J. steroid Biochem. Vol. 23, No. 6A, pp. 955–965, 1985
Printed in Great Britain. 

0022-4731/85 $3.00 + 0.00


# HYDROPHOBIC INTERACTION CHROMATOGRAPHY (HIC) FOR THE SEPARATION OF PROTEIN-BOUND AND FREE STEROIDS. APPLICATION TO BINDING PROTEIN AND RECEPTOR ASSAYS

TH. STEIMER*, G. E. THEINTZ†, P. C. SIZONENKO† and W. L. HERRMANN*
*Department of Obstetrics and Gynecology and †Department of Pediatrics and Genetics, Division of Biology of Growth and Reproduction, University Hospital, 1211 Geneva 4, Switzerland

(*Received* 22 *April* 1985)

**Summary**—Protein-bound steroids can be separated from free steroids using microcolumns of silica gel coated with an hydrophobic (octadecyl) solid phase. The bound fraction is eluted in the assay buffer, whereas the free fraction is retained quantitatively on the column in the first step and can be recovered in methanol. Both fractions can be quantitated directly (e.g. by liquid scintillation spectrometry when using radioactive ligands) or kept for further analysis (e.g. by TLC, HPLC etc.). Separation of the bound and free fractions is rapid, accurate and reproducible; intra- and inter-assay coefficients of variation are lower than 5 and 10%, respectively. Recovery of radioactive steroids is high (usually over 85%) and can be estimated separately for each sample. Since assay blanks are very low (typically less than 0.1% of input), this new method, which could be termed "hydrophobic interaction chromatography" (HIC), should prove especially useful for the development of sensitive binding assays, particularly in the field of steroid receptors. The HIC method compared well with three methods currently used for steroid binding assays, namely adsorption of unbound steroids on dextran-coated charcoal, gel filtration on Sephadex LH-20 and adsorption of steroid–protein complexes on DEAE-cellulose filters. Examples of application described here include studies on human plasma sex hormone binding globulin (SHBG) and $SP_2$ placental protein (saturation analysis, binding specificity etc.), the separation of antibody-bound steroids in a radioimmunoassay and the estimation of androgen binding to rat epididymal androgen binding protein (rABP). Receptor assays are illustrated by saturation analysis of the mouse uterine oestrogen receptor and of the androgen receptor in the human genital skin.

## INTRODUCTION

Steroid action often requires non-covalent binding of the hormone to specific proteins, e.g. carrier proteins in biological fluids and intracellular receptors [1–3]. The binding characteristics of these proteins (capacity, affinity, specificity) are usually studied using radioactive tracers. A critical step in these assays is the separation of the bound (B) and free (F) fractions, which should occur without disturbing the binding equilibrium. In practice, however, separation under equilibrium conditions is rarely achieved, except in the case of equilibrium dialysis [4]. This method is rather cumbersome and time-consuming, especially when large numbers of samples have to be processed. Therefore, most current methods rely on a rapid separation of the bound and free fractions, which results in minimal disturbance of equilibrium conditions if the time required (a few minutes) is short as compared to the dissociation rate of the steroid–protein complex [5]. The physico-chemical principles involved include selective precipitation of the steroid–protein complex with ammonium or protamine sulphate [6, 7], adsorption on dextran-coated charcoal [8] or hydroxyapatite [9], size exclusion (LH-20 [10] or G-25 [11] Sephadex) or ion-exchange (DEAE-cellulose filter assay [12]) chromatography. The only method so far that has made use of the hydrophobic properties of the ligand is that of Kawamoto [13], in which free steroids are adsorbed onto Amberlite XAD-2 resin. Recently, new hydrophobic solid phases, such as $C_{18}$-silica gel derivatives, have been developed and used for the extraction and chromatographic separation ('reverse phase chromatography') of lipophilic compounds [14]. During evaluation of these new materials for the extraction and purification of steroids prior to HPLC analysis, we discovered that protein-bound steroids were only partly retained on microcolumns of octadecyl silica gel. However, when these columns were used after recycling (see Experimental section), or pre-treated with biological fluids (e.g. plasma) or a buffer containing proteins (albumin or gelatin), the recovery of steroid-protein complexes in the first eluate was found to be quantitative, probably because unspecific adsorption sites on the silica gel matrix were thus inactivated. These observations prompted us to develop a new method for the separation of protein-bound and free steroids, which could be termed "hydrophobic interaction chromatography", or HIC for short. The purpose of this paper is to describe this new procedure in detail, to compare it to well-established methods and to provide a few examples of application in the field of steroid biochemistry. Since the HIC method offers many advantages over existing procedures, particularly as regards accuracy

and sensitivity, it should prove useful in the field of steroid receptor assay, where maximal sensitivity must be achieved in order to detect low levels of these macromolecules in biological samples.

## EXPERIMENTAL

### *Materials*

Sepralyte®, preparative grade (40 $\mu$m), a $C_{18}$-(octadecyl)-bonded silica gel, and plastic micro-columns (1 ml capacity) fitted with 20 $\mu$m plastic frits were obtained from Analytichem Int. (Harbor City, CA 90710, U.S.A.). Radioactive steroids were obtained either from Amersham Int. (U.K.) or NEN (Boston, MA 02118, U.S.A.). Unlabelled steroids and biochemicals were from Sigma (St Louis, MO 63178, U.S.A.), except for phenylmethylsulfonyl fluoride (Serva, Heidelberg, F.R.G.). Lumagel scintillation cocktail was from Lumac (6372 AD, Schaesberg, The Netherlands), GF/A and GF/B glass microfiber filters as well as DE-81 filters from Whatman (Maidstone, Kent, U.K.) and LH-20 Sephadex from Pharmacia (Uppsala, Sweden).

### *Preparation of reagents*

(1) *Assay buffers.* (a) Tris buffer (TB)—10 mM Tris–HCl, pH 7.4 at 25°C, was prepared from a 50 mM stock solution (7.58 g/l Trizma® 7.4 in boiled bidistilled water); (b) Tris–gelatin buffer (TGB)—10 mM Tris–HCl, pH 7.4, 0.1% (w/v) gelatin (Sigma, type II), gelatin was dissolved by gentle stirring at 37°C; (c) receptor assay buffer 1 (RAB 1)—50 mM Tris–HCl, pH 7.4, 1 mM $Na_2EDTA$, 10 mM sodium molybdate, 1 mM dithiothreitol (DTT), 10% (v/v) glycerol; (d) receptor assay buffer 2 (RAB 2)—50 mM Tris–HCl, pH 7.4, 1 mM $Na_2EDTA$, 12 mM monothioglycerol (MTG), 10 mM sodium molybdate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10% (v/v) glycerol; (e) receptor assay buffer 3 (RAB 3)—20 mM Tris–HCl, pH 7.8, 0.25 M sucrose, 1 mM $MgCl_2$, 1 mM mercaptoethanol, 0.001% sodium azide.

(2) *Radioactive tracers.* An aliquot of the stock solution in toluene/ethanol (9/1, v/v) was evaporated quickly at 37°C under air or nitrogen and immediately taken up in assay buffer. Tracer solutions were always prepared fresh just before the experiment. Tracer purity was checked at regular intervals and purification carried out by TLC or HPLC when required. The various concentrations used for saturation analysis were obtained after serial dilution of the original solution in assay buffer.

(3) *Unlabelled steroids for competition experiments.* These were dissolved in methanol (1 mg/ml) and diluted in assay buffer as appropriate (final methanol concentration was always lower than 0.1% v/v).

### *Preparation of biological samples*

Human heparinized plasma was used as a source of sex hormone binding globulin (SHBG). Small (50 $\mu$l) aliquots were kept at −20°C and diluted 1/30 (male) or 1/60 (female) with TGB just before use. Denaturation for the estimation of non-specifc binding was carried out for 60 min at 60°C [3]. Human placental protein $SP_2$ was kindly supplied by Dr H. Bohn (Behring, Marburg, F.R.G.). This standard preparation (OP 51 176) containing 20.35 mg pure $SP_2$/ml was diluted 1/500 with TGB just before the assay. The rat androgen binding protein (rABP) preparation was obtained after homogenization of pooled rat epididymes in RAB 1 buffer and centrifugation for 60 min at 105,000 ***g***. The mouse uterine receptor assay was carried out on the high-speed supernatant (105,000***g*** × 60 min) after homogenization of uteri from adult albino mice in buffer RAB 3. Human foreskin samples obtained at circumcision from prepubertal boys were pulverized in liquid nitrogen and homogenized in RAB 2 buffer. The androgen receptor assay was carried out either on the low-speed supernatant (1000***g*** × 10 min), or on the cytosol. Results were similar in either case.

### *Experimental procedure*

Hydrophobic interaction chromatography (HIC) for steroid binding assays was carried out on micro-columns of Sepralyte® ($C_{18}S$) with the experimental setup shown in Figs 1a and 1b. Plastic reservoirs (1 ml capacity) fitted with 20 $\mu$m plastic frits (see Materials section for supplier) were packed with approx. 20 mg dry $C_{18}S$ using a calibrated spoon. Small disks punched out of GF/A (or GF/B) glass microfibre filters were put on top of the gel to avoid re-suspending the fine silica gel particles when pipetting solutions onto the gel. The assay procedure is outlined in Table 1. After pre-conditioning the columns with methanol and water, the wet gel was treated with TGB to occupy non-specific binding sites on the silica gel matrix which would otherwise prevent quantitative recovery of steroid–protein complexes. These first three steps (conditioning of the gel) were carried out at room temperature; columns were then equilibrated with the assay buffer in the cold [at 4°C in the cold room or at 0°C in the special ice-bath, see Fig. 1b] for at least 15 min. The sample was carefully applied on top of the gel. When it had gone through, the column was rinsed twice with the elution buffer. These first three fractions (e.g. the initial volume displaced following sample application and the two buffer rinses) were collected as a pool and constitute the protein-bound steroid fraction (B). In most applications involving radioactive ligands, protein-bound steroids were collected directly into scintillation vials and measured by liquid scintillation spectrometry. The gel was then washed with 1 ml of bidistilled water and buffer or salt solution without proteins to remove traces of protein on the columns, which otherwise could have been precipitated with methanol; this appeared to be essential for further use of the columns (recycling). The free steroid fraction (F) was collected with two successive washes of methanol.

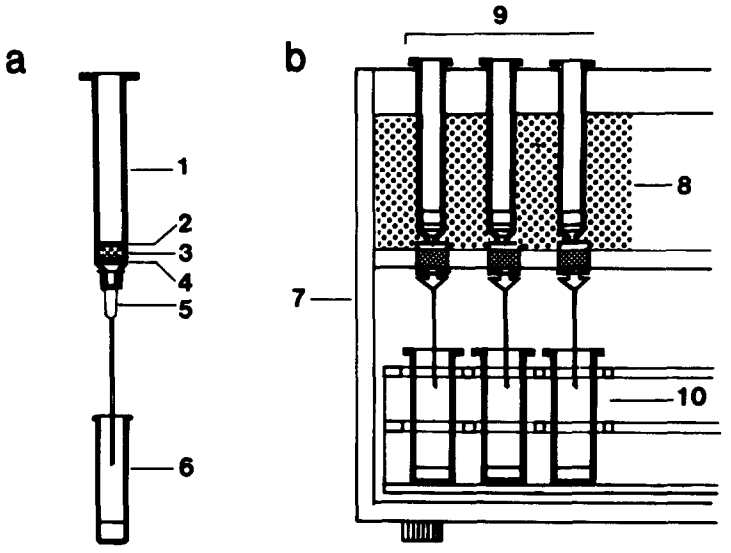


Fig. 1. (a) A $C_{18}S$ microcolumn for HIC: 1, 1 ml plastic reservoir; 2, Whatman GF/A or GF/B glass microfiber filter disk; 3, Sepralyte® (20 mg); 4, 20 $\mu$m plastic frit; 5, 18G × $1\frac{1}{2}''$ hypodermic needle; 6, collection vial. (b) Experimental setup for binding assay at 0°C: 7, column rack; 8, ice-bath with crushed ice/water; 9, $C_{18}S$ microcolumns; 10, test-tube rack with collection vials.

Finally, the gel was rinsed with 1–3 ml of methanol. Radioactivity in the effluent after the last rinse was equivalent to counter background level. When assaying diluted plasma or the high-speed supernatant from tissue homogenates, columns were generally re-used many times without repacking (column recycling, see Table 1). Viscous solutions or suspensions (e.g. the low-speed supernatant from tissue homogenates) tend to clog the top filter and the flow rate was markedly decreased even after a single use. In this case, new columns had to be prepared and pre-treated with TGB for each assay batch. This cannot be considered as a major drawback of the method, however, because repacking of the columns is not time-consuming (50 columns can easily be set up in less than 30 min).

*Quantification of bound and free fractions and calculation of results*

A major advantage of the HIC separation procedure is that both protein-bound and free steroids can be measured directly as experimental variables. However, since the bound and free fractions were generally counted under different conditions, i.e. as aqueous or organic phases and often in different sample volumes, counting efficiency had to be determined in each case to establish a proper calibration curve and to convert raw counts into dpm. Alternatively, chromatographic conditions, e.g. elution volumes, were manipulated so that counting efficiency was the same for the bound and free fractions. Thus, under the standard assay conditions shown in Table 1, 500 $\mu$l of the buffer solution and 500 $\mu$l of the methanolic eluate were both counted with a 30.5% efficiency for tritium (4 ml of Lumagel in 5 ml scintillation vials, Packard Tri-Carb 3255 spectrometer with pre-set 3H/3HQ window). The amount of ligand bound to protein (B) was calculated as:

$$B = L \times \frac{b}{f + b}, \quad (1)$$

where L = amount of ligand/assay tube (e.g. fmol

Table 1. Flow-chart for standard assay procedure[a]

| | |
|---|---|
| 1. *Column conditioning* | |
| 1.1 Wash 1 ml methanol | |
| 1.2 Wash 1 ml dist. water | |
| 1.3 Wash 1 ml Tris–gelatin buffer (TGB) | |
| 1.4 Wash 1 ml elution buffer (assay buffer) | |
| 2. *Binding assay* | |
| 2.1 Apply sample (50–200 $\mu$l) | Collect: *B fraction* |
| 2.2 Wash 200 $\mu$l elution buffer | |
| 2.3 Wash 200 $\mu$l elution buffer | |
| 2.4 Rinse 1 ml dist. water, buffer or salt solution, discard | |
| 2.5 Wash 250 $\mu$l methanol | Collect: *F fraction* |
| 2.6 Wash 250 $\mu$l methanol | |
| 2.7 Rinse 1 to 3 ml methanol, discard | |
| 2.8 Recycle (back to step 1.4) | |

[a]See text for procedural details, buffer composition etc.

steroid), b = dpm in the protein-bound fraction, f = dpm in the free steroid fraction (dpm were corrected for counter background). Total binding ($B_T$) was defined as the amount of ligand bound when the radioactive tracer only was present. Non-specific binding ($B_{NS}$) was determined in the presence of an excess (100- to 500-fold) of unlabelled hormone or after denaturation of the specific binding site (e.g. by heat inactivation, see SHBG protocol). The amount of hormone bound to specific, saturable binding sites ($B_S$) was then obtained as:

$$B_S = B_T - B_{NS}. \quad (2)$$

Each experimental variable was usually determined in duplicate, especially in single-point assay under saturating conditions. However, because of the excellent reproducibility of the method (duplicate values within 5% of the mean), a single measurement was often found to be sufficient, particularly when examining binding as a function of protein concentration (linearity tests) or time (estimation of association and dissociation rates).

## RESULTS

### *Validation of the method*

*Elution patterns under various conditions.* Typical elution patterns from $C_{18}S$ microcolumns are shown in Fig. 2. When steroid binding proteins were denaturated after incubation with the radioactive tracer, e.g. by acidification of the sample, or prior to incubation, e.g. by heat inactivation of the specific binding sites, the first peak (B) representing the protein-bound fraction was abolished or greatly reduced (Fig. 2a, central panel, cf. top panel). When the radioactive tracer was incubated with buffer only under otherwise identical conditions (assay blank), the B peak was totally absent (Fig. 2a, lower panel): radioactivity level in the B peak region was negligible, i.e. about twice the counter background (25 cpm). These chromatographic patterns show two important features of the new separation procedure, namely: (a) the efficient, clear-cut separation between protein-bound and free steroid (columns can be washed extensively with aqueous solutions without releasing the free steroids adsorbed onto the solid hydrophobic matrix); and (b) the very low assay blank (residual radioactivity in the B fraction is negligible in the absence of binding proteins).

*Recovery of protein-bound and free steroids.* When columns were used without pre-treatment, the recovery of protein-bound steroids in the B fraction was poor (Fig. 2b) and not linearly related to binding capacity (the lower the protein concentration, the

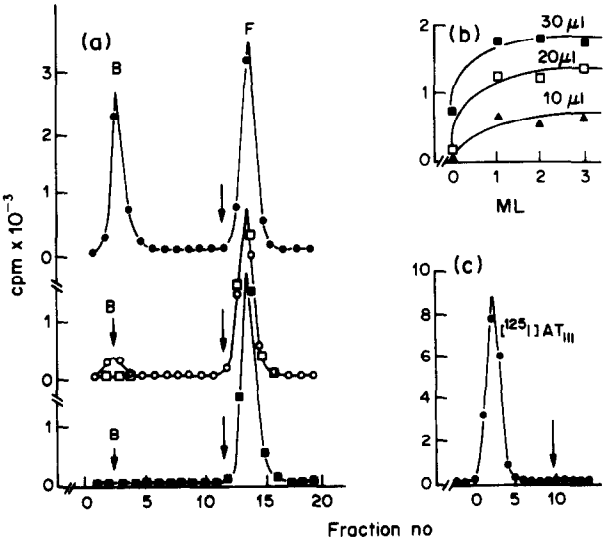


Fig. 2. (a) Typical $C_{18}S$ chromatographic profiles showing [$^3$H]DHT binding to SHBG (human plasma, 1 : 20 final dilution). Samples (30 µl diluted plasma in 50 µl total assay volume) were incubated for 30 min at 37°C. Elution was carried out at 0°C using 100 µl aliquots of 10 mM Tris–HCl pH 7.4 followed by methanol. *Top panel*, Total binding (untreated sample, ●); *middle*, estimation of non-specific binding—SHBG pre-incubated for 60 min at 60°C (○) to destroy specific binding activity (residual radioactivity in the B fraction due to low affinity/high capacity binding to plasma albumin) and after addition of 20 µl 1 M HCl to the incubate (□) prior to separation; *lower panel*, incubation with buffer only (assay blank, ■)—radioactivity in the B peak region was just above counter background (25 cpm). Elution with methanol is indicated by arrows. (b) Effect of $C_{18}S$ pre-treatment with 0.1% gelatin buffer: cpm [$^3$H]DHT bound to SHBG as a function of gelatin buffer volume (ml) used for conditioning, at three different plasma dilutions (10, 20 and 30 µl of human male plasma in 50 µl assay volume). (c) HIC of [$^{125}$I]-antithrombin III (mol. wt 58,000, pre-purified on Sephadex G-25 F to remove free iodine)—the radioiodinated protein was completely eluted in the first 500 µl of 10 mM Tris–HCl buffer (pH 7.4 at 25°C) corresponding to the B fraction. Recovery: 83.5 ± 1.3% (6 separate experiments with 6 different $C_{18}S$ columns pre-treated with 1 ml gelatin solution).

Table 2. Reliability of binding assays based on HIC

| Test system[a] | Total counts per assay tube (TC) | Average cpm specifically bound ($B_S$) | Assay blank cpm mean ± SD ($N$) | Intra-assay[b] variation CV% ($N$) | Inter-assay[c] variation CV% ($N$) |
|---|---|---|---|---|---|
| SHBG–DHT binding assay (plasma) [single-point assay] | $5 \times 10^4$ | $6 \times 10^3$ | $65 \pm 4(6)$[d] | 3.4 (24) | 4.4 (38) |
| Androgen receptor assay (human skin) [saturation analysis] | $5 \times 10^3$ to $6 \times 10^4$ | $1.5 \times 10^2$ | $10 \pm 1$ (16)[e] | 7.0 (18) | 8.8 (5) |
| 4-Androstenedione binding to specific antibody (RIA) | $1 \times 10^4$ | $3 \times 10^3$ | — | 3.1 (6) | — |

[a]See text (results) for details.
[b]SHBG assay—6 replicates in 4 experiments, average CV% (range 2.2–4.1); androgen receptor assay—average variation for 18 duplicate measurements at 4 different ligand concentrations, from 0.1 to 4 nM R 1881.
[c]SHBG assay—Mean variation in the concentration of the internal standard ("Seronorm") processed in 38 separate assay batches; androgen receptor assay—mean variation in the assay standard from 5 independent experiments.
[d]TGB buffer containing gelatin (0.1%).
[e]RAB 2 buffer (protein-free).

poorer the recovery). Various column conditioning procedures were tested: columns were rinsed with diluted plasma, albumin (BSA) or gelatin solutions in buffer. All these treatments were effective and increased the yield of bound steroids in the B fraction. Gelatin (0.1%, w/v) was eventually chosen because interference with binding measurement was found to be minimal (see Table 2, SHBG assay blank). Figure 2b shows that washing the column with 1 ml of the gelatin solution was sufficient; further washes were without effect. Protein recovery in the B fraction was also estimated directly using an iodinated protein (Antithrombin III, AT III, mol. wt 58,000) solubilized in 1/30 diluted human male plasma. The elution pattern of Fig. 2c shows that this protein was completely eluted in the B fraction (first 500 $\mu$l of buffer). The overall recovery was found to be $83.5 \pm 1.3\%$ (mean ± SD, $N = 6$). This figure was not significantly different from that obtained for the overall recovery of tritiated steroids from $C_{18}$S microcolumns (B + F fractions), i.e. $80.7 \pm 3.0\%$ ($N = 10$) in a typical SHBG assay. These data indicate that there is no preferential loss of steroid–protein complexes on hydrophobic silica gel and that overall tritium recovery can be used as a reliable estimate of assay efficiency in routine experiments.

*Comparison with previously described procedures.* Separation of protein-bound and free steroids by the HIC procedure was compared to well-established techniques in order to validate the method for the measurement of steroid binding sites. We used [$^3$H]testosterone binding to human plasma proteins (mainly SHBG) as a first test system. The dissociation constant ($K_D$) of the steroid–protein complex is in the same range as the $K_D$s of steroid receptors (nM range), but the rate of dissociation is much faster with a half-life of approx. 1 h. As this will maximize any deviation from equilibrium conditions, this test system should be discriminatory. All experiments were carried out in parallel under strictly identical conditions, apart from the separation step.

Gel filtration on microcolumns of Sephadex LH-20 is now widely used in steroid receptor assays (e.g. oestrogen receptors in the brain [10]). We employed a modification of previously published procedures in which the free steroid is eluted in 70% methanol to increase sharpness of the peak and ensure complete desorption of the free steroid from the gel. Binding was measured as a function of protein concentration [$\mu$l of 1/10 diluted male plasma per assay tube]. The results (Fig. 3a) show that the assay was linear over the range of protein concentration tested.

The DEAE–cellulose filter assay was carried out essentially according to the method of Santi *et al.*[12], which has also been applied to plasma proteins [15]. Under our experimental conditions, this assay was not found to be strictly linear, but the results (Fig. 3b) were in the same range as those obtained in the gel filtration assay.

Adsorption of free steroids on dextran-coated charcoal (DCC) is probably the most frequent experimental approach to the problem of steroid separation. Despite its limitations [16], it is still widely used, mainly because it is simple and convenient when large number of samples have to be processed. As shown in Fig. 3c, the binding capacity was underestimated when using DCC in the present system, probably because the time required for separation (approx. 30 min at 4°C) was too long as compared to the dissociation rate of the steroid–protein complex.

Results obtained using the HIC procedure are shown in Fig. 3d. These results were strictly identical with those obtained using the LH-20 gel filtration assay.

The applicability of HIC to the separation of steroids bound to macromolecules was also investigated in another test system using steroid antibodies, as illustrated by data in Fig. 4a. A standard curve for the radioimmunoassay of 4-androstenedione according to the method currently used in our laboratory [17] was set up in quadruplicate. Half the samples were submitted to the usual DCC separation procedure, whereas the other half was applied onto $C_{18}$S microcolumns (the elution profile of radioactive androstenedione, in free and antibody-bound

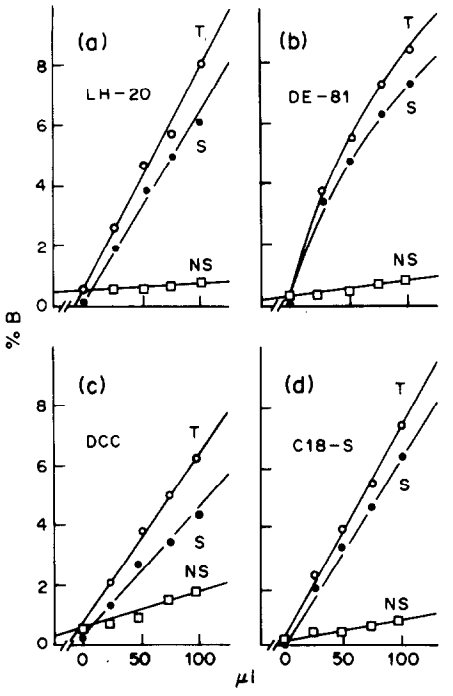


Fig. 3. Comparison between HIC and other procedures currently used for steroid binding assays. Test system—testosterone binding to human plasma proteins. Various volumes (0–100 µl) of human plasma (dilution 1 : 10) were incubated with [1β, 2β $^3$H]testosterone (sp. act. 50.4 Ci/mmol, $2 \times 10^4$ cpm/assay tube) in 100 µl final volume for 30 min at 23°C. Separation of protein-bound and free steroids was carried out using four different methods in parallel experiments. (a) Separation on LH-20 Sephadex microcolumns (1.5 ml bed volume). The bound fraction (B) was eluted at 4°C with 10 mM Tris–HCl buffer, pH 7.4, the free fraction (F) with 70% methanol. Testosterone binding is shown at various plasma dilutions (0–100 µl): T, total binding (○); NS, non-specific binding (□, displacement by a 125-fold excess of unlabelled testosterone); S, specific binding (T-NS, ●). (b) Separation using the DEAE–cellulose filter assay [15]. DE-81 filters (Whatman), soaked overnight in 50 mM Tris–HCl, pH 7.4, were washed with 10 1-ml aliquots of ice-cold 10 mM Tris buffer, pH 7.4, 1 min after application of the sample, dried and counted in 8 ml of Lumagel following addition of 0.5 ml methanol. (c) Separation using DCC: samples were equilibrated for 10 min at 0°C and 200 µl of the DCC solution (0.5% charcoal, 0.05% dextran) were added. After 10 min incubation, samples were centrifuged for 10 min at 2000*g* (4°C), the supernatant decanted and counted for the determination of bound steroids. (d) Separation on $C_{18}$S microcolumns (standard protocol, see Table 1); results are strictly comparable to those obtained with LH-20 Sephadex chromatography.

form, is shown in the insert, Fig. 4a). The two calibration curves thus obtained (Fig. 4a) were almost identical.

In conclusion, results obtained using the HIC separation procedure compared well with those obtained using other current procedures, such as DCC, LH-20 gel filtration or the DEAE–cellulose filter assay in two different test systems. As demonstrated in the following sections, HIC offers additional advantages which could make it a method of choice for the quantification of steroid binding sites in biological systems.

*Examples of application*

*Androgen binding to rat epididymal rABP.* The high-speed supernatant (105,000*g* × 60 min) obtained after homogenization of rat epididymes, which is known to contain a specific rABP [18], was incubated for 30 min at 30°C with radioactive dihydrotestosterone ([$^3$H]DHT). Under these conditions, binding to the intracellular androgen receptor was negligible [19]. The elution profile of labelled rABP on $C_{18}$S microcolumns is shown in Fig. 4b; labelled DHT was displaced by an excess of unlabelled DHT, thus demonstrating the presence of saturable androgen binding sites.

This rABP preparation was also selected to investigate the possibility of using hydrophobic silica gel for "stripping", i.e. for the removal of endogenous ligand from biological samples prior to the measurement of binding activity. This is sometimes achieved by pre-treatment of the sample with activated charcoal, but some loss of binding activity usually occurs at this stage. In the present experiment, an aliquot of the original tissue extract (high-speed supernatant) was first processed through a $C_{18}$S column to remove free steroids and the fraction collected in the B volume was then labelled with [$^3$H]DHT under conditions identical with those used in the previous experiment. The results (Fig. 4c) showed that there was a slight increase in specific binding activity. During pre-treatment of the sample for the removal of engodenous ligand, some dilution (approx. 50% in this case) of the original sample is inevitable. However, dilution can be estimated in a parallel assay after spiking the sample with tritiated water: the drop in radioactive concentration after the first chromatographic step is a reliable index of sample dilution, because tritiated water is completely eluted in the B fraction. Alternatively, binding capacity can be directly related to protein concentration in both treated and untreated samples. This application of HIC seems promising, because it offers a most valuable alternative to other procedures such as dialysis or charcoal treatment. Removal of endogenous steroids is very rapid (a few minutes) and takes place under mild conditions, thus minimizing the risk of degradation of labile macromolecules.

*Studies on human plasma SHGB and placental $SP_2$ protein.* In this section, we would like to give only a brief account of these studies, which are being published in detail elsewhere [20, 24]. Despite the high binding capacity of SHBG and $SP_2$ in biological samples, quantitative measurement of their binding sites is often difficult. These proteins are relatively stable as compared to intracellular steroid receptors (SHBG in undiluted plasma can be kept at 4°C for at least 48 h without significant loss of binding activity), but the half-life of the steroid-protein complex is

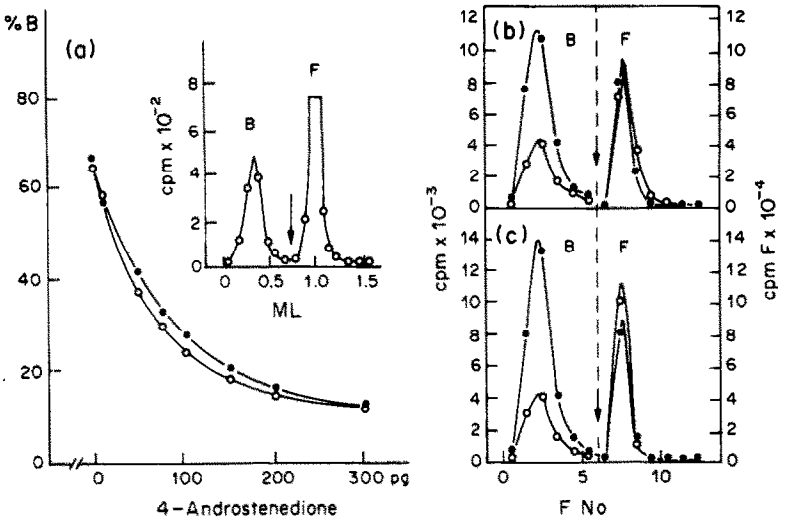


Fig. 4. (a) Binding of [$^3$H]4-androstenedione to a specific antiserum (standard curve for 4-androstenedione RIA). Separation of B and F by the DCC procedure (○) and by HIC (●) at 0–4°C. Insert, elution profile of 4-androstenedione in antibody-bound (B) and free (F) form on a $C_{18}$S microcolumn, Arrow—elution with methanol. (b) Androgen binding to rABP in the 105,000*g* supernatant from rat epididymis. This preparation was incubated for 30 min at 30°C with 5 nM [$^3$H]DHT with (○) or without (●) a 200-fold excess of unlabelled DHT. (c) Binding to stripped epididymal cytosol. Aliquots of the high-speed supernatant were first applied onto $C_{18}$S microcolumns to remove endogenous steroids and the B fractions were then labelled with [$^3$H]DHT as above. The results indicate that rABP was recovered quantitatively after $C_{18}$S stripping of the cytosol. Specific binding amounted to 436 and 457 fmol/mg protein for the untreated and stripped cytosol, respectively.

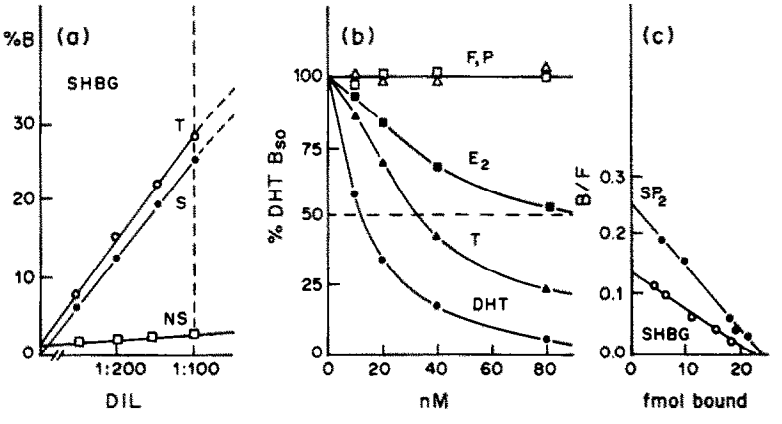


Fig. 5. (a) Linearity of the SHBG–DHT binding assay. Human female plasma at various dilutions was incubated with $6 \times 10^4$ cpm [$^3$H]DHT (sp. act. 153 Ci/mmol) for 30 min at 37°C. SHBG-bound and free DHT were separated on $C_{18}$S microcolumns at 0°C according to the standard protocol (see Table 1). Non-specific binding to other plasma proteins was estimated after heat-denaturation of specific binding sites (60 min at 60°C [3]). T, total binding (○); NS, non-specific binding (□); S, specific binding (●). This assay is linear up to 1 : 100 dilution (results expressed as % total DHT bound). (b) Displacement of [$^3$H]DHT bound to SHBG by various unlabelled steroids (0–80) nM concentration, results expressed as % DHT bound initially, $B_{so}$). DHT, testosterone (T) and oestradiol-17$\beta$ ($E_2$) competed effectively for specific binding sites (relative binding affinities [RBA] were 1.0, 0.34 and 0.10, respectively), whereas C-21 steroids, progesterone, P (□) and cortisol, F. (△) had no effect. (c) Scatchard plot for oestradiol-17$\beta$ binding to human plasma SHBG (○) and placental protein $SP_2$ (●) with apparent $K_D$s of 1.75 and $3.22 \times 10^{-9}$M, respectively. Incubation conditions and estimation of non-specific binding were as for Fig. 5a.

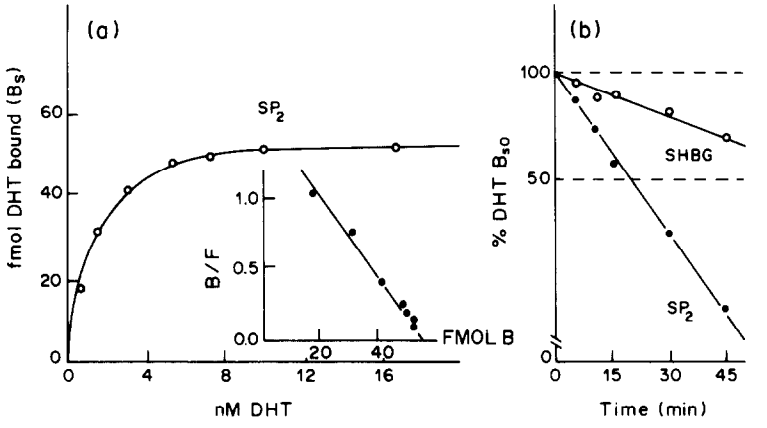


Fig. 6. (a) Saturation analysis for [$^3$H]DHT binding to placental protein $SP_2$: direct plot for specific binding (○) and Scatchard analysis (insert) of the data gave a $K_D$ of $0.6 \times 10^{-9}$M. Same experimental conditions as for SHBG (see legend to Fig. 5). (b) Dissociation rates for [$^3$H]DHT binding to $SP_2$ (●) and SHBG (○). After incubation at 37°C, samples were left on $C_{18}$S columns for the time indicated ($x$-axis) at 0°C before elution of the B fraction with $2 \times 100$ µl of assay buffer. Data are expressed as % DHT specifically bound at $t = 0$ ($B_{S0}$). Semi-log plots of these data yielded half-lives ($t_{1/2}$) of 19 and 73 min for the $SP_2$–DHT and SHBG–DHT complexes, respectively.

very short (less than 120 min at 0°C, see Fig. 6b). This means that the separation of protein-bound and free steroids must be very rapid to avoid significant dissociation of the ligand. We have developed a microassay for human SHBG which is based on its specific binding characteristics. Tritiated dihydrotestosterone (DHT) is used as the ligand because: (a) DHT displays greater affinity for SHBG than any other steroid tested so far; (b) DHT is available at high specific activity (increased assay sensitivity); and (c) DHT does not bind to transcortin (CBG) [21], unlike testosterone or oestradiol (increased assay specificity). Figure 5a shows a calibration curve obtained using human female plasma at various dilutions. The assay is linear up to 1:100 dilution and sensitive enough to permit SHBG measurements in 1:400 diluted plasma. Consequently, less than 5 µl of heparinized plasma are required for a duplicate determination of SHBG in human peripheral blood. Figure 5b shows the effect of various concentrations of competing steroids on [$^3$H]DHT binding to SHBG. These displacement curves can be used to estimate the relative binding affinities (RBA) of these steroids to SHBG (see legend to Fig. 5). Oestrogen binding to SHBG is shown in Fig. 5c (Scatchard plot).

The β-globulin $SP_2$, isolated from the human placenta [22], appears to be closely related to plasma SHBG [23]. We have studied its steroid binding characteristics in detail [24] and part of these data are also presented here to illustrate the applicability of HIC to the separation of short-lived steroid–protein complexes. Saturation analysis of DHT binding to $SP_2$ (Fig. 6a) yielded a $K_D$ of $0.6 \times 10^{-9}$M at 37°C which did not differ significantly from that of SHBG ($0.52 \pm 0.11 \times 10^{-9}$M, mean ± SD of 4 determinations), whereas the same parameter for oestradiol-17β($E_2$) binding to these proteins (Fig. 5c) was found to be different ($3.2 \times 10^{-9}$M for SHBG vs $1.8 \times 10^{-9}$M for $SP_2$).

Dissociation rates for both $SP_2$ and SHBG were also determined using the HIC separation procedure. Samples labelled with [$^3$H]DHT were applied onto $C_{18}$S columns and eluted after various time intervals. When data were expressed as log of % DHT bound initially, a linear relationship was obtained (Fig. 6b), which allowed evaluation of the respective half-lives ($t_{1/2}$, see legend to Fig. 6b).

The above results show that HIC is an appropriate separation method to perform classical binding studies including displacement and saturation analysis, measurement of association and dissociation rates etc. especially when the binding characteristics require a rapid separation of protein-bound and free steroids (steroid–protein complex with a short half-life). The accuracy and reproducibility of the method is evident from the low scatter of experimental values, as clearly shown on Scatchard plots (e.g. Figs 5c and 6a; see also Table 2).

*Measurement of oestrogen and androgen receptors.* In this section, two examples are provided to show that HIC also performs very well in steroid receptor assays.

In the first study, cytosolic oestrogen receptors were measured in the mouse uterus according to a standard protocol (modified from [25]). Separation of bound and free steroids was carried out using the DCC procedure from the original method and HIC in parallel experiments. The HIC data (Fig. 7a, direct plot and Woolf plot [26]) compared very well with those obtained after DCC separation (apparent $K_D$, $1.6 \times 10^{-10}$ M).

In the second study, androgen receptors were

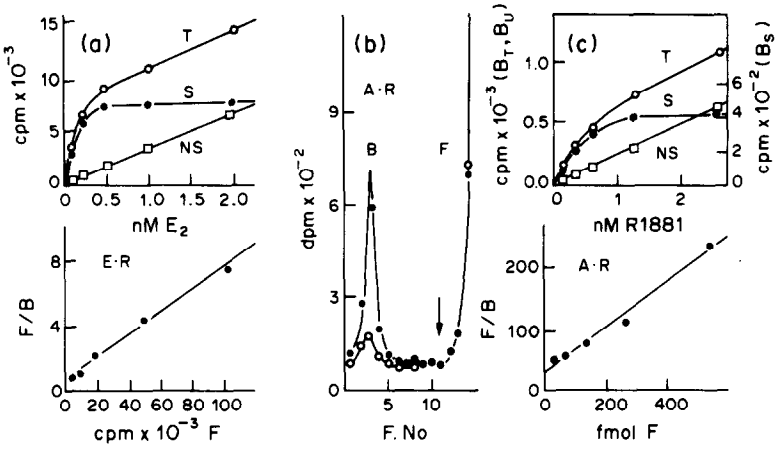


Fig. 7. (a) Oestradiol-17$\beta$ binding to cytosolic oestrogen receptors in the mouse uterus. Aliquots of the 105,000*g* supernatant obtained from mouse uteri were incubated in duplicate with [$^3$H]$E_2$ (0–2 mM); non-specific binding was estimated in the presence of a 100-fold excess of unlabelled diethylstilboestrol (DES). *Upper panel*: total binding (T, ○), non-specific binding (NS, □) and specific binding (S, ●). *Lower panel*: Woolf plot of the same data, apparent $K_D$ of $0.16 \times 10^{-9}$M. (b) Elution profile of the androgen receptor from human foreskin cytosol on a $C_{18}$S microcolumn. ●, total binding; ○, non-specific binding obtained after displacement of [$^3$H]R 1881 by unlabelled DHT (150-fold excess); the arrow indicates elution with methanol (100 $\mu$l fractions). (c) Saturation analysis of the androgen receptor in human foreskin cytosol. *Upper panel*: total binding (T, ○), non-specific binding (NS, □) after displacement by a 150-fold excess of unlabelled DHT, specific binding (S, ●). *Lower panel*, Woolf plot of the same data; apparent $K_D$ of $0.5 \times 10^{-9}$M, $B_{max}$ 9.6 fmol/mg protein in the 800*g* supernatant.

measured in human foreskin samples essentially according to the method of Coulam *et al.*[27]. The radioactive ligand for this assay was [$^3$H]methyltrienolone (R 1881). This synthetic steroid does not bind to plasma proteins, notably SHBG, and can be used as a specific probe for intracellular binding sites [28]. Non-specific binding was estimated after displacement of bound [$^3$H]R 1881 by unlabelled DHT [27] (Fig. 7b). Saturation analysis was carried out with ligand concentrations varying between 0.1 and 4 nM (Fig. 7c) and gave an apparent $K_D$-value of $5 \times 10^{-10}$M (Fig. 7) by Woolf plot analysis [26], a value identical with previously published data [29, 30].

These last two examples demonstrate that HIC may prove particularly useful in the field of steroid receptor assay. The very low assay blanks (see Table 2) increase the accuracy of binding sites measurement, the limit of detection being lower than 0.5 fmol of steroid bound per assay tube.

*Reliability of binding assays based on HIC*

A statistical evaluation of the reliability of assays based on HIC for the separation of protein-bound and free steroids was carried out on data obtained in our laboratory during routine application of this procedure (Table 2). The amount of radioactive tracer applied onto $C_{18}$S microcolumns varied from approx. $5 \times 10^3$ to $6 \times 10^4$ cpm, depending on the type of experiment (saturation analysis vs single-point assay). The assay blank (control tubes containing buffer and tracer only) was higher when the assay buffer included a protein component (e.g. gelatin in TGB) showing some non-specific binding activity (SHBG assay). When expressed as a function of total counts (TC), however, this blank was still very low (0.1% of TC). It was negligible (equivalent to counter background) when protein-free buffers were used, e.g. RAB 2 in the androgen receptor assay. This remarkable feature of the HIC separation procedure can increase the sensitivity of binding assays dramatically. As shown in Table 2, even low capacity binding can be measured with accuracy: both intra- and inter-assay coefficients of variation (CV) were lower than 10% in the androgen receptor assay. The limit of detection can be as low as 0.2 fmol of steroid bound under appropriate conditions (sp. act. of ligand, 50–100 Ci/mmol). In this respect, HIC appears to perform better than any of the current procedures used for steroid separation.

The intra-assay CVs varied between 3 and 7%, whereas the inter-assay CVs were lower than 10%. These figures compare well with those usually found in the literature for similar assays.

## DISCUSSION

When developing new procedures for the separation and quantitation of protein-bound and free steroids, the following criteria should be considered:

(a) the method should be rapid and economical to allow processing of large numbers of samples;
(b) separation should be accurate and reproducible for precise quantitation of binding

sites and determination of binding parameters, such as $K_D$, association and dissociation rates etc.;

(c) assay blanks should be as low as possible to achieve optimal sensitivity of the assay.

Although most of these criteria are usually met in the existing methods, further improvement resulting in increased sensitivity and reproducibility can be critical for many applications. The new method described here, based on HIC, offers many advantages over current procedures. Two important features of this chromatographic technique are: (a) the efficient and rapid separation of protein-bound and free steroids, which allows measurement of labile steroid–protein complexes; and (b) the very low assay blanks and the good recovery of both free and bound steroids, which result in increased sensitivity. As illustrated by examples of application presented in this paper, separation of protein-bound and free steroids by HIC is also accurate and reproducible. Therefore, HIC appears to be ideally suited to the measurement of steroid binding sites in low capacity biological systems, such as intracellular steroid receptors. Another interesting aspect of HIC is the versatility of the procedure: both bound and free steroids can be recovered quantitatively for further analysis. Therefore, specific binding can be measured even when active steroid-metabolizing enzymes are present in the preparation, because the exact nature of the bound and free steroids can be determined after further chromatographic analysis. This possibility is worth considering when studying interactions between enzymes and receptors in target cells and multiple binding sites. Moreover, since separation occurs rapidly under mild conditions, the binding protein itself can be recovered in an active form for further study, e.g. for examination of the binding characteristics in the absence of competing endogenous steroids etc.

We hope that this new, efficient and reliable procedure will find many applications in the field of steroid biochemistry. Further development can be envisaged for the separation of protein-bound and free lipophilic molecules other than steroids, such as prostaglandins, vitamins etc. When combined with the use of radioiodinated tracers of high specific activity, HIC may prove one of the most sensitive and accurate methods available at present for the quantitation of specific binding sites on soluble macromolecules.

*Acknowledgements*—The technical assistance of Ms L. Mankovsky, M. Thiébaud and J. Cicotti is gratefully acknowledged. We thank Drs A. M. Riondel, P. Bischof and J. B. Hutchison for their comments on the original manuscript, Professor A. Cuendet and Dr C. Lecoultre for providing human skin samples obtained at circumcision, Ms F. Urner for her contribution to the oestrogen receptor assay and Ms D. Roiron for typing successive versions of the paper. This work was supported by the Fonds National Suisse de la Recherche Scientifique (Grant No. 3.833.81).

## REFERENCES

1. Westphal U.: Steroid–protein interaction from past to present. *J. steroid Biochem.* **19** (1983) 1–15.
2. Jensen E. V., Greene G. L., Closs L. E., DeSombre E. R. and Nadji M.: Receptors reconsidered: a 20-year perspective. *Recent Prog. Horm. Res.* **38** (1982) 1–34.
3. Siiteri P. K., Murai J. T., Hammond G. L., Nisker J. A., Raymoure W. J. and Kuhn R. W.: The serum transport of steroid hormones. *Recent Prog. Horm. Res.* **38** (1982) 457–503.
4. Goertz G. R., Guérin M. A., Crépy O. C., Longchamp J. E. and Jayle M. F.: Détermination d'un indice d'affinité des stéroïdes sériques, fondée sur une méthode de mesure de la liaison à l'équilibre. *Eur. J. Biochem.* **35** (1973) 311–317.
5. Munck A.: General aspects of steroid hormone receptor interactions. In *Receptors and Mechanism of Action of Steroid Hormones* (Edited by J. R. Pasqualini), Marcel Dekker, New York, Part I (1976) pp. 1–40.
6. Verhoeven G., Heyns W. and De Moor P.: Ammonium sulfate precipitation as a tool for the study of androgen receptor proteins in rat prostate and mouse kidney. *Steroids* **26** (1975) 149–167.
7. Blondeau J. P., Corpéchot C., Le Goascogne C., Baulieu E. E. and Robel P.: Androgen receptors in the rat ventral prostate and their hormonal control. *Vitam. Horm.* **33** (1975) 319–344.
8. Odell W. O. and Daughaday W. H. (Eds): *Principles of Competitive Protein-Binding Assays*. Lippincott, Philadelphia, Pa (1971) pp. 310–311.
9. Erdos T. and Bessada R.: The hydroxylapatite-column assay of estrogen receptors: the routine analyses of many samples and the calculation of the equilibrium association constant. *J. steroid Biochem.* **10** (1979) 267–275.
10. Roy E. J. and McEwen B. S.: An exchange assay for estrogen receptors in cell nuclei of the adult rat brain. *Steroids* **30** (1977) 657–669.
11. Barkai U., Sherizly I. and Kraicer P. F.: A rapid assay for progesterone receptors in rat uterine cytosol: technique, changes at induction of decidualization. *J. steroid Biochem.* **14** (1981) 713–720.
12. Santi D. V., Sibley C. H., Perriard E. R., Romkins G. M. and Baxter J. D.: A filter assay for steroid hormone receptors. *Biochemistry* **12** (1973) 2412–2416.
13. Kawamoto H.: Estimation of testosterone binding capacity in the serum with hydrophobic resin. *Endocr. jap.* **23** (1976) 173–178.
14. Snyder L. R. and Kirkland J. J.: *Introduction to Modern Liquid Chromatography*. Wiley, New York (1974) pp. 200 ff.
15. Mickelson K. E. and Petra P. H.: A filter assay for the sex steroid binding protein (SBP) of human serum. *FEBS Lett.* **44** (1974) 34–38.
16. Peck E. J. and Clark J. H.: Effect of ionic strength on charcoal adsorption assays of receptor–estradiol complexes *Endocrinology* **101** (1977) 1034–1043.
17. Abraham G. E., Manlimos F. S. and Garza R.: Radioimmunoassay of steroids. In *Handbook of Radioimmunoassay* (Edited by G. E. Abraham). Marcel Dekker, New York (1977).
18. Lobl T. J.: Androgen transport proteins: physical properties, hormonal regulation, and possible mechanism of TeBG and ABP action. *Archs Androl.* **7** (1981) 133–151.
19. Kirchoff J., Soffie M. and Rousseau G.: Differences in the steroid-binding site specificities of rat prostate androgen receptor and epididymal androgen-binding protein (ABP). *J. steroid Biochem.* **10** (1979) 487–497.
20. Steimer Th., Mankovsky L. Theintz G. E. and Sizonenko P. C.: A new micro-assay for sex hormone binding globulin (SHBG) in human plasma. Unpublished data.

21. Hansson V., Larsen J. and Reusch E.: Physicochemical properties of the 5α-dihydrotestosterone binding protein in human male serum. *Steroids* **20** (1972) 555–574.
22. Bohn H.: Isolierung, Charakterisierung und quantitative immunologische Bestimmung des Steroid-bindenden β-globulins. *Blut* **29** (1974) 17–31.
23. Bohn H. and Kranz Th.: Untersuchungen über die Bindung von Steroidhormonen an menschliche Schwangerschaftsproteine. *Archs Gynak.* **215** (1973) 63–71.
24. Steimer Th., Theintz G. E., Bischof P., Sizonenko P. C. and Herrmann W. L.: Sex steroid binding to placental protein $SP_2$: A comparison with plasma sex hormone binding globulin (SHBG). Unpublished data.
25. Bayard F., Damilano S., Robel P. and Baulieu E. E.: Cytoplasmic and nuclear oestradiol and progesterone receptors in human endometrium. *J. clin. Endocr. Metab.* **46** (1978) 635–648.
26. Keightley D. D., Fisher R. J. and Cressie A. C.: Properties and interpretation of the Woolf and Scatchard plots in analysing data from steroid receptor assays. *J. steroid Biochem.* **19** (1983) 1407–1412.
27. Coulam C. B., Razel A. J., Kelalis P. P., Svensson J. and Spelsberg C.: Androgen receptor in human foreskin. *Am. J. Obstet. Gynec.* **147** (1983) 513–520.
28. Bonne C. and Raynaud J. P.: Methyltrienolone, a specific ligand for intracellular androgen receptors *Steroids* **26** (1975) 227–232.
29. Brown T. R., Rothwell S. W. and Migeon C. J.: Comparison of methyltrienolone and dihydrotestosterone binding and metabolism in human genital skin fibroblasts. *J. steroid Biochem.* **14** (1981) 1013–1022.
30. Kaufman M., Pinsky L., Hollander R. and Bailey J. D.: Regulation of the androgen receptor by androgen in normal and androgen-resistant genital skin fibroblasts. *J. steroid Biochem.* **18** (1983) 383–390.