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A SIMPLE NON-CHROMATOGRAPHIC RADIOIMMUNOASSAY FOR PLASMA ANDROSTENEDIONE

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SUMMARY

A simple non-chromatographic radioimmunoassay method for the measurement of androstenedione in plasma is described. Polyethylene glycol is employed to separate the bound fraction, which is counted directly by adding scintillator to the precipitate in the assay tube. This simplifies the assay and improves the precision at low steroid concentrations. The method gives results which show a good correlation with a previously validated method.

INTRODUCTION

Methods for measuring androstenedione in plasma have usually employed a chromatographic purification stage to achieve adequate specificity. Various procedures have been used including alumina column chromatography [1, 2], Sephadex LH20 column chromatography [3, 4], celite column chromatography [5], and thin layer chromatography [6]. The inclusion of a chromatographic purification stage, prior to radioimmunoassay of the extract makes it more difficult and tedious to handle a large number of samples. The aim of the present method was to develop an assay for androstenedione, in which no chromatographic purification stage was necessary, but which still retained the specificity required to obtain accurate and reliable results.

The method described below involves a simple extraction stage with hexane:ether, followed by evaporation and incubation of the residue with the antiserum, separation of the bound and free fractions with polyethylene glycol and finally counting the bound fraction in a liquid scintillation counter.

MATERIALS

Androstenedione was supplied by Steraloids Ltd, Croydon, England. [1,2,6,7, (*n*) ^{3}H]-androstenedione (104 Ci/mmol) was obtained from the Radiochemical Center Ltd., Amersham, England. All chemicals, except where stated were supplied by Fisons Scientific Apparatus Ltd., Loughborough, Leicestershire. Human gamma globulin, HG 11, from Sigma London Chemical Company Ltd., Poole, Dorset. Bovine plasma albumin from Armour Pharmaceutical Co., Eastbourne, Sussex. 0.05M Borate buffer pH 8.0 was prepared from boric acid, 6.2 g in distilled water made up to 2 l. containing 2% methanol and the pH adjusted. Scintillation solution was prepared by dissolving 6 g *p*-terphenyl and 0.08 g dimethyl POPOP in 2 l. of sulphur free toluene containing 2% methanol. Extraction tubes manufactured by Quickfit & Quartz Ltd., Stone, Staffordshire, type MF 24/0/4 with stoppers type SB 10. Glass assay tubes, 50 mm × 10 mm with plastic closures, controlled neck shell vial No. 3/B/D/08/015/01/A manufactured and supplied by F. B. G. Trident Ltd, Temple Cloud, Bristol, were rinsed with methanol before use. Racks for holding assay tubes during decanting stage are Analmatic racks type 401/2113 fitted with adaptors type 401/2113/12 supplied by Searle Instrumentation, P.O. Box 53, Lane End Road, High Wycombe, Buckinghamshire. Constriction pipettes manufactured by H. E. Pedersen, 7 Sommerstedgade, DK1718, Copenhagen V, Denmark. Compu-Pet dispenser, dual micro model from William R. Warner Ltd., Eastleigh, SO5, 3ZQ, England.

ASSAY METHOD

An aliquot of plasma (0.3 ml), previously mixed on a vortex mixer was pipetted into an extraction tube using a constriction pipette. After adding 4 ml of *n*-hexane–diethyl ether (4:1, v/v) the tubes are stoppered and shaken horizontally on a mechanical shaker for 10 min. The two phases are allowed to separate and 2 × 0.5 ml aliquots of the extract transferred to assay tubes using a constriction pipette. 0.1 ml of 10% v/v propylene glycol in methanol was added to all tubes and the contents of the tubes evaporated at 45°C under vacuum until only the propylene glycol residue remained.

Standard quantities of androstenedione 10, 20, 40, 80, 160, 320 pg in 0.1 ml of methanol were pipetted into assay tubes from individual working solutions. Into these tubes, as well as two tubes for the estimation of non-specific binding and two zero antigen tubes was added 0.1 ml of 10% propylene glycol in methanol. The contents of the tubes were evaporated as above.

The working solution of antiserum was prepared in borate buffer at 1:30,000 dilution containing 0.2%

(v/v) bovine plasma albumin; 0.1% (v/v) human γ-globulin and tritiated androstenedione (approximately 40,000 d.p.m. per ml). After mixing gently, 0.25 ml was added to all tubes, using the Compu-Pet automatic dispenser. The amount of radioactivity added to each tube was determined by dispensing the same volume of antiserum solution into two extra tubes and counting after the addition of scintillator (1.2 ml).

To the two non specific binding tubes 0.25 ml of buffer solution was added containing all the constituents with the exception of the antiserum.

The walls of the assay tubes were rinsed with antiserum by gently mixing on a vortex mixer for 1 or 2 s. All tubes were incubated overnight at 4°C or for half an hour at room temperature followed by one hour at 4°C.

After incubation, the tubes were stood in an ice/water bath during the addition by displacement pipette of an equal volume (0.25 ml) of 30% polyethylene glycol in borate buffer.

Polyethylene glycol may be added more conveniently to large numbers of assay tubes using the Compu-Pet. Due to the very high viscosity of the reagent, and because the Compu-Pet is normally calibrated to dispense aqueous solutions, adjustments have to be made to the Compu-Pet settings and concentration of the polyethylene glycol solution such that the final concentration of polyethylene glycol in the assay tube is 15%. This may best be achieved by leaving the instrument calibrated for aqueous solutions but depressing the buttons to deliver 0.4 ml and reducing the concentration of the working solution of polyethylene glycol to 27%. This arrangement has proved quite satisfactory and was arrived at by using a solution of polyethylene glycol in buffer containing a radioactive marker (e.g. tritiated testosterone), in order to compare the volumes dispensed automatically against those dispensed manually.

After the addition of the polyethylene glycol, the contents of each tube were mixed thoroughly on a vortex mixer for several seconds, left to stand for 10 min in the ice/water bath and finally centrifuged at 1500 *g* for 30 min at 4°C.

The tubes, after replacing in the racks, were inverted to decant the supernatant (free) to waste and left to drain, inverted on several layers of absorbent paper for 10 min. Scintillator (1.5 ml) was added and the stoppered tubes shaken horizontally on a mechanical shaker, for 10 min. All the tubes were then placed in standard glass counting vials whose caps had been drilled out such that the assay tubes were suspended centrally and vertically by the rim of their stoppers. They were then counted in a liquid scintillation counter at ambient temperature for 10 min or to an error of 2%.

CALCULATION

The bound c.p.m. (B) of the duplicate standards were calculated as a percentage of the mean bound c.p.m. (Bo) of the zero tubes. These percentages B/Bo were plotted against the mass in pg of androstenedione on a logarithmic scale to give the standard curve as shown in Fig. 1. Similarly the mean bound c.p.m. for the plasma aliquots were calculated and the relative mass of androstenedione found from the standard curve.

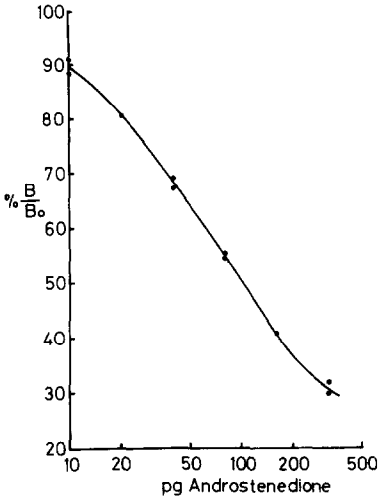

Fig. 1. A typical standard curve. The bound c.p.m. are expressed as a percentage of the bound c.p.m. in the zero tube, plotted against the mass of androstenedione on a log. scale.

VALIDATION

The precision of the assay was assessed from within batch analyses of duplicate plasma samples. This gave a within assay variation with coefficient of variation (C.V.) of 8.8% (N = 24). The between assay variation was assessed by assaying aliquots of two plasma pools in successive batches. This gave a C.V. of 10% (N = 23) with a mean value of 4.2 nmol/l and a C.V. of 9.8% (N = 12) with a mean value of 1.9 nmol/l.

When the following volumes 0.1, 0.2, 0.3, 0.4 and 0.5 ml of one plasma sample were assayed good linear relationship was obtained as shown in Fig. 2.

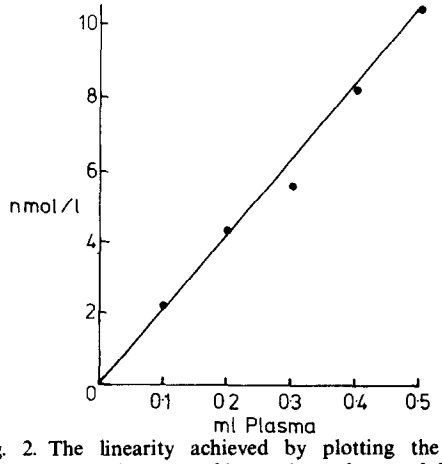

Fig. 2. The linearity achieved by plotting the values obtained from the assay of increasing volumes of the same plasma specimen.

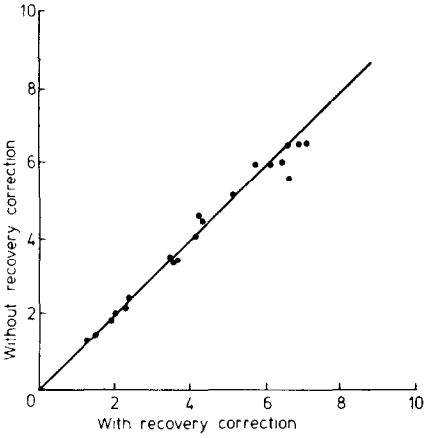

Fig. 3. The results obtained with and without making a correction for methodological losses. Values expressed as n.mol./l.

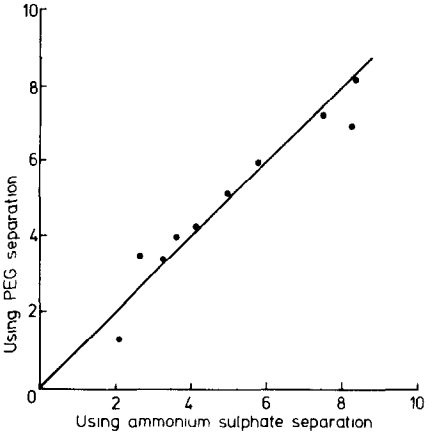

Fig. 4. The values obtained when 10 plasma specimens were assayed by the method using polyethylene glycol separation and that using ammonium sulphate precipitation after purification of the plasma extracts by alumina column chromatography. Values expressed in nmol/l.

The recovery from plasma was estimated by adding 0.5 ng and 1.0 ng of unlabelled androstenedione to a previously assayed plasma sample and reassaying. In three experiments, where 0.5 ng was added, recoveries of 96, 101 and 104% were obtained. In four experiments where 1.0 ng was added, a mean recovery of 101% was obtained with a range of 93–106%.

In addition, confirmation that the extraction step was quantitative was obtained from an experiment in which radioactive androstenedione, added to plasma samples, was extracted and counted. This gave a mean recovery of 96.6% and a C.V. of 4.1% ($N = 28$). Furthermore, when 10 plasma samples were assayed in duplicate and the value corrected for methodological losses by measuring the recovery of an added amount of radiolabelled androstenedione, originally added to the plasma sample, the comparison of these results with those obtained when this correction was not applied, gave very good agreement, as shown in Fig. 3. The C.V. for these duplicates with the correction was 9.0% and without the correction 9.5%. Thus, it was considered justifiable to omit a correction for recovery from the method.

Ten plasma samples were assayed using the method described above and also by the method described previously [1] which used ammonium sulphate precipitation for the separation of the bound and free fractions and required an initial purification of the plasma extracts on an alumina column. As Fig. 4 shows, a good correlation ($r = 1.0$) has been obtained between these two methods.

The non specific blank of the radioimmunoassay over 15 batches gave a mean value of 8.7 with a range of 6.4–12.5 as a percentage of the total counts. The blank value of the method was assessed by substituting distilled water into the assay procedure. This gave a value which was determined to be 3 pg from nine estimations. The sensitivity of the assay was estimated as being 5 pg, by calculating two standard deviations from a series of 10 zero tube determinations and estimating this value from a standard curve drawn on a linear graph paper.

DISCUSSION

The method described here has a number of advantages over other published radioimmunoassays for androstenedione in plasma. It has sufficient specificity to eliminate the need for a chromatographic stage. This is partly due to the specificity of the antiserum employed, but it depends in part, as has been found for other methods [13, 14] on the choice of the separation technique. In the method previously described [1], in which ammonium sulphate precipitation of the bound fraction was employed with the same antiserum, it was still essential to carry out further purification of the hexane–ether extract of the plasma by alumina column chromatography in order to achieve sufficient specificity. By using polyethylene glycol separation, this chromatographic step can be dispensed with, without sacrificing the specificity as shown by the close similarity of the results obtained when plasma samples were analysed by both methods.

Similar results have been obtained in our radioimmunoassay for testosterone [7]. An aliquot of plasma is extracted with ether and after evaporation, the residue is incubated with a specific antiserum. The bound fraction is precipitated using polyethylene glycol, as for the androstenedione plasma assay, and then counted in the incubation tube after the addition of scintillator.

The use of polyethylene glycol to separate bound and free fractions was first described by Desbuquois and Aurbach [8] in peptide hormone assays. Since then several authors have employed the technique and its use has been extended to steroid radioimmunoassays (e.g. testosterone [1, 9], oestriol [10] and progesterone [11]). Tresguerres *et al.*[12] also

employed this reagent in their assay for testosterone glucosiduronate.

By counting the precipitated bound fraction directly in the incubation tube, the use of expensive counting vials is eliminated, and the volume of scintillator needed is reduced to 1.5 ml. Disposal costs of the used scintillator are also minimised. As with other methods where the bound fraction is counted, in preference to the free, samples containing a low mass of steroid have high count rates facilitating improved counting conditions. Although there are several advantages to the technique in this form, the only other report, to our knowledge, of a method in which scintillator is added directly to the incubation tube is that by Hennam *et al.*[4]. The disadvantages of the method are the need for a large centrifugal force and the longer centrifuge times to separate the precipitate. However, this ensures firm packing of the precipitate to the tube and ensures that it is not easily disturbed. The supernatant fluid may then easily be removed by decanting, which enables large batches of samples to be handled.

This method has now been in use in our laboratory for over a year and has proved to be robust and reliable, features which are highly desirable when large numbers of samples have to be processed. The use of polyethylene glycol and the direct counting of the precipitate as described has been applied to other steroid assays and offers major advantages over other separation techniques of separation and counting.

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