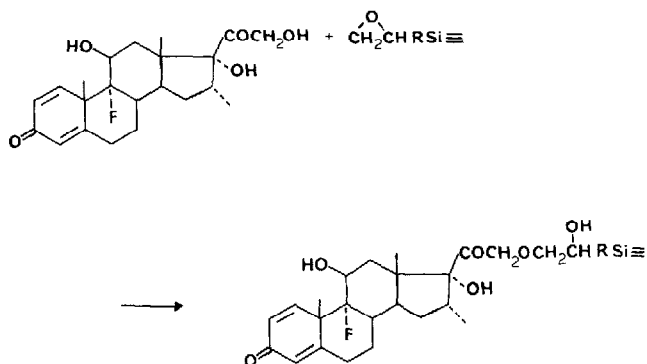
Fig. 1. Covalent binding of dexamethasone to epoxy-silica beads.

RESULTS AND DISCUSSION

Coupling of dexamethasone to the epoxy silica

The Ultraffinity-EP column and kit contained an already activated silica, pore size 300 Å and particle diameter 10 μm, bearing epoxide functions. Dexamethasone appeared to react fairly easily in an organic solvent with this activated silica to yield a covalent linkage, probably by reaction of the epoxide with the primary 21-OH group of the steroid, the reactivity of which was probably increased by the adjacent 20-keto group (Fig. 1). However, we observed a striking difference in the coupling efficiencies of dexamethasone to the ultraffinity-EP column capacity kit and to the ultraffinity-EP column itself. With the kit the silica-bound dexamethasone was only 0.21 μmol per gram of silica, whereas it reached 4.1 μmole with the column. Both the coupling procedures were performed on the same activated silica, bearing 69.1 μmol of titratable epoxy groups according to the manufacturer, using the same solvent and coupling time at only slightly different temperatures. The much higher derivatization yield obtained with the column procedure probably resulted from the fact that, using the closed circuit process, the coupling solution was repeatedly forced through the compacted silica and penetrated all the pores of the beads, whereas this certainly occurred to a far lesser extent using the batch procedure. To obtain improved results with the latter process would probably require working at higher temperature, with sonication of the beads during the coupling step. On the other hand, the ligand substitution level obtained on the column was in the micromolar range already reported by others[1,12,13] and was considered satisfactory for our purpose.

Purification of antidexamethasone antibodies on the dexamethasone column

The overall results of six successive purification experiments performed on the column starting from various dexamethasone antisera samples obtained from the same rabbit are summarized in Table I. The column performances may be discused with respect to both the adsorption and elution steps.

Adsorption step. Adsorption appeared to be very rapid, as shown in experiment 1 where 89% of the specific immunoglobulins present in the sample were retained on the column injected at a 1 ml min^{-1} flow-rate, *i.e.*, a contact time with the stationary

phase of only 30 s (the 50 × 4.6 mm I.D. column corresponded to a total solvent diffusion volume of about 0.5 ml). This result was in excellent agreement with pre-liminary experiments performed with the column capacity kit (data not shown). The maximal capacity of the column was tested by injecting large sample amounts in order to saturate the affinity matrix in experiments 2 and 3. This maximal capacity was in the range 0.7–1.0 mg of specific immunoglobulins, *i.e.*, 4–7 nmol, for a column of total volume 0.83 ml, or 4.8–8.4 nmol per ml of gel. This capacity was very similar to that obtained after a longer adsorption time on a conventional "soft" gel (of the agarose type) derivatized with dexamethasone in a similar fashion (data not shown).

The rapidity of the antibody binding on the dexamethasone–silica is illustrated in Fig. 2, which shows the residual specific antidexamethasone activity found in the effluent of the affinity column during the injection in experiment 3. It can be seen that the first fraction was almost totally cleared of specific antidexamethasone anti-bodies and that the column then became rapidly saturated, as the subsequent effluent displayed the same antidexamethasone activity as the injected sample. Here, the in-jection could have been stopped from the fourth fraction and a sample load reduced to 3 ml would have yielded the same gel saturation as the 6 ml used. Moreover, the column washing procedure used between two sucessive injections appeared to be of utmost importance in order to obtain reproductible purification. In our first attempts we used only a simple washing with 6 *M* urea, a procedure that appeared almost satisfactory during our routine purification procedure using a conventional agarose
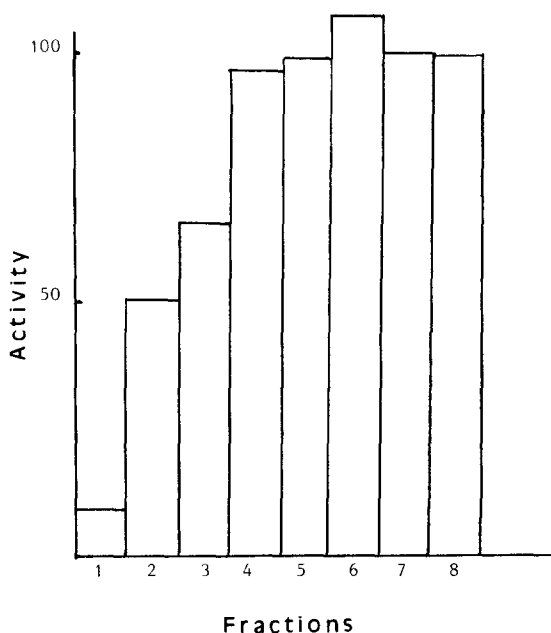

Fig. 2. Residual dexamethasone specific binding activity in the effluent of the dexamethasone derivatized column (50 × 4.6 mm I.D.) during the injection of a 6-ml sample of rabbit antidexamethasone antibodies. Flow-rate, 0.2 ml min^{-1}. Fractions of 0.8 ml were collected and assayed for [^{3}H]dexamethasone binding. Results are expressed as a percentage of the binding activity of the injected sample.

TABLE I

COMPARATIVE DATA OF SIX SUCCESSIVE PURIFICATION EXPERIMENTS PERFORMED ON THE SAME 50 × 4.6 mm I.D. COLUMN CONTAINING DEXAMETHASONE DERIVATIZED ULTRAFFINITY-EP SILICA BEADS

Antidexamethasone antiserum pre-fractionated by ammonium sulphate precipitation was loaded on the column. Between two successive experiments the column was washed with 6 M urea, except after experiment 5, where an additional wash with 0.1 M acetic acid containing 2 M NaCl and then with formic acid and propanol was used.

Step	Parameter	Experiment					
		1	2	3	4	5	6
Adsorption	Amount of specific immunoglobulins present in the sample (μg)	275	42 768	2864	1061	1261	4303
	Sample volume (ml)	4	27	6	3	5.8	10
	Injection flow-rate (ml min^{-1})	1	1	0.2	0.5	0.4	0.5
	Amount of specific immunoglobulins adsorbed (μg)	225	1029	686	281	230	673
	Adsorption yield (% of injected specific material)	89	2.4	24	26	18.2	15.6
Elution	Total amount of active specific immunoglobulins eluted (μg)	71	324	266	40	70	202
	Elution yield (% of adsorbed Ig)	32	31.5	39	14	30	30
	% of the total eluted immunoglobulins found in the main 0.5-ml eluate fraction	65.4	49.7	48.3	51	52	37
	Apparent homogeneity of the purifed immunoglobulins (%)	51	27	86	–	–	–

TABLE II

QUANTITATIVE DATA CONCERNING THE ELUTION OF EXPERIMENT 3 (SEE TABLE I), DETAILED IN FIG. 2

The concentration of the active specific antidexamethasone immunoglobulins was calculated from the [^{3}H]dexamethasone binding activity assayed in saturating conditions. Total protein content was assayed by the Coomassie blue method.

Fraction	Volume (ml)	Protein (μg ml^{-1})	Binding activity (cpm · 10^{-6} ml^{-1})	Active specific immunoglobulins		Eluted activity (% of the total)	Specific activity (cpm · 10^{-6} per μg of protein)	Apparent homogeneity (%)	Purification (fold)
				μg ml^{-1}	Total μg				
Starting serum extract	6	5200	229.6	477	2864	—	0.044	9.2	1
Affinity eluate fractions:									
1–3	1.5	0	0	0	0	0	0	0	0
4	0.5	56	21.5	45.4	22.7	8.3	0.384	80	8.7
5	0.5	82	34.8	73.2	36.6	13.6	0.424	88	9.6
6	0.5	92	42.0	88.3	44.2	16.4	0.456	96	10.4
7	0.5	302	123.6	260.0	130.0	48.3	0.409	86	9.3
8	0.5	82	29.3	61.6	30.8	11.4	0.357	75	8.1
9	0.5	29	5.2	10.9	5.5	2.0	0.179	38	4.1
Total					270	100			

gel. However, with the silica matrix this washing procedure was insufficient, especially after the massive loads applied in experiments 2 and 3, and this could explain the relative decrease in performance in terms of column capacity observed during experiments 4 and 5, where, according to the sample load, we expected to retain a larger amount of immunoglobulins on the column. However, an additional wash of the column using 0.1 *M* acetic acid containing 2 *M* sodium chloride and then 0.1 *M* propionic acid–propanol allowed the column capacity to be restored to its previous value (experiment 6). In our opinion, the column was probably contaminated by hydrophobic components adsorbed on the hydrophobic steroid stationary phase. The relatively high derivatization level of the matrix, in comparison with the agarose gel, reinforced this hypothesis.

Elution. Non-biospecific elution was obtained with an almost aceptable yield (30–39%), owing to the drastic physico-chemical conditions used (acidic buffer of pH 3.1, containing 20% acetonitrile). The severity of these conditions was required because of the high affinity for dexamethasone displayed by the γ-globulins to be purified. The same buffer supplemented with acetonitrile has already been used with success by others for the purification by affinity chromatography of anticortisol antibodies[33]. The elution yield obtained appeared to be reproducible, except in experiment 4 where its low value could perhaps be ascribed to the inadequate column washing which had already hindered the adsorption step in this trial. In each exper-
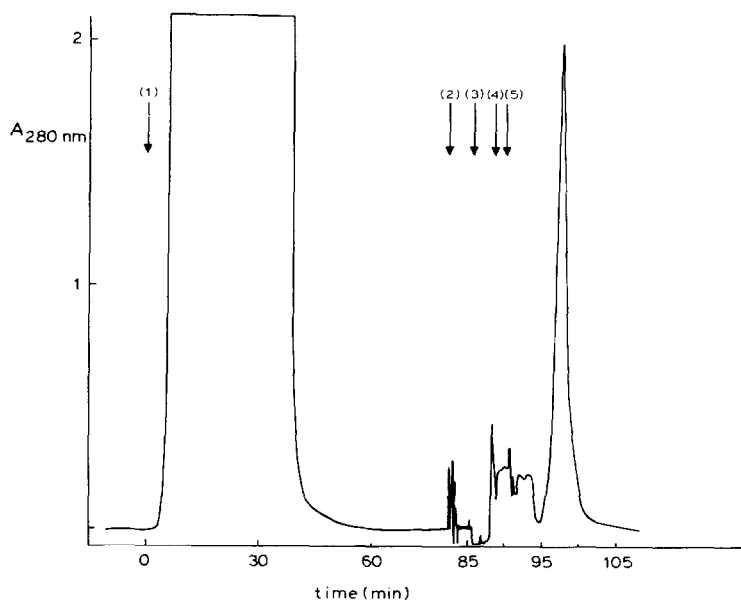

Fig. 3. Absorbance at 280 nm recorded during the purification experiment 3 (see Table I). A 6-ml sample containing the antidexamethasone immunoglobulins to be purified was injected on to the column at 0.2 ml min^{-1} and 20°C (1). The column was washed with phosphate buffer saline at 1 ml min^{-1}, then switched off the solvent line to a stopped-flow position (2) and immersed in an ice-bath, whereas the solvent line was rapidly equilibrated with the cold acidic elution buffer at 4 ml min^{-1}. The flow-rate was then reduced to 0.2 ml min^{-1} and the column switched to the line (3), 0.4-ml fractions being collected. After 4 min the column was switched to the stopped-flow position (4) for 2 min and then switched to the line again (5).
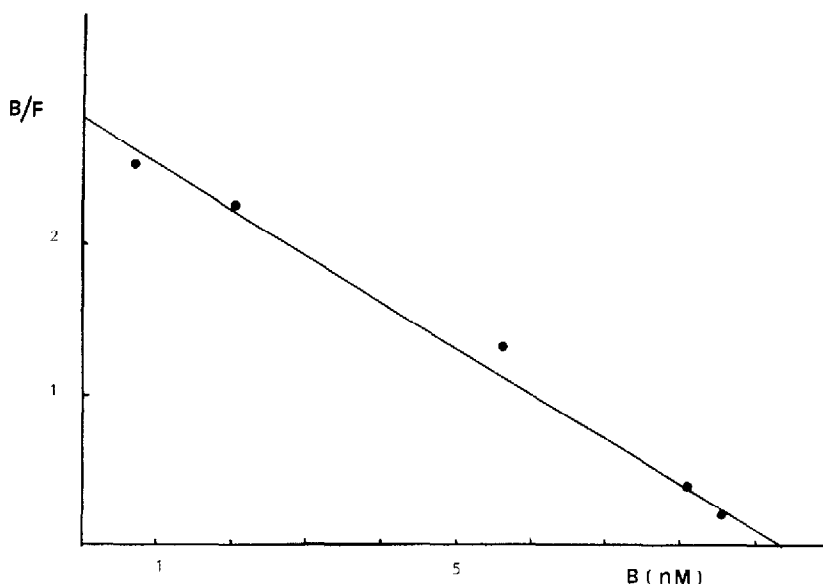
Fig. 4. Scatchard plot obtained by incubating the affinity-purified antidexamethasone immunoglobulins (from experiment 2 in Table I) with various concentrations of [³H]dexamethasone in the nanomolar range for 16 h at 0°C. B, Concentration of antibody specifically bound [³H]dexamethasone; F, concentration of the free [³H]dexamethasone. Separation of bound from free steroid was performed by charcoal assay.

iment it could be assessed that the missing specific antibodies were either still retained on the column or already eluted, but in a denatured form that could not be restored to an active form by the rapid readjustment of the pH of the collected fractions. The assay of the protein content of each fraction and the determination of its specific activity helped to clarify the choice between these two hypotheses.

Indeed, when calculated on the basis of the antidexamethasone specific activity (with the assumption that we were dealing only with immunoglobulin G displaying a molecular weight of 150 kD and able to bind two molecules of dexamethasone per molecule of antibody), the level of homogeneity of the purified immunoglobulins was very satisfactory, as in experiment 3 the eluate was made of almost pure active anti-dexamethasone antibodies (86% apparent homogeneity). The high purity of the antibodies obtained is significant, and appeared impressive with regard to the non-specific mode of elution used here. Table II gives the detailed results of this experiment, the absorbance line at 280 nm of which is depicted in Fig. 3. We used chromatographic conditions similar to those used by Muller and Carr[7], using a configuration that allowed us to switch the column to the solvent line or to isolate it in a stopped-flow position. This configuration appeared very useful in saving time and allowing, if required, stopped-flow elution. The elution peak was obtained rapidly in a concentrated form (260 µg of specific immunoglobulins per ml of eluate in fraction 7), clearly more concentrated (4–6-fold more) than that usually obtained with a conventional agarose gel (data not shown). Generally as high as 50% of all the eluted material was found in a single 0.5-ml fraction (see Table I). The more efficient diffusion of the eluting solution through the pores of a "high-performance" stationary
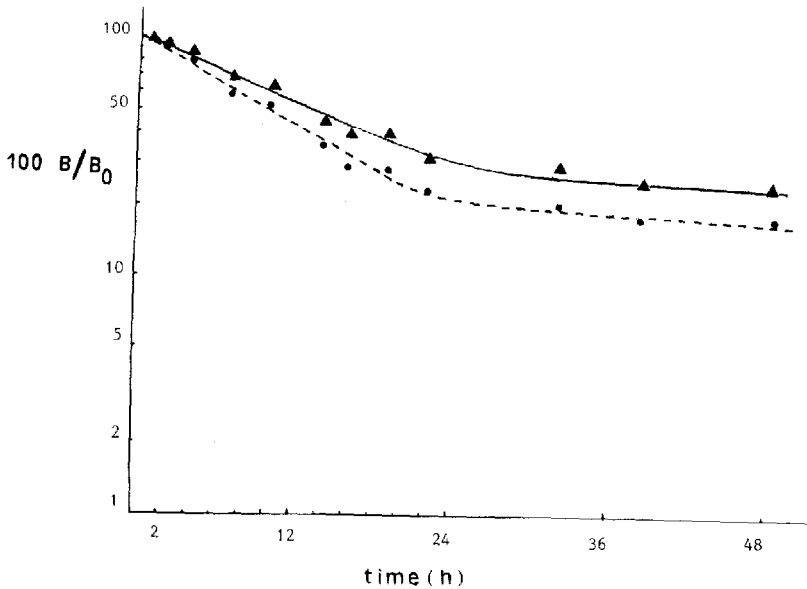
Fig. 5. Dissociation kinetics of both crude antidexamethasone antiserum (●) and of the affinity-purified γ-globulins (▲). Each sample was pre-incubated with 30 nM [³H]dexamethasone and then supplemented with a 500-fold excess of unlabelled dexamethasone. The evolution of the [³H]dexamethasone binding activity B was followed by periodic charcoal assay and expressed as a percentage of the initial activity B_0 assayed just before the addition of the unlabelled steroid.

phase made of rigid beads of small diameter probably explains this result. Moreover, the HPLC hardware used in the system precluded any post-column dilution effects, which were more difficult to avoid when handling conventional agarose gels and columns devices.

Characterization of the affinity-purified antidexamethasone antibodies

A crucial point to be checked after a purification by affinity chromatography using a drastic non-specific elution procedure is the biological quality of the eluted material. We therefore measured the binding parameters of the purified antibodies towards tritiated dexamethasone and these parameters were then compared with those of the starting antisera. The dissociation constant assayed at equilibrium using a Scatchard plot (Fig. 4) was roughly the same before and after purification (3.0 nM *versus* 3.5 nM). Moreover, the dissociation rate constant measured by isotopic dilution was significantly lower for the purified immunoglobulins than for the starting antiserum, corresponding to a half-life 1.4-fold longer in the former instance (Fig. 5). Hence the affinity chromatography step seemed to have allowed isolation from various polyclonal antidexamethasone immunoglobulins present in the plasma of the rabbit, if not the most avid then at least those forming the most stable complexes with the steroid. This constitutes a very interesting result and was one of the aims of the study.

CONCLUSIONS

Dexamethasone has been shown to bind covalently to commercial activated silica beads bearing epoxide functions. The resultant chromatographic adsorbent appeared to be suitable for the rapid purification of antidexamethasone antibodies. The yield and the level of purification were excellent. HPLAC compared very favorably with conventional affinity chromatography on agarose beads and allowed a more rapid purification of small amounts of purified immunoglobulins, obtained in a very small volume. However, the purification factor required for complete purification in the application reported here was relatively modest (11-fold for the affinity step). It would be of great interest to see if HPLAC would be a satisfactory alternative in more difficult purification problems such as that of cellular steroid receptors, for which we recently described an improved purification by affinity chromatography on a dexamethasone-bearing agarose gel[34].

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