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Note

Dihydrotestosterone binding capacity of androgen-binding protein in tissue extract using high-performance size exclusion chromatography

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Androgen-binding protein (ABP) was first discovered in rat epididymis. This glycoprotein is synthesized by Sertoli cells from rat testis. Most of it is released into the lumen of the tubules. The function and the fate of ABP in seminiferous tubules and in epididymis remain uncertain. We have recently purified this protein from rat testis and demonstrated that it is internalized by the principal cells of the caput of epididymis, using electron microscopy radioautography [1]. The purification as well as the study of the metabolism of ABP requires a reliable method for determination of its binding capacity in biological fluids and tissue extracts. Various methods have been used to quantitate ABP. The electrophoretic methods of Corvol et al. [2] and Ritzen et al. [3] determine the dihydrotestosterone (DHT) binding capacity of ABP from β-counting of

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slices corresponding to bound and free [^{3}H]DHT. Other methods, such as radioimmunoassay and radioisotope dilution assay with dextran-coated charcoal, are less reliable [4,5]. Recently, another method was described which uses ion exchange on mini-columns [6].

This paper describes the use of high-performance liquid chromatography (HPLC) to determine the [^{3}H]DHT binding capacity of ABP from tissular extracts.

EXPERIMENTAL

Chemicals and reagents

5α-Dihydro-[1,2,5,6,7-^{3}H]testosterone (180 Ci/nmol) was obtained from the Radiochemical Centre (Amersham, U.K.) and Δ_6-testosterone (17Δ-4,6-hydroxy-[1,2-^{3}H](N)-androstanedien-3-one) (48.6 Ci/mmol) was obtained from NEN Research Products (Boston, MA, U.S.A.).

Scintran® (for scintillation counting) was from BDH (Poole, U.K.) and glycerol and guanidine hydrochloride from Merck (Darmstadt, F.R.G.). All other reagents were obtained as described previously [7].

Preparation of tissue extracts

Testes were obtained from 250-g adult Wistar rats. Organs were decapsulated and homogenized in ice-cold 20 m*M* Tris–HCl buffer (pH 7.4) containing 10% (v/v) glycerol (TG buffer) with a tissue ratio of 1:2, using a Polytron homogenizer. The suspension was centrifuged at 105 000 *g* for 1 h at 4°C. ABP was precipitated from the supernatant by slowly adding ammonium sulphate to a final concentration of 0.36 g/ml. The mixture was incubated overnight at 4°C under rotative agitation, and it was centrifuged at 17 000 *g* for 2 h at 4°C. The pellet was dissolved in buffer and centrifuged again. The supernatant was used as a testis extract for the determination of the DHT binding capacity of ABP. One part of this sample was dialysed against 20 volumes of TG buffer. The other part was dialysed in an additional step, against 5 *M* guanidine hydrochloride, in order to remove the endogenous androgens, which could saturate ABP in the tissue extract, according to a procedure previously described [8]. The sample was then renaturated against 20 volumes of TG buffer.

High-performance size exclusion chromatography

The injected samples were prepared by incubating 400 μl of tissue extract with [^{3}H]DHT in a total volume of 500 μl of TG buffer for 18 h at 4°C under rotative agitation. The experiment was repeated under the same conditions with the amount of [^{3}H]DHT increased from 11 to 176 pmol. The binding capacity of ABP and its dissociation constant (K_D) for [^{3}H]DHT were determined using a Scatchard plot [9].

The HPLC analysis was performed using a two-pump gradient system

(Waters Assoc., Milford, MA, U.S.A.). The injector was a Rheodyne 655-0890 valve fitted with a 1-ml loop. The separation of bound and free tritiated androgens was performed on a Diol 200 (7 μm, 250 mm×7 mm I.D.) column (Merck). The mobile phase was TG buffer containing 10% (v/v) ethyleneglycol, and the flow-rate was 1.0 ml/min. The eluted tritiated androgens were collected in 1-ml fractions using a Frac-100 fraction collector (Pharmacia, Uppsala, Sweden). Tritiated androgens were quantified by liquid scintillation spectrometry (Packard Model 5780), using 5 ml Scintran for 50 μl of each fraction, with a counting efficiency of 30%.

Gel permeation fast protein liquid chromatography (FPLC)

The fractions of ABP-[^{3}H]testosterone eluted from the Diol 200 column were pooled and poured onto a Superose 6^{TM} column (30 cm×1.0 cm I.D.). The column was eluted with TG buffer at a flow-rate of 0.5 ml/min using an FPLC system (Pharmacia) with two P-2500 pumps. The fractions were collected and treated as described above for liquid scintillation spectrometry. The column was calibrated using tritiated water, dextran blue 2000 and standard proteins, as described previously [10].

Electrophoresis

Disc polyacrylamide gel electrophoresis (PAGE) was used for determination of bound and free tritiated androgens according to the method of Corvol et al. [2]. A 50-μl volume of tissue extract was incubated with [^{3}H]DHT (0.5 μCi, 2.7 pmol) for 2 h. A dense solution (saccharose at saturation) and a drop of bromophenol blue were added. A 50-μl aliquot of this mixture was poured onto the top of the gel. Migration was performed at 4°C, at 1 mA per gel. The gel was cut, and each slice was incubated with 1 ml of toluene and 4 ml of Scintran prior to liquid scintillation spectrometry. Analysis of the protein content of the tissue extract and of the fractions of [^{3}H]DHT-ABP collected from the Diol 200 column were performed in sodium dodecyl sulphate (SDS) PAGE using an automated Phast System^{TM} (Pharmacia). The polyacrylamide gradient was 8–25%, and 2.5% SDS and 5.0% β-mercaptoethanol were added to each sample. The sample was heated at 100°C for 5 min and poured onto the gel after the addition of bromophenol blue. Reference proteins for molecular mass estimation were phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and α-lactalbumin.

Photoaffinity labelling

A 10-ml volume of the dialysed testicular extract were incubated overnight with 1 nmol Δ_6[^{3}H]testosterone at 4°C under rotative agitation. After exposure to UV light (345 nm) for four 15-min periods under controlled temperature, the sample was ready for injection, in order to separate the covalently bound Δ_6[^{3}H]testosterone from the remaining free Δ_6[^{3}H]testosterone. The separation was performed on a preparative Diol 200 column (10 μm, 250 mm×25 mm I.D.) at a flow-rate of 10.0 ml/min.

RESULTS AND DISCUSSION

An example of an HPLC elution profile of [^{3}H]DHT from the Diol 200 column is given in Fig. 1. When [^{3}H]DHT was incubated with the tissue extract, [^{3}H]DHT bound to ABP was eluted in two peaks with retention times of 6.8 ± 0.6 and 10.6 ± 0.7 min (Fig. 1). The retention times of these peaks did not change when samples of different concentrations were injected. The minor peak was the first to be eluted and it could correspond to a specific binding of DHT to a carrier protein rather than to absorption of [^{3}H]DHT on albumin, because it disappeared when [^{3}H]DHT was incubated with the tissue extract

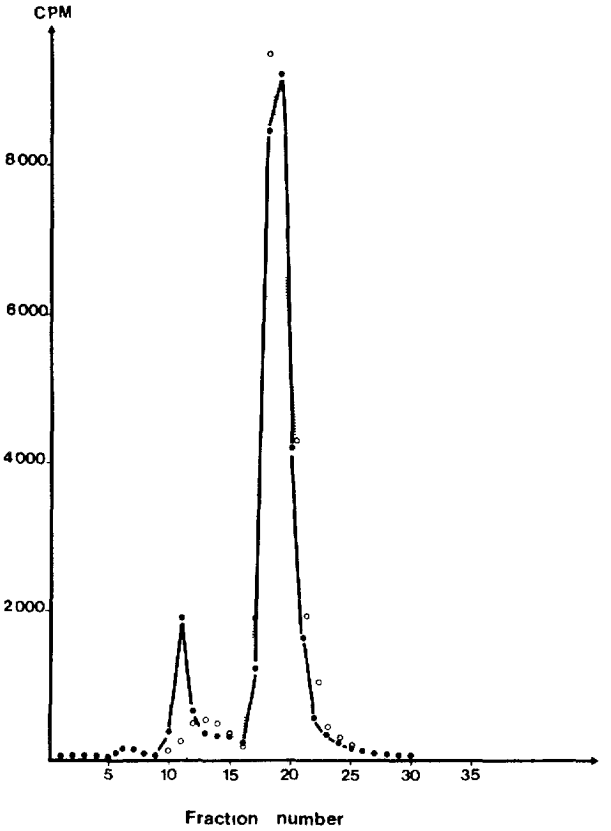

Fig. 1. Chromatogram (Diol 200 column, 250 mm × 7 mm I.D.) obtained with [^{3}H]DHT incubated with 400 μl of rat testicular cytosol extract (●) [^{3}H]DHT bound to ABP and free [^{3}H]DHT are eluted at 10.6 and 18.1 min, respectively. A control analysis is shown (○) for the injection of [^{3}H]DHT alone, in which no peak for [^{3}H]DHT appears at 10–11 min. The same elution profile was obtained when 400 μl of rat testicular cytosol extract were incubated with labelled and unlabelled DHT in a molar ratio of 1.200.

in excess of non-labelled DHT (Fig. 1). Its DHT binding capacity was estimated to ca. 0.13 pmol/mg of protein, and its K_D ca. 47.6 n*M*.

When a part of the second peak of bound [^{3}H]DHT eluted from the Diol 200 column was injected on to a Superose 6 gel permeation column, it was eluted as a main peak corresponding to an estimated relative molecular mass of 45 000. A minor peak corresponding to an estimated molecular mass of 95 000 was also observed. It was therefore concluded that the peak of bound [^{3}H]DHT which was eluted with a retention time of 10–11 min from the Diol 200 column corresponded mainly to a monomer of ABP. The dissociation of dimeric ABP was not complete, since a minor peak corresponding to the dimer was observed in Superose 6 gel permeation. The dissociation may be the result of unspecific interaction of the protein with the solid phase of the column. The retardation of elution could be also due to an interaction of ABP with the matrix.

Free [^{3}H]DHT was eluted from the Diol 200 column with a retention time of 18.1 ± 0.9 min. The between-run coefficients of variation of the retention times of bound and free [^{3}H]DHT were estimated as 6.5 and 4.8%, respectively ($n=10$). When [^{3}H]DHT and tissue extract were successively injected and filtered through the Diol 200 column, no radioactivity was removed in the second analysis. This was carried out to verify that no [^{3}H]DHT remained adsorbed on the column after each analysis.

The Scatchard plot demonstrated that [^{3}H]DHT was specifically bound to ABP with a binding capacity of 3.3 pmol/mg of protein, using the guanidine hydrochloride dialysed testis extract (Fig. 2). This result was higher than the binding capacity of 2.6 pmol/mg estimated with the method of Corvol et al. [2]. In fact, the method of Corvol et al. [2] is known to underestimate the DHT binding capacity [3]. The [^{3}H]DHT binding capacity of ABP was estimated to be 0.54 pmol/mg in the non-treated testis extract. A similar result has been obtained by Larrea et al. [11] for the specific activity of ABP in rat testis extract treated by ammonium sulphate precipitation, using a radioisotopic dilution assay with dextran-coated charcoal.

The enhancement of ABP specific activity in testis extract after dialysis with guanidine hydrochloride can be explained by the desaturation of ABP [8]. Guanidine hydrochloride dialysis has a mild effect on the binding site of ABP after renaturation of the extract since the K_D was double that determined with the untreated extract: K_D was estimated to be 71.4 and 35.7 n*M* for ABP from the testis cytosol extract treated with guanidine hydrochloride and from the untreated extract, respectively. These values are ten-fold higher than those obtained by other techniques [5,6], but the tissue extract was different because it was obtained after ammonium sulphate precipitation of the ABP fraction. One may assume that such a procedure modifies the affinity of DHT for ABP, either by denaturation of ABP or by enhancement of the ionic strength of the medium; a part of dimeric ABP is dissociated and eluted as a monomer in our

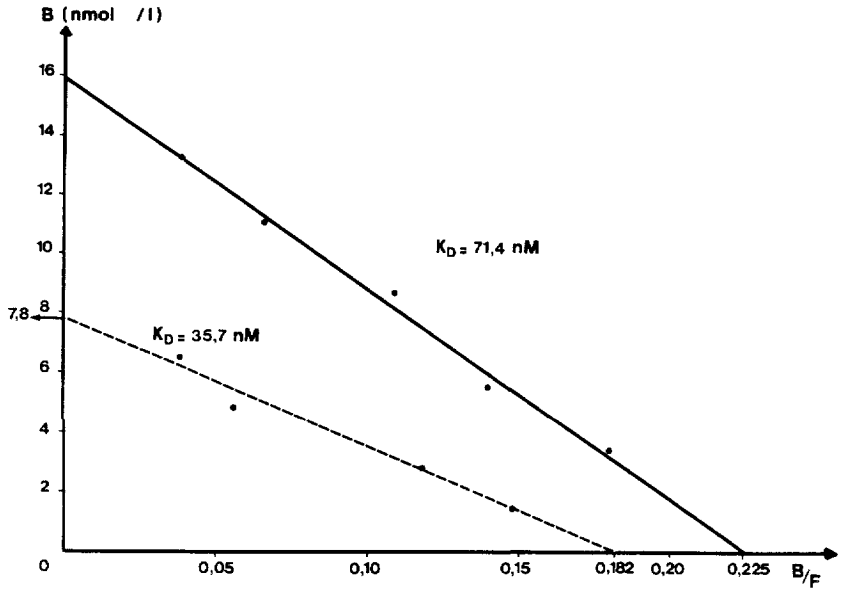

Fig. 2. Scatchard plot of dialysed (——) and non-dialysed (- - -) cytosol extract from rat testis incubated with increasing amounts of [^{3}H]DHT.

HPLC method (Fig. 1). Another explanation for the low K_D of the ABP–DHT complex could be that DHT has a lower affinity for monomeric ABP than for the dimer.

A Diol 200 column (with a preparative column) was also used to separate Δ_6-testosterone bound the ABP from free Δ_6-testosterone after photoaffinity labelling of ABP from tissue extract. Two peaks of bound Δ_6-testosterone were eluted with retention times of 6 and 10 min (Fig. 3). When free Δ_6-testosterone alone was injected under these conditions it was eluted with a retention time of 18 min. When a part of the fractions corresponding to the peaks 1 and 2 (Fig. 3) were injected on a Superose 6 gel permeation column, they were eluted in positions corresponding to estimated relative molecular masses (M_r) of 95 000 and 49 000, respectively. Peaks 1 and 2 therefore corresponded to the monomer and the dimer of photoaffinity-labelled ABP.

The photoaffinity labelling of ABP in crude tissue extracts has been previously described by Taylor et al. [12]. These authors used dialysis to separate free from bound Δ_6-testosterone. The Diol chromatography is more rapid and easier to perform.

SDS-PAGE of the fraction of monomeric Δ_6-testosterone-ABP eluted from the Diol 200 column showed that proteins and peptides were simultaneously present in the fractions (Fig. 4); two protein bands (46 800 and 52 500) had M_r values close to that of the monomers of ABP [7,11]. Using the Diol 200 column, ABP was well separated from albumin, which had a retention time of

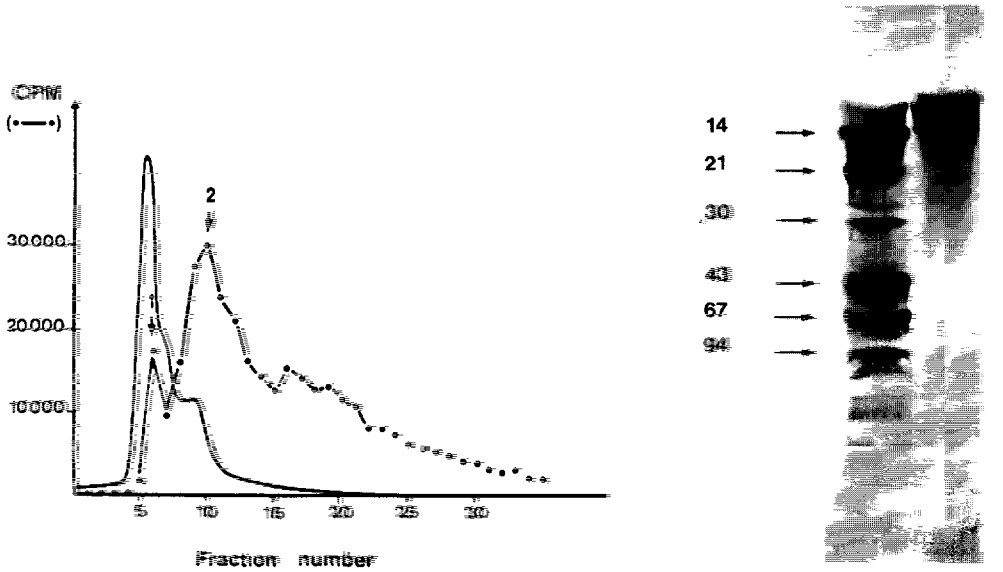

Fig. 3. Chromatogram (preparative Diol 200 column, 250 mm × 25 mm I.D.) obtained with testosterone incubated with 3 ml of rat testicular cytosol extract. Photoaffinity labelling was performed as described in the text. The preparative column was eluted at a flow-rate of 10 ml/min. The photoaffinity-labelled ABP was eluted as both dimer and monomer, with respective retention times of 6 and 10 min. Absorbance detection (ABS, ——) was performed at 280 nm (1.0 a.u.f.s.).

Fig. 4. SDS electrophoresis of the fraction of [^{3}H]DHT-ABP complex collected from Diol 200 chromatography. Two protein bands have M_r values (46 800 and 52 500) close to that of a monomer of ABP. The presence of abundant contaminating peptides was noticed. M_r values were estimated using phosphorylase b, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and α-lactalbumin as reference molecules (right lane). Their M_r (10^{-3}) values are indicated by arrows.

ca. 6.6 min, even though its M_r is close to that of albumin. From calibration of the Diol 200 column with standard molecules, it was also observed that ABP was eluted in a position corresponding to that of glucagon [13].

In conclusion, we have improved a rapid and reliable HPLC method for separating the free androgens from androgens bound to ABP in tissue extracts. This method has been used to measure the binding capacity of ABP and to prepare semi-purified photolabelled ABP from rat testis extract.

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