J. steroid Biochem. Vol. 17, pp. 453 to 457, 1982
Printed in Great Britain. All rights reserved

0022-4731/82/100453-05$03.00/0
Copyright © 1982 Pergamon Press Ltd

AN ENZYME-IMMUNOASSAY (ELISA) FOR THE SEX STEROID-BINDING PROTEIN (SBP) OF HUMAN SERUM*

SANDRA BORDIN†, RAUL TORRES and PHILIP H. PETRA‡
Departments of Obstetrics and Gynecology and Biochemistry, University of Washington, Seattle, WA 98195, U.S.A.

(Received 29 December 1981)

SUMMARY

An enzyme-linked immunosorbent assay (ELISA) for the Sex Steroid-Binding Protein of human serum (hSBP)§ has been developed using purified monospecific hSBP antibodies labelled with alkaline phosphatase. The enzyme assay is as sensitive as the radioimmunoassay and at least ten times more sensitive than the radioligand assay and the "rocket" immunoelectrophoresis assay. The standard criteria of precision and reproducibility are satisfied. The assay employs stable reagents, inexpensive equipment, and is applicable to routine determinations of serum hSBP in the clinical and research laboratory.

INTRODUCTION

The plasma of humans and other species contains a protein, SBP§, which specifically binds 5α-dihydrotestosterone and testosterone with high affinity (see Ref. [1] for review). In the case of primates, the protein also specifically binds estradiol-17 β [2, 3]. Extensive specificity studies have revealed the most important features for achieving maximum binding to human and monkey SBP [2, 4, 5, 6]. These are: A planar steroid structure, an oxygenated function at the 3-position, and a free hydroxyl group at the 17β position. The protein was discovered in 1965 by incubating plasma samples with radioactive steroids [7, 8] and various assay procedures were devised to quantitate it using radioligands [9–12]. By consensus the protein was named SBP in 1969.‖ Recent availability of monospecific antibodies prepared against homogeneous human SBP [13, 14] allowed the development of a radioimmunoassay [14]. Although this assay has proven to be useful in our laboratory we are no longer using it. Our recent investigations have indicated that iodination of homogeneous SBP results in extensive losses in binding capacity to the antibodies (approximately 50%, unpublished data). The protein contains about two tyrosine residues per mole monomer [15] and iodination of these residues perhaps affect antibody binding affinity. In order to improve the immunoassay and to circumvent the frequent need of handling hazardous gamma-emitting radioisotopes, we have developed a non-isotopic immunoassay based on enzymatic activity measurements. This approach now widely used in immunochemistry [16], consists of covalently linking an enzyme to purified monospecific immunoglobulins, and measuring bound-antigen enzymatically with an appropriate substrate. In this report, we describe the determination of SBP concentration in human serum by using alkaline phosphatase-linked antibodies in conjunction with p-nitrophenylphosphate as the substrate.

* This work was supported by National Institutes of Health Grant HD-07190 and HD-13956.

† Present address: Department of Pathology, University of Washington, Seattle, WA 98195, U.S.A.

‡ To whom reprint requests should be addressed.

‖ *IVth meeting of the International Study Group for Steroid Hormones*, Rome, 1969. Other terms such as TeBG and SHBG were introduced later.

§ *Abbreviations:* SBP, Sex Steroid-Binding Protein; hSBP, human SBP, Tween-20, polyoxyethylenesorbitan monolaurate; PBS, phosphate buffered saline; BSA, bovine serum albumine; DHT, 5-αdihydrotestosterone.

MATERIALS AND METHODS

Reagents

Disposable polystyrene microtiter plates (8 × 12 cm) were purchased from Flow Laboratories, McLean, VA: alkaline phosphatase (type VII-S, specific activity 1100 units/mg protein, 8 mg/ml of ammonium sulfate suspension), Tween 20, p-nitrophenylphosphate and glutaraldehyde (grade II) from Sigma Chemical Co., St. Louis, MO.; diethanolamine from Eastman Organic Chemicals, Rochester, N.W.; Sepharose 6B from Pharmacia Ltd (Uppsala, Sweden), and bovine serum albumin (BSA) from Miles Research Laboratories, Elkhart, Indiana. [1,2-^{3}H]-DHT (50 Ci/mmol) was purchased from Research Products International.

Preparation of antibodies

Human SBP is purified to homogeneity using a recently modified procedure [17], 5 mM $CaCl_2$ is now added after the affinity chromatography step. Antisera

are prepared in rabbits as previously described [13]. Monospecific hSBP antibodies are obtained by immunoaffinity chromatography using homogeneous hSBP covalently attached to agarose [18].

Preparation of alkaline phosphatase anti-hSBP conjugate

The enzyme-antibody conjugate is prepared essentially as described previously [19]. An aliquot of 0.2 ml (1.6 mg) enzyme is centrifuged at 120 *g* for 10 min at 4°C. The supernatant (0.1 ml) is discarded and 0.38 ml of monospecific hSBP antibodies (1.4 mg/ml) is added. The resulting protein concentration of the solution is 4.4 mg/ml at 3:1 weight ratio of alkaline phosphatase to antibody. The solution is dialysed against PBS overnight at 4°C. An aliquot (4 microliters) of 25% glutaraldehyde is added and the solution is allowed to incubate for 2 h at 25°C. The solution is diluted to 1 ml with PBS and dialysed overnight at 4°C against PBS. The enzyme–antibody conjugate is purified by gel filtration on a column (1.5 × 30 mm) of Sepharose 6B, the conjugate elutes in the void volume with 0.05 M Tris–Cl, pH 8.0, [20]. The conjugate is concentrated by ultrafiltration (PM-10 membrane) to 4 ml and stored in the dark at 4°C in 0.05 M Tris–Cl, pH 8.0 in the presence of 1% bovine serum albumin and 0.02% NaN_3.

Enzyme-Immunoassay procedure

A "two-site" immunoenzymometric method is used [21]. In this method, the wells of microtiter plates are first sensitized by adding 0.2 ml of purified monospecific hSBP antibodies in coating buffer (0.05 M sodium carbonate buffer pH 9.6, 0.02% NaN_3) at a concentration of 5 μg/ml. The plates are incubated overnight at 25°C. The wells are then washed three times. The washing procedure is performed by "flooding" the plates with a solution of PBS containing 0.05% Tween-20. Plates are gently agitated for 3 min and the contents of the wells are decanted by the inversion of the plates. After the third wash, the plates are shaken dry. To control non-specific adsorption of the antigen to the plate, 0.2 ml aliquots of PBS-tween-BSA buffer (0.05% tween-20, 1mg/ml BSA) is added to each well and the plates are incubated for 30 min at 37°C. The plates are washed again with PBS containing 0.05% tween-20, 1 mg/ml BSA and 0.02% NaN_3. Aliquots of 0.2 ml of diluted serum (100–1000fold) containing standard or unknown concentrations of hSBP are added to each well. Plates are incubated overnight at 25°C. After washing 0.2 ml of the stock conjugate (diluted 1:100 with PBS-tween-BSA buffer) is added into each well. Plates are incubated 3 h at 37°C. Excess conjugate is washed out and the amount of alkaline phosphatase bound to the wells is determined by adding 0.2 ml of *p*-nitrophenylphosphate substrate (1 mg/ml in 1 M diethanolamine buffer at pH 9.8 containing 0.5 mM MgCl). This substrate concentration is sufficient to keep the enzyme conjugate complex saturated during the extent of the reaction; the hydrolysis reaction of *p*-nitrophenylphosphate catalysed by the enzyme–conjugate–hSBP complex exhibits linear zero-order kinetics for at least 1 hr. After a 20 min-incubation at room temperature, the reaction is stopped by adding 50 μl of 2 M NaOH in each well. The content of the wells are diluted to 1 ml water and absorbances are measured at 403 nm in a Beckman Model DB-GT spectrophotometer. All assays are done in triplicate. The optimum conditions for the assay are as described, however, reliable results have been obtained by performing the original antibody adsorption at 37°C for 90 min instead of overnight at room remperature. This approach may be used to save time.

Radioligand assay procedure

The concentration of hSBP in serum is determined by the DEAE cellulose filter paper assay using [^{3}H]-DHT [12].

RESULTS

The principle and experimental design behind the enzyme-immunoassay is shown in Fig. 1. Purified monospecific antibodies are first adsorbed onto wells in a microtiter plastic plate. Albumin is then added to make sure that all sites not previously saturated with antibodies are occupied. This is important to prevent nonspecific adsorption of the antigen. A sample containing SBP (black circles) along with other proteins (other symbols) is added to each well. The plates are incubated, washed, and enzyme–antibody conjugate is added. The amount of enzyme–conjugate is then directly proportional to the amount of SBP present in each well. The data presented here indicate that in the case of SBP such a procedure will yield linear standard curves from which unknown concentrations of SBP can be determined.

Figure 2 shows a typical standard calibration curve using diluted samples of human serum. Depending upon the sensitivity of the spectrophotometer, the limit of detection of about 0.1 nM SBP per assay. This sensitivity is well within the range of the radio-immunoassay (14, and unpublished results). The standard calibration curve is shown to be linear from the limit

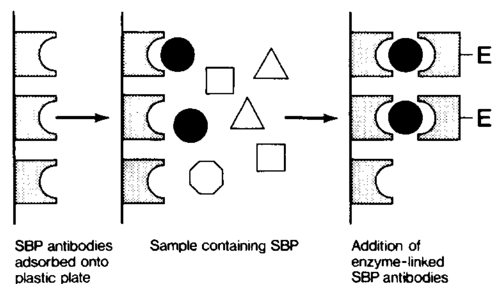

Fig. 1. Schematic diagram representing the principle and experimental design of the enzyme-immunoassay. (●) SBP; (, △, □) other proteins present in the sample.

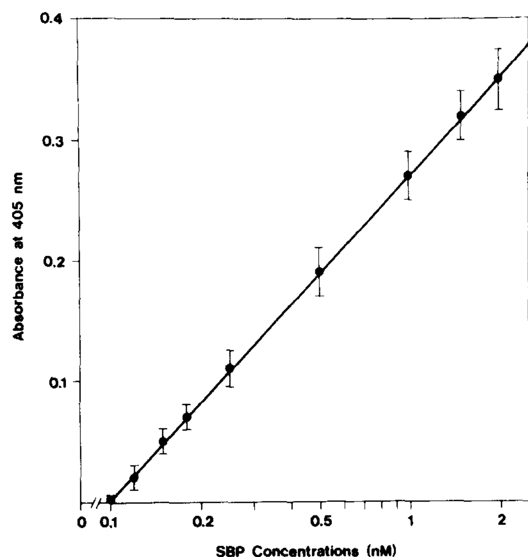

Fig. 2. Calibration curve for the enzyme immunoassay of hSBP. Each point shows mean ± SD of six runs done in triplicate. The SBP concentration was determined by the filter assay.

of sensitivity up to 2 nM SBP (36 ng SBP per assay) and actually remains linear up to 5 nM SBP. A blank of only 0.02–0.04 absorbance units at 405 nm is obtained when serum is replaced by buffer with all other components remaining the same. Since a double-beam spectrophotometer is used for the data in Fig. 2 this blank value is automatically subtracted.

Figure 3 represents the correlation between the filter assay and the enzyme-immunoassay carried out on twelve individual male and female sera using the standard curve of Fig. 2. The data indicate complete correlation between the radio-ligand assay and the enzyme-immunoassay indicating that the two assays may be used interchangeably. Table 1 shows the reproducibility of the assay when performed on the

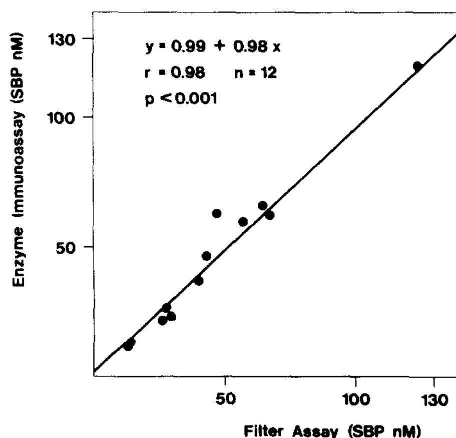

Fig. 3. Comparison between the enzyme immunoassay and the radioligand assay. Each point represents the mean of triplicates.

Table 1. Coefficient of variation (C.V.) of the enzyme immunoassay*

hSBP (nM)	C.V.† "within-run" (%)	C.V.‡ "between-run" (%)
0.10	9.9	26
0.25	11	19
1.00	3.1	28

*The standard curve of Fig. 2 was used to attain SBP values. †Ten replicates on the same day. ‡Three replicates on each of six different days.

same or different days using different ranges of hSBP concentrations. The precision is adequate in the case of intra-assay conditions; however, the inter-assay data indicate that the assay cannot be compared from day to day with the use of the same standard curve. This variation is due to the different adsorbative capacity which each microtiter plastic plate exhibits. It is therefore necessary to standardize each plate by "running" a standard curve in parallel with the unknowns applied on the same plate. We do not consider this a drawback for the assay since such precautionary measures should be taken anyway, particularly when measuring extremely low concentrations of an unknown protein such as hSBP.

DISCUSSION

The sensitivity of the enzyme-immunoassay depends mainly on the purity of the monospecific antibodies. This is particularly important in this case because monoclonal SBP antibodies are not yet available. The SBP antibodies are purified by adsorbing the isolated rabbit gamma-globulins onto an immuno-adsorbent consisting of homogenous human SBP covalently attached to agarose [18]. This procedure allows the isolation of antibodies of very high specific activity (1% of the total gamma-globulin fraction) for the preparation of the enzyme–antibody conjugate. Once this condition has been met, the enzyme-immunoassay is very simple and clearly has a definite advantage over the radio-immunoassay for two reasons. *First*, the pure antigen, SBP, is not required for constructing the standard curve as in the case of RIA. Commonly available pregnancy serum or normal serum can be used for that purpose as shown in Fig. 2. *Second*, frequent iodination of the antigen with gamma-emitting isotopes is therefore eliminated. In contrast, the enzyme–antibody complex is very stable and can be kept frozen at −20°C for at least a year. The relative ease in the routine performance of the enzyme-immunoassay is due to the fact that the major efforts are placed prior to the performance of the assay in obtaining pure and monospecific SBP antibodies. This is done easily now by first purifying about 12–15 mg of homogenous human SBP in one preparation according to our published pro-

cedure [17]. About half of the SBP obtained is used to synthesize the immunoadsorbent which is stable for at least a year when kept in the presence of 0.1% sodium azide at 4°C. The rest of the SBP is kept frozen at −20°C for antibody production. This amount of SBP is sufficient to produce at least a year-supply of monospecific antibodies.

A few words should be mentioned about the "rocket" immunoelectrophoresis assay which has been used for measuring SBP directly. For a number of years this laboratory has been using this assay for studying the cross-reactivity of SBP from various species [13] as well as for quantitating SBP in plasma (unpublished procedures). Although this assay has proven valuable, it is 10 times less sensitive than the enzyme-immunoassay and the RIA and about half to three times less sensitive than the standard radio-ligand assays such as the filter assay [12]. For instance, depending upon the antibody titer, "rockets" of not more than 1 cm in height are obtained when estimating SBP in human male plasma (30 nM SBP and lower). The heights of these immunoprecipitates are too small to be able to detect small changes accurately at such concentrations of SBP. Recently, these observations have been confirmed [22]. Since the enzyme-immunoassay and radio-ligand assay have now been correlated (Fig. 3), we recommend for now the use of the radio-ligand assays for measuring SBP levels below 30 nM when either RIA or ELISA are not available.

It should be realized that the availability of an immuno-assay for SBP should not preclude the use of radioligand assays for measuring SBP concentrations. Results obtained with immunoassays cannot help in distinguishing between active and inactive SBP molecules because the assay is not based on, or dependent upon, a functioning steroid-binding site. We have found, for instance, that native and denatured SBP are both recognized by the antibodies. This finding should be expected because the steroid-binding site represents only a small portion of the entire protein surface. We suspect that these conclusions will also apply to other steroid-binding proteins including steroid receptors for which immunoassays are being developed [24]. In particular, an ELISA method for measuring estrogen receptors in human breast tumors will soon be available [25]. As in the case of SBP, measurements of receptors by ELISA alone will most likely reflect the entire population of receptor molecules without distinguishing between active, inactive, or partially active species. Radio-ligand assays may have to be included in special cases when the receptor status of a particular tumor is being determined only by immunochemical methods.

Despite these limitations, the development of the enzyme-immunoassay will nevertheless permit to explore new avenues of investigation concerning the physiological role of SBP. Recently, we have presented evidence that SBP can cross the plasma membrane and enter cells [18]. Furthermore, the first experimental evidence for the presence of SBP in normal liver as well as other tissues has been presented [18] setting the stage for studying the biosynthesis of SBP and its control in normal tissues. Since the evidence is primarily immunochemical in nature, questions were raised on the possible cross-reactivity of the SBP-antibodies with ABP, the Androgen Binding Protein [18]. Although this may be true for male reproductive tissues from various species where ABP is thought to exist [26], we still detect the presence of SBP in tissues not primarily involved in reproductive functions, such as the liver, as well as tissues of the female [23] where ABP does not exist. Because of the anticipated fact that SBP in tissues will be present in very low amounts, we view the availability of the enzyme-immunoassay as instrumental in being able to pursue physiological studies leading to a further understanding of SBP function.

REFERENCES

1. Westphal U.: *Steroid–Protein Interactions* Springer-Verlag, New York (1971).
2. Petra P. H. and Schiller H. S.: Sex steroid-binding protein in the plasma of *Macaca nemestrina*. *J. steroid Biochem.* **8** (1977) 655–661.
3. Renoir J.-M., Mercier-Bodard C. and Baulieu E.-E.: Hormonal and immunological aspects of the phylogeny of sex-steroid binding plasma protein. *Proc. natn. Acad. Sci., U.S.A.* **77** (1980) 4578–4582.
4. Kato T. and Horton R: Studies of testosterone binding globulin. *J. clin. Endocr. Metab.* **28** (1968) 1160–1168.
5. Vermeulen A. and Verdonck L.: Studies on the binding of testosterone to human plasma. *Steroids* **11** (1968) 609–635.
6. Mickelson K. and Petra P. H.: Purification and characterization of the sex steroid-binding protein of rabbit serum. *J. biol. Chem.* **253** (1978) 5293.
7. Mercier-Bodard C., Alfsen A. and Baulieu E. E.: A testosterone binding globulin. *Proc. 2nd Symp. Ster. Horm.* Ghent, Belgium, 1965. *Excerpta Med. Intern. Congr. Serv.* No. 101 (1966) p. 212.
8. Rosenbaum W., Christy N. P. and Kelly W. G.: Electrophoretic evidence for the presence of an estrogen-binding β-globulin in human plasma. *J. clin. Endocr. Metab.* **26** (1966) 1399–1403.
9. Pearlman W. H. and Crepy O.: Steroid–protein interaction with particular reference to testosterone binding by human serum. J. biol. Chem. **242** (1967) 182–189.
10. Rosner W.: A simplified method for the quantitative determination of testosterone-estradiol-binding globulin activity in human plasma. *J. clin. Endocr. Metab.* **34** (1972) 983.
11. Ritzen E. M., French F. S., Weddington S. C., Nayfeh S. N. and Hanson Y.: Steroid binding in polyacrylamide gels. *J. biol. Chem.* 249 (1974) 6597–6604.
12. 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.
13. Bordin S., Lewis J. and Petra P. H.: Monospecific antibodies to the sex steroid-binding protein (SBP) of human and rabbit sera. *Biochem. biophys. Res. Commun.* **85** (1978) 391–401.
14. Mercier-Bodard C., Renoir J. M. and Baulieu E. E.: Further characterization and immunological studies of human sex steroid binding plasma protein. *J. steroid Biochem.* **11** (1979) 253–259.

15. Petra P. H.: The serum sex steroid-binding protein. Purification, characterization and immunological properties of the human and rabbit proteins. *J. steroid Biochem.* **11** (1979) 245–252.
16. Engvall E. and Perlman P.: Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-labelled anti-immunoglobulin in antigen-coated tubes. *J. Immunol.* **109** (1972) 129–135.
17. Petra P. H. and Lewis J.: Modification in the purification of the sex steroid binding protein of human serum by affinity chromatography. *Anal. Biochem.* **105** (1980) 165.
18. Bordin S. and Petra P. H.: Immunocytochemical localization of the sex steroid-binding protein of plasma in tissues of the adult monkey. Macaca nemestrina. *Proc. natn. Acad. Sci., U.S.A.* **11** (1980) 5678–5682.
19. Engvall E. Jonsson K. and Pearlman P.: Enzyme-linked immunosorbent assay. II. Quantitative assay of protein antigen, immunoglobulin G, by means of enzyme-labelled antigen and antibody-coated tubes. *Biochem. biophys. Acta.* **251** (1971) 427–434.
20. Avrameas S.: Coupling of enzymes to proteins with glutaraldehyde. *Immunochemistry* **8** (1969) 1175–1179.
21. Clark M. F., and Adams A. N.: Characteristics of the microplate method of ELISA for the detection of plant viruses. *J. gen. Virol* **34** (1977) 475–483.
22. Egloff M., Vranchky R. Tardivel-Lacombe J. and Degrelle H.: Immunochemical characterization and quantitation of human sex steroid binding plasma protein. *Steroids* **37** (1981) 455.
23. LoBue G., Bordin S. and Petra P.H.: Immunocytochemical localization of sex steroid-binding protein (SBP) of human plasma in tissues of the human female reproduction tract. *Twenty-eight Meeting of the Society for Gynecological Investigation.* Abstract No. 81, (1981).
24. Greene G. L.; Closs L. E., Fleming H, DeSombre E. R. and Jensen E. V.: Antibodies to estrogen receptor. Immunochemical similarity of estrophilin from various mammalian species. *Proc. natn. Acad. Sci. U.S.A.* **74**, (1977) 3681–3685.
25. Jensen E. V.: Hormone dependency of breast cancer. *Cancer* **47** (1981) 2319–2326.
26. Hansson V., Ritzen E. M., French F. S. and Nayfeh S. N.: *Handbook Physiol.* **5** (1975) 173–201.