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SHORT COMMUNICATION

A SOLID-PHASE RADIOIMMUNOASSAY OF SERUM DEHYDROEPIANDROSTERONE SULPHATE USING A MONOCLONAL ANTIBODY

R. E. KNYBA, D. Y. WANG[1] and V. E. FANTL
Imperial Cancer Research Fund, P.O. Box 123, Lincoln's Inn Fields, London WC2A 3PX, U.K.

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Summary—A monoclonal antibody directed against dehydroepiandrosterone, but with high affinity for dehydroepiandrosterone sulphate (DHA-S), has been used to develop a solid phase radioimmunoassay for measuring serum DHA-S. The antibody was covalently linked to polyacrylamide microbeads with no change in binding characteristics. The procedure requires only the chromatography of serum on anion-exchange cellulose before assaying the equivalent of 0.25 μl serum. The method is precise, accurate and specific and can detect 19.5 pg of DHA-S. Serum DHA-S levels measured by this method were in good agreement with those found in a validated radioimmunoassay method involving hydrolysis. The method is quick and one operator could assay 50 blood specimens per day. DHA-S levels in serum from 50 men and 86 women were in agreement with those in the literature. With the availability of theoretically limitless quantities of consistently high quality monoclonal antibodies the advantages of developing solid phase radioimmunoassays for steroids is discussed.

INTRODUCTION

Blood dehydroepiandrosterone sulphate (DHA-S) is widely measured as an aid in the clinical assessment of adrenal steroid secretion and has largely replaced the traditional estimation of 24 h urinary 17-oxosteroid excretion [1, 2]. Methods of determining DHA-S are, therefore, needed which are accurate and quick to perform.

This laboratory has produced various monoclonal antibodies to steroids [3–5] amongst which are antibodies with high affinity for DHA-S [6]. This has provided an opportunity to develop radioimmunoassay methods which have advantages over conventional methods, but which entail the use of large quantities of relatively pure antibody. Most of the present methods for determining steroids rely on charcoal for the separation of bound from free ligand. Covalent bonding of antibody to a solid phase support not only avoids the necessity of using charcoal but ensures that the separation of bound from free ligand occurs under equilibrium conditions.

This paper deals with the covalent binding to polyacrylamide microbeads of a monoclonal antibody raised to dehydroepiandrosterone (DHA) and its use to measure serum DHA-S.

EXPERIMENTAL

Monoclonal antibody

The production and binding characteristics of the monoclonal antibody (7D4) have been previously described [6]. Although this antibody was directed towards DHA it had a high affinity for DHA-S ($K_a = 2 \times 10^{10}\ M^{-1}$) and was therefore potentially suitable for measuring DHA-S.

Chemicals

[1,2,6,7-^{3}H]Dehydroepiandrosterone (64 Ci/mmol) and [7-^{3}H]dehydroepiandrosterone sulphate (24 Ci/mmol) were obtained from Amersham International plc, Bucks, U.K. and New England Nuclear Boston, U.S.A., respectively. Polyacrylamide microbeads (Immunobeads) and coupling reagents were purchased from Biorad, Watford, U.K. Protein A-sepharose and anion-exchange cellulose DE23 were bought from Pharmacia, Sweden and Whatman Chemical Separation Ltd, Kent, U.K. respectively. Lipidex-5000 and scintillation fluid (Picofluor 15) were obtained from Packard Instrument Co. U.S. Other chemicals were obtained from B.D.H., U.K. Screw-topped polypropylene tubes with conical bottoms [cat. no. 72.693] were supplied by Sarstedt Ltd, Leicester, U.K. Isotonic phosphate buffered saline (pH 7.4) containing gelatine (0.1%) and sodium azide (0.1%) was used for radioimmunoassay (RIA buffer).

Preparation of solid phase antibody

Ascites fluid (1 ml) containing 7D4 was mixed with 1 ml of 0.1 M phosphate buffer (pH 8) and centrifuged for 15 min at 2000 ***g***. The resulting supernatant was filtered (Millipore filter, 0.45 μ) and the monoclonal antibody purified on a Protein A-sepharose column by the method of Ey *et al.* [7]. Column fractions were monitored for DHA-S binding activity. The peak binding was found to occur in the fractions eluted at pH 6 and corresponded to the murine immunoglobulin of subclass G_1 to which 7D4 belongs [6]. Purified 7D4 antibody (1.8 mg) was coupled to 100 mg of Immunobeads using 1-ethyl-3[3-dimethylamino propyl] carbodiimide hydrochloride by the method recommended by the manufacturers of Immunobeads. In terms of steroid binding activity about 10% of the purified antibody had been linked to the microbeads. Aliquots of the antibody-coated microbeads were lyophilised and their activity has not changed after over a year in storage.

Preparation of serum

Lack of specificity of the monoclonal antibody 7D4 [6] required the isolation of DHA-S by passing the serum (50 μl) through a column (3 × 0.5 cm) of Whatman DE23 advanced ion-exchange cellulose. After loading the serum directly onto the column, the column was washed with 2 ml

[1]To whom correspondence should be addressed.

of water and the DHA-S was eluted in 2 ml of 0.2 MNaCl. For normal levels of DHA-S, 10 μl of this fraction was sufficient for assay. Recovery from the columns measured for 10 assays was 75.4 ± 0.9% (±SE) and DHA-S levels were adjusted for this.

Assay

Each assay was carried out in a screw-topped polypropylene tube containing tritiated DHA-S (10,000 dpm), solid-phase antibody (sufficient to give 50% binding at zero concentration) 25 μg of unreacted microbeads, 12.5 μg Nonidet P40 and standard, or unknown, in a final volume of 500 μl using RIA buffer. The tubes were incubated at 4°C for at least 2 h, or when convenient overnight. After centrifugation at 4°C the supernatant liquid was decanted, the microbead pellet resuspended in water (50 μl) and phosphor (1.25 ml) added. The tubes were stoppered, shaken, supported in glass counting vials and left for 3 h before the tritium content was determined.

RESULTS

Radioimmunoassay

Addition of unreacted microbeads to the incubation mixture permitted a visible pellet of microbeads to be formed on centrifugation of the assay tubes. The increase in non-specific binding that this produced was decreased by using Nonidet P40 in the incubation mixture. Incubation of the reaction mixture at 4°C for 2 h (which the manufacturers claim is the least period that the microbeads will remain in suspension) was sufficient time for the assay system to reach equilibrium, but normally the reaction was allowed to continue overnight. The use of polypropylene assay tubes which could eventually be tightly capped permitted incubation and scintillation counting to be performed in the same tubes. It was found necessary to resuspend the microbead pellet, after centrifugation, in 50 μl of water before adding phosphor and to allow this mixture to stand for 3 h to ensure maximum removal of radioactivity from the microbeads.

Validation of the assay

Specificity. The cross-reaction of the monoclonal antibody with DHA (25%), DHA glucuronide (12%) and androsterone sulphate (31%) remained unchanged after linking it to the microbeads. Only this last steroid is likely to interfere in the assay since its concentration in blood is 25–50% of that of DHA-S [8, 9]. However, addition of 100 pg of androsterone sulphate to an assay tube containing 125 pg DHA-S gave an apparent titre of 136 pg DHA-S indicating that under normal circumstances androsterone sulphate is unlikely to cause significant interference in the assay.

The DHA-S fractions from 28 serum samples were prepared as described and portions of these extracts solvolysed [10] and chromatographed on Lipidex-5000. Comparison of assay values before and after this extra purification step (correction being made for the loss of the sulphate group) showed a high degree of correlation ($r = 0.99$). The linear regression equation was $y = 1.016x - 1.74$ where y and x are the titre before and after Lipidex-5000 chromatography.

Different amounts of female serum extract (5, 10 and 20 μl) were assayed and the inhibition curves compared with that of the standard. The lines for the serum assays were essentially parallel to that of the standard curve. This parallelism results in the close agreement between the titres using 5, 10 and 20 μl of extract. Taking the 5 μl assay values as 100% the titres in 10 and 20 μl are 93 ± 4% (±SE) and 104 ± 4% (±SE), respectively. The titre range was 50–223 μg/100 ml serum.

The results of this assay were compared with those using a previously validated radioimmunoassay method which involved acid hydrolysis at 120°C and solvent extraction [11]. The linear regression equation on 20 sera was $y = 0.98x + 12.8$ where x are results previously obtained and y those obtained with the present method. The correlation was $r = 0.94$.

Accuracy, precision and sensitivity

The equivalent of 25, 50 and 100 μg of DHA-S per 100 ml serum was added to serum pools. The amount of DHA-S recovered is seen to be satisfactory (Table 1).

Intra-assay variation was assessed by comparing duplicate samples. The coefficients of variation are shown in Table 2.

Inter-assay variation was assessed by comparing the results of two quality control pools measured over 32 routine assays. The titres were 177 and 232 μg/100 ml, respectively with both having a coefficient of variation of 9.9%.

Estimations were done on 12 zero concentration tubes and the smallest quantity which could be detected with 95% certainty was 19.5 pg. This represents a titre of 10.2 μg/100 ml in the method as described although this could be considerably lowered if larger volumes of DHA-S extract were used.

Serum DHA-S levels in normal males and females

The concentration of DHA-S was measured in 50 men and 86 women, respectively. There was a statistically significant negative correlation between the amount of serum DHA-S and age for both sexes. The linear regression equations were:

$$y = 403 - 3.03x \text{ for males and,}$$
$$y = 288 - 2.40x \text{ for females}$$

where y is the serum DHA-S concentration and x is age. The regression coefficients were 0.29 and 0.23, respectively, and both correspond to significance levels of $P < 0.05$.

DISCUSSION

The method described provides a specific, quick and convenient method for estimating the concentration of DHA-S in blood. The use of cellulose anion-exchange chromatography avoids the necessity of hydrolysing the sulphate moiety from DHA-S and therefore the need of an organic solvent to prepare extracts suitable for assay. The advantage of the solid-phase assay is that the separation of bound from free ligand does not involve a change in equilibrium and is, therefore, not critically time dependent as in the more conventional methods employing charcoal.

Table 1. Assessment of accuracy

Addition	Before addition	After addition	Difference
25 μg	184.6 ± 16.5	210.0 ± 19.6	25.4
50 μg	173.7 ± 14.6	222.6 ± 7.5	48.9
100 μg	179.0 ± 15.5	280.7 ± 22.6	101.7

Known amounts of DHA-S were added to sera and DHA-S measured before and after addition. The results are expressed as mean ±1 SD ($n = 11$) and the units for all columns are μg/100 ml serum.

Table 2. Assessment of precision

Titre	C.V.	N
0–100	10.0%	47
101–150	6.0	50
151–200	6.3	42
201–250	6.7	33
>250	4.9	34

DHA-S has been measured in N duplicates and coefficient of variation (C.V.) calculated for various ranges of titres (μg/100 ml serum).

The levels of serum DHA-S found by this method agree with those reported in the literature [12, 13, 14]. The significant negative correlation between age (x) and blood DHA-S levels (y) is in accord with previous studies [8, 9]. The regression equations found in this investigation are similar to those published earlier from this laboratory [9]. Thus for males the equation is $y = 402 - 3.03x$ compared with $y = 402 - 4.45x$. Similarly in females the equation is $y = 288 - 2.4x$ compared with $y = 301 - 3.41x$.

Although 50 samples can be easily assayed in a day, nevertheless, it is a disadvantage to employ a chromatographic step. It was hoped that by using a monoclonal antibody its specificity would have been sufficient to permit direct measurement of DHA-S in diluted serum samples. Our experience with monoclonal antibodies raised against steroids is that only a small proportion of antibodies are suitable, in terms of specificity and affinity, for radioimmunoassay. Since there are polyclonal antibodies which are suitable for the direct assay of DHA-S [12] it is presumably only a matter of raising a sufficient number of monoclonal antibodies to identify a clone producing an antibody of similar high specificity. Such an antibody would enable the measurement of large numbers of samples such that data handling could become the rate limiting factor using the present method. The method could also be improved in terms of cost by the use of I^{125}-labelled ligands, this would avoid the use of scintillation fluid and reduce the assay-time.

This paper highlights the considerable potential of solid phase systems for measuring steroids in a large number of samples. This has now been made more attractive by the advent of monoclonal antibodies which ensures the indefinite supply of a consistently high quality antibody.

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