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RADIOIMMUNOASSAY OF PLASMA OESTRIOL

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SUMMARY

A relatively specific antiserum to oestriol-16-17-dihemisuccinate-bovine serum albumin was raised in white New Zealand rabbits and a radioimmunological method developed for the quantitative determination of ether-extractable oestriol in the peripheral venous plasma (0·1 ml) of pregnant women. The method has been evaluated and applied to the determination of plasma oestriol in 400 blood samples collected from women with uncomplicated pregnancies.

INTRODUCTION

OESTRIOL has been measured in human blood plasma by spectrophotometric, fluorimetric and gas–liquid chromatographic techniques[1–6]. However, the limited sensitivity of these procedures made them unsuitable for the assay of pregnancy plasma before mid-term and necessitated the use of relatively large volumes of plasma. In 1971, Gurpide *et al.*[7] used an antigen to oestriol-16,17-dihemisuccinate linked to bovine serum albumin for the radioimmunoassay of oestrogens in human pregnancy urine, plasma and amniotic fluid. The antiserum cross-reacted with many phenolic steroids other than oestriol but there was a linear correlation between the concentration of plasma oestrogens and urinary oestriol. Tulchinsky and Abraham[8] raised an antiserum in ewes to hemisuccinates of oestriol coupled with human serum albumin and developed a more sensitive radioimmunological method in which oestriol was separated from oestradiol-17β and oestrone on celite columns. The method was applied to the analysis of small volumes of plasma (0·1 ml–1·0 ml) throughout pregnancy.

Recently, Loriaux *et al.*[9] used an antiserum raised to oestrone-17-oxime-bovine serum albumin and separation on LH 20 Sephadex columns for the radioimmunoassay of oestrone sulphate, oestrone, oestradiol-17β and oestriol in pregnancy plasma. The present report describes a sensitive and convenient procedure for the determination of oestriol in small volumes of peripheral venous plasma (0·1 ml) throughout pregnancy.

MATERIAL

Solvents and reagents

Acetone, benzene, methanol and diethyl ether were all Analar grade, redistilled before use. Water was distilled and deionized. Tri-n-butylamine was obtained from B. D. H. Chemicals Ltd. Isatin Analar grade was from Koch Light Laboratories Ltd., Colnbrook, Bucks., England. Sephadex LH 20 was obtained from Pharmacia, Uppsala, Sweden; succinic anhydride from Eastman Organic Chemicals, Rochester, New York, U.S.A.; isobutylchloroformate from Pfaltz and Bauer Inc., Flushing, New York, U.S.A.; and bovine serum albumin was obtained from Sigma Chemicals Co., St. Louis, Mo., U.S.A. The source of the reagents, buffer, and their preparations not mentioned in this paper have been reported elsewhere.

Standards

Oestriol, oestradiol-17β, and oestrone were obtained from Sigma Chemical Co. Ltd., Kingston-upon-Thames, Surrey, England. [2,4,6,7-^{3}H]-oestriol (S.A. 98·7 Ci/mmol) was obtained from New England Nuclear, 6072 Dreieichenhain bei Frankfurt/Main, Siemensstrasse 1, Germany. 17-epioestriol, 16-epioestriol, 16,17-epioestriol and 16α-hydroxyoestrone were donated by the Fondation de Recherche en Hormonologie, Fresnes, France. Oestetrol (1,3,5(10)-oestratrien-3, 15α,16α,17β-tetrol) was a gift from Professor S. Solomon, McGill University, Montreal, Canada.

Antigen

One mmol of oestriol (288·4 mg) and 10 mmol of succinic anhydride (1·0 g) were dissolved in 10 ml of dry pyridine, refluxed for 4·5 h, evaporated to dryness under reduced pressure. The semi-solid residue was dissolved in chloroform, washed three times with water, dried over Na_2SO_4, evaporated to dryness and recrystallised in acetone. One mmol of steroid hapten was dissolved in dioxane. Two mmol (0·47 ml) of tri-n-butylamine were added, solution cooled and treated with 6 mmol (0·1 ml) of isobutyl-chloroformate. The reaction was allowed to proceed in the cold for 30 min. The mixture was added to a cooled solution of 500 mg of bovine serum albumin in 30 ml of water, 20 ml of dioxane and 1,2 ml of 1 M NaOH, then stirred in an ice bath for four hours. The solution was dialysed against running water overnight and brought to pH 4·6 with 1 M HCl. The precipitate was collected by centrifugation and suspended in 40 ml of water, bringing the pH to 7·0 with 1 M NaOH and reprecipitated at pH 4·6 with 1 M HCl in 50 ml of cold acetone. This treatment was repeated twice. The conjugate was dissolved in 50 ml of water and the pH was adjusted to 7·8 with 1 M NaOH, then dialysed against running water for eight hours and lyophilised.

Antiserum

Ten white New Zealand rabbits were immunized as follows:–1 mg of antigen was dissolved in 0·5 ml of sterile isotonic saline; 0·5 ml of Freund's complete adjuvant was added and the mixture was emulsified and injected subcutaneously and intradermally at multiple sites into each rabbit, every 7 days for 4 weeks, then once every two weeks for two months; rabbits were bled from the third month, ten days after every injection. Antisera were tested and the most specific antiserum with the highest titer was used for the procedure at a final dilution of 1:20,000 (v/v) with phosphate buffer saline pH 7 containing 0·1 % gelatine.

The preparation of dextran-coated charcoal and the measurement of radioactivity were as already described [9].

METHODS

2000 d.p.m.(5 pg) of tritiated oestriol were added to 0·1 ml of plasma in an extraction tube (Quickfit MF 24/3); a vortex mixer was used for the extraction with diethyl ether (2 × 10 ml), the extracts were transferred to a pointed tube (Quickfit BC 24/C 14T) with a Pasteur pipette and dried at 50°C under a stream of nitrogen; the residues were removed with four drops of benzene–methanol 85:15 (v/v) containing isatin (0·1 %) and transferred to a column (Pasteur pipette) of Sephadex LH 20 equilibrated with benzene–methanol 85:15 (v/v). The tubes were washed with a further three drops of the same solvent and transferred to the

same column. Elution was performed with the same solvents. The yellow fraction containing oestradiol was discarded and, immediately, a fraction of 4 ml containing oestriol was collected from the column in a counting vial and dried at 15°C under nitrogen, then redissolved in 1 ml of acetone. A sample of 0·2 ml was removed in duplicate from the counting vial to a disposable glass test tube (75 × 10 mm), dried under nitrogen at 50°C and used for assay. The remainder was also dried, liquid scintillation fluid added and the amount of radioactivity was determined to correct for losses.

Standard curve and unknowns were prepared in duplicate, dried under nitrogen at 50°C, cooled to room temperature and equilibrated with 100 μl of diluted antiserum (1/10,000) in phosphate buffer (pH 7) for 30 min.

After addition of 100 μl of ^{3}H-oestriol (20,000 d.p.m./50 pg) in phosphate buffer, the solutions were mixed and equilibrated for 2 h at 4°C. The free steroids were removed by the addition of dextran-coated charcoal [9–11].

CALCULATION OF RESULTS

A standard curve (0–200 pg), where the amount of tritiated oestriol bound to antibody is expressed as d.p.m., is shown in Fig. 1. For every point on the stan-

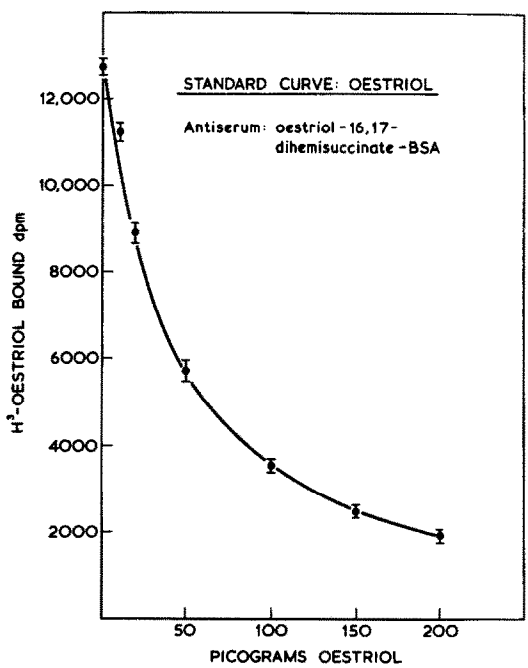

Fig. 1. Standard curve for oestriol using an antiserum to oestriol hemisuccinate bovine serum albumin at a dilution of 1 : 20,000 (v/v).

dard curve, the mean number of d.p.m. in the samples removed for assay was calculated. The corresponding values in pg were read from the standard curve and correlated for the aliquots taken, the experimental losses and volume of plasma extracted. A desk-top computer was used to calculate the results [10].

(i) *Accuracy and precision*

The accuracy was assessed by replicate analysis of oestriol added to distilled deionized water. The amounts added were similar to the mean found in pregnant women. The mean values ± S.D. and the coefficients of variation are shown in Table 1.

Table 1. Evaluation of a radioimmunological procedure for the determination of oestriol in peripheral venous plasma

Parameter		No. of	Oestriol
Int. Std. Rec. (% mean ± S.D.)		400	75 ± 10
Steroid equiv. of blank (μg/100 ml)		20	0·03
Precision (coef. of var. %, limits)		40	8–12
Accuracy (recovery %, mean ± S.D.)		20	92 ± 10
Sensitivity (limit of detection; μg/100 ml)			0·05
Random levels (mean and range, μg/100 ml)			
Pregnancy (weeks)	16–20	40	0·31 (0·07–0·54)
	20–24	80	0·43 (0·12–0·82)
	24–28	100	0·69 (0·61–1·5)
	28–32	80	0·90 (0·6–3·2)
	32–40	100	1·61 (0·53–6·6)

The precision was assessed from serial determinations on plasma from healthy women during pregnancy. The coefficients of variation are shown in Table 1. The recovery of ^{3}H-oestriol after extraction and chromatography in 400 analyses was 75 ± 10 (% mean ± S.D.).

(ii) *Sensitivity*

The lower limit of steroid which may be determined with reasonable precision depends upon the volume of plasma extracted; the sample removed for the assay and the method blank. As the standard deviation of 5 pg on the curve never overlaps the standard deviation of the corresponding 0, a reading of 5 pg may be defined as the lower limit of sensitivity. This means that the lower limit of sensitivity, when the method is applied to pregnancy plasma, is 50 ng/100 ml. The corresponding blank values for the procedure fall well below this figure.

(iii) *Specificity*

The specificity of the method depends upon the characteristics of the antiserum and the chromatographic step on Sephadex LH 20. This separates oestriol from oestradiol-17β and oestrone. The ability of several steroids with similar chemical structures and chromatographic mobilities to compete for binding sites on the antibody to oestriol hemisuccinate-BSA has been investigated. Using larger amounts of each steroid to determine the amount required to decrease the initial binding of ^{3}H-oestriol by 40% has shown that the antiserum has minimal cross-reaction (< 5%) with oestrone (Fig. 2). Cross-reaction with oestradiol-17β or oestetrol is less than 20%; with 17-epioestriol and 16α-hydroxyoestrone, cross-reaction is of the order of 60% whereas standard curves for 16-epioestriol and 16,17-epioestriol are very similar to those for oestriol.

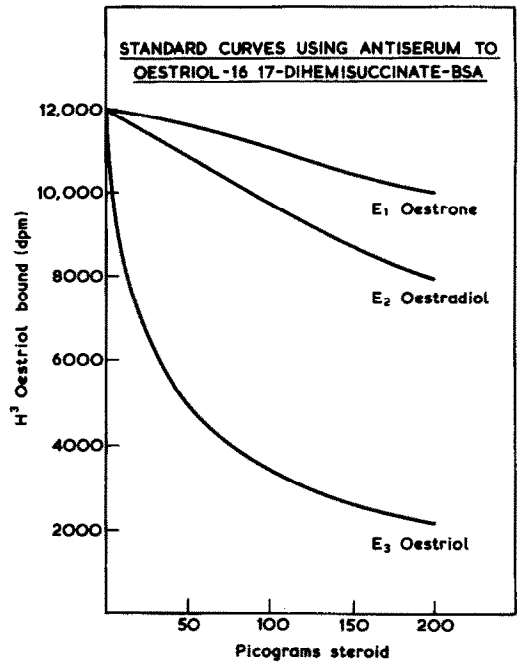

Fig. 2. The ability of E_1, E_2 and E_3 to compete with ^{3}H-oestriol for binding sites on an antiserum to oestriol hemisuccinate bovine serum albumin.

Plasma concentrations in uncomplicated pregnancies

Random samples of peripheral venous blood (400) were collected from healthy women with uncomplicated pregnancies. The blood was transferred to a lithium heparin tube and centrifuged within 20 min; the plasma was decanted and stored at − 15°C. The mean and range of values are shown in Table 1.

DISCUSSION

This method is similar to those previously developed in this department for the radioimmunoassay of gonadal steroids [10–13] and has similar characteristics in terms of sensitivity, accuracy and precision. The use of antisera to oestriol hemisuccinate-BSA enables the method to be applied without derivative formation and with only one simple and fast chromatographic step. The antiserum differs from that used by Tulchinsky and Abraham [8] where there was very high degree of cross-reaction between oestriol, oestrone and oestradiol-17β. Furthermore, the Sephadex micro-columns used in the present technique are more convenient and rapid than the celite columns used by these workers or the collection of eluates of large volume from the Sephadex columns employed by Loriaux *et al.* [9].

With regard to the results obtained in uncomplicated pregnancy, it is of interest, in view of the relatively large number of analyses, that the rise in the mean concentration of plasma oestriol during pregnancy is less striking than that obtained by the other radioimmunological techniques. It should be emphasised that nycterohemeral variations in plasma oestriol concentration have not yet been studied by the present technique and that the analysis is confined to the determination of ether-extractable oestriol.

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