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THE RADIOIMMUNOASSAY OF PREGNANEDIOL-3α-GLUCURONIDE

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SUMMARY

An antibody was prepared against pregnanediol-3α-glucuronide-BSA. The hapten (5β-pregnane-20α-ol-3α-yl-glucuronide) used for the preparation of the immunogen and as 'cold' standard for RIA was synthesized by an unambiguous chemical synthesis. The corresponding [6,7-^{3}H]-labelled conjugate was prepared and the cross-reactions of the antiserum against other glucuronides and free steroids were examined. Application of RIA to menstrual cycle urines and pregnancy urine is discussed. The results of our own studies carried out throughout the menstrual cycle from seven women showed the mean and range for the follicular phase were 3.8 ± 0.67 (3.0 – 5.4) μmol/24 h and for the luteal phase 17.7 ± 6.9 (6.9 – 29.0) μmol/24 h.

INTRODUCTION

Rationale

Increased excretion of 5β-pregnane-3α,20α-diol ('Pregnanediol') during the luteal phase of the ovarian cycle is usually accepted as evidence of the formation of a functioning corpus luteum and likewise the progressive rise in the excretion of this compound in pregnancy is associated with the normal development of the placenta [1–3]. Although the measurement of urinary pregnanediol is acknowledged as a useful parameter for the detection of ovulation or for monitoring the progress of a difficult pregnancy, the determination of this unique metabolite of progesterone by existing methods is tedious and time consuming. As far as is known the metabolite is excreted exclusively as the 3α-glucuronide (5β-pregnane-20α-ol-3α-yl-glucuronide) and routine methods of measurement invariably begin with the hydrolysis of this conjugate as a means of isolating the hydrophobic steroid. There are several disadvantages to this procedure. Acid hydrolysis is non-specific and may lead to destruction of the molecule [4], while enzyme hydrolysis, although more specific is subject to inhibition by endogenous urinary substances [5]. Apart from this source of error there is no unique chemical colour reaction which can be used to assay this diol, and existing chemical methods depend for accuracy on extensive chromatography of the sterol and its diacetate [6]. More recently gas liquid chromatography (glc) methods have been applied to facilitate the purification but isomeric diols are troublesome and the hydrolysis step is not avoided. In this communication we describe the production of an antiserum against pregnanediol-3α-glucuronide. This antiserum was used for the development of a direct radioimmunoassay procedure to measure pregnanediol-3α-glucuronide concentration in the urine of normal menstruating women.

EXPERIMENTAL

Materials and methods

General-purpose solvents, except ethanol (R.R. grade; James Burrough, London S.E.11, U.K.) were obtained from BDH, Poole, Dorset, U.K., and were distilled before use. Bulk steroids were purchased from Diosynth, Morden, Surrey, SM4 5DZ, U.K. Isobutyl chloroformate and tributylamine were supplied by Kodak, Kirkby, Liverpool, L33 7UF, U.K., and activated charcoal (Norit A) was from Hopkin and Williams, Romford, Essex, RM1 1HA, U.K. Bovine serum albumin (crystallized and freeze-dried) was obtained from Sigma (London) Chemical Co., Kingston, Surrey, KT2 7BH, U.K., and Freund's complete adjuvant was from Difco Laboratories, West Molesey, Surrey, KT8 0SE, U.K.

Celite 535 (Johns-Manville, London S.E.1., U.K.) for partition chromatography was extensively washed with concentrated HCl, water and methanol before being dried at 60°C. Thin-layer chromatography was carried out on prepared silica gel plates (Kieselgel 60 F_{254}; Merck) supplied by BDH. Sephadex G-25 was obtained from Pharmacia (G.B.) Ltd., Paramount House, London W5 5SS, U.K. Components of the liquid scintillant (2,5,diphenyloxazole (PPO), 1,4-di-2-(5-phenyloxazolyl) benzene (POPOP), Triton X-100 were purchased from Fisons Scientific Apparatus, Loughborough, Leics, LE11 0RG, U.K. Unless otherwise indicated chemical reagents were of analytical grade and were obtained from BDH. Tritium gas was supplied by the Radiochemical Centre, Amersham.

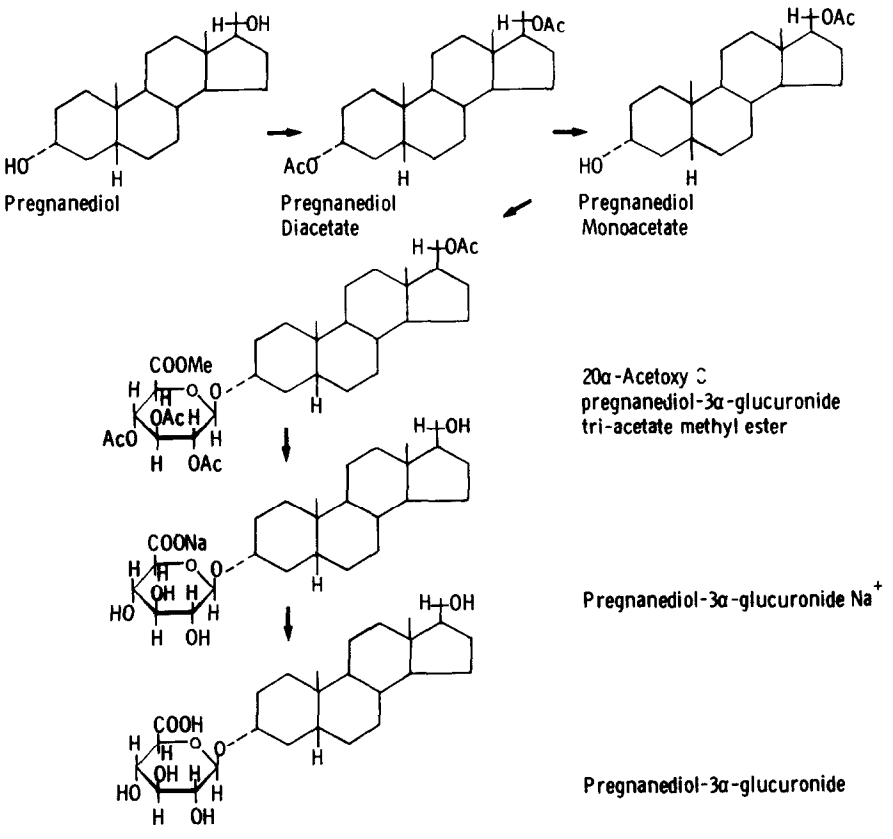

Fig. 1. Synthesis of pregnanediol-3α-glucuronide.

Synthesis of 5β-pregnanediol-3α-glucuronide (hapten)

Figure 1 illustrates the synthesis of pregnanediol-3α-glucuronide starting from pregnanediol. 3α,20α-Diacetoxy-5β-pregnanediol was prepared by conventional acetylation and subjected to differential hydrolysis to give the C-20α-monoacetate which was purified by Celite partition chromatography. The system employed for partition was *n*-hexane–benzene–methanol–water (9:1; 4:1, by vol.). 20α-Acetoxy-5β-pregnane-3α,20α-diol-3α-glucuronide triacetate methyl ester was prepared by the method of Conrow and Bernstein (1971)[7], and hydrolysed with methanolic NaOH at room temperature to yield the required 5β-pregnane-20α-ol-3α-yl-glucuronide [Na^+]. The sodium salt was converted into the glucuronide free acid by adsorption and elution from a column of the Amberlite ion-exchange resin XAD-2 [8, 9]. M.P. 183–185°C.

Synthesis of labelled 5β-pregnanediol-3α-glucuronide (radioligand) [6,7-^3H]5β-pregnane-20α-ol-3α-yl-glucuronide

The synthesis of tritium labelled pregnanediol-3α-glucuronide was carried out according to the method of Conrow and Bernstein starting with Δ^6-pregnanediol-20-acetate (5β-pregn-6-ene-3α-ol-20-acetate) (Fig. 2). Catalytic reduction of the resulting Δ^6-pregnanediol-3α-glucuronide with carrier free tritium in the presence of 10% palladium on charcoal resulted in the isotope labelled glucuronide [6,7-^3H]5β-pregnane-20α-ol-3α-yl-glucuronide (Fig. 3). The starting material Δ^6-pregnane-diol-20-acetate was prepared by the method of Kirk and Leonard (1973) [10] starting with progesterone. The specific radioactivity of the tritium labelled pregnanediol-3α-glucuronide estimated to be 97% pure was 42 Ci/mmol.

The immunogen

5β-pregnane-20α-ol-3α-yl-glucuronide in the free acid form was joined covalently to BSA by the mixed acid anhydride reaction Erlanger *et al.*, 1957; 1959[11, 12] whereby the hapten was joined as a peptide bond via the glucuronide carboxylic acid residue to ε-amino groups of lysine residues in the polypeptide chain of the protein [13]. The reaction mixture containing the pregnanediol-3α-glucuronide-BSA complex was purified by gel-filtration on a column of Sephadex G-25 and the product was freeze-dried to yield a white feathery powder which was stored at −20°C. Analysis of the immunogen formed by using a trace of [6,7-^3H]-5β-pregnanediol-3α-glucuronide indicated a molar incorporation of the steroid glucuronide into the serum albumin of 15 mol/mol of protein corresponding to 26% of the theoretical maximum (60 amino residues).

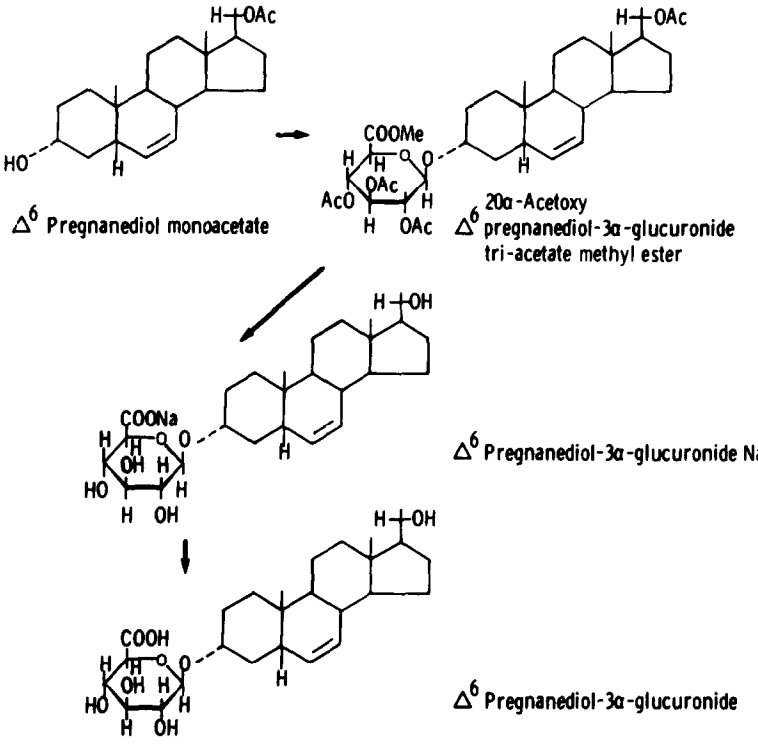

Fig. 2. Synthesis of Δ^6 pregnanediol-3α-glucuronide.

RESULTS

The antiserum

Antisera to 5β-pregnane-20α-ol-3α-yl-glucuronide-BSA immunogenic complex were raised in 3 adult male New Zealand white rabbits (body weight ~3 kg) as previously described [14]. Each test antiserum sample was examined for antibody titre i.e. the reciprocal of the antiserum dilution binding 50% of the radioligand under assay conditions, and this titre rose steadily in all rabbits over the first 6 months and maintained a plateau between 6 and 9 months corresponding to serum dilution of 1/8,000–1/18,000.

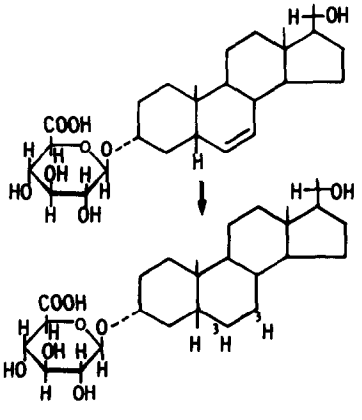

Fig. 3. Synthesis of [6,7-^{3}H]-5β-pregnane-20α-yl-glucuronide.

Characterization of the antiserum; Serum-dilution curve—anti-5β-pregnane-20α-ol-3α-yl-glucuronide-BSA serum

The antibody titre of the serum samples obtained from the blood of the immunized rabbits were examined by constructing a conventional antiserum dilution curve in the presence and in the absence of non-radioactive 5β-pregnane-20α-ol-3α-yl-glucuronide. The anti-5β-pregnane-20α-ol-3α-yl-glucuronide serum was serially diluted with phosphate-gelatine buffer [0.1M, pH 7.0 containing 0.9% NaCl] 5×10^2–3×10^5. Two sets of radioimmunoassay tubes (10 mm × 75 mm) were prepared containing (1) serially diluted antiserum (100 μl), ^{3}H-labelled ligand solution (100 μl) and buffer (100 μl); (2) serially diluted anti-serum (100 μl) ^{3}H-labelled ligand solution (100 μl) and non-radioactive ligand solution (100 μl). After incubating for 1 h at 37°C, the bound and free forms were separated by adding dextran-coated charcoal suspension. The tubes were centrifuged at 10,000 *g* for 5 min (M.S.E. Mistral 62) and a portion of the supernatant (250 μl) was transferred into counting vials containing 2.5 ml of Triton-X100 scintillant. Radioactivity measured in a Packard Tricarb liquid scintillation spectrometer (Model 3375; 3385).

The results were expressed graphically by constructing a serum dilution curve as shown in Fig. 4 in which the percentage binding of the radioligand is plotted against the log of the antiserum dilution. The upper curve shows the change in percentage binding of the radioligand (~10 nCi) as the antiserum

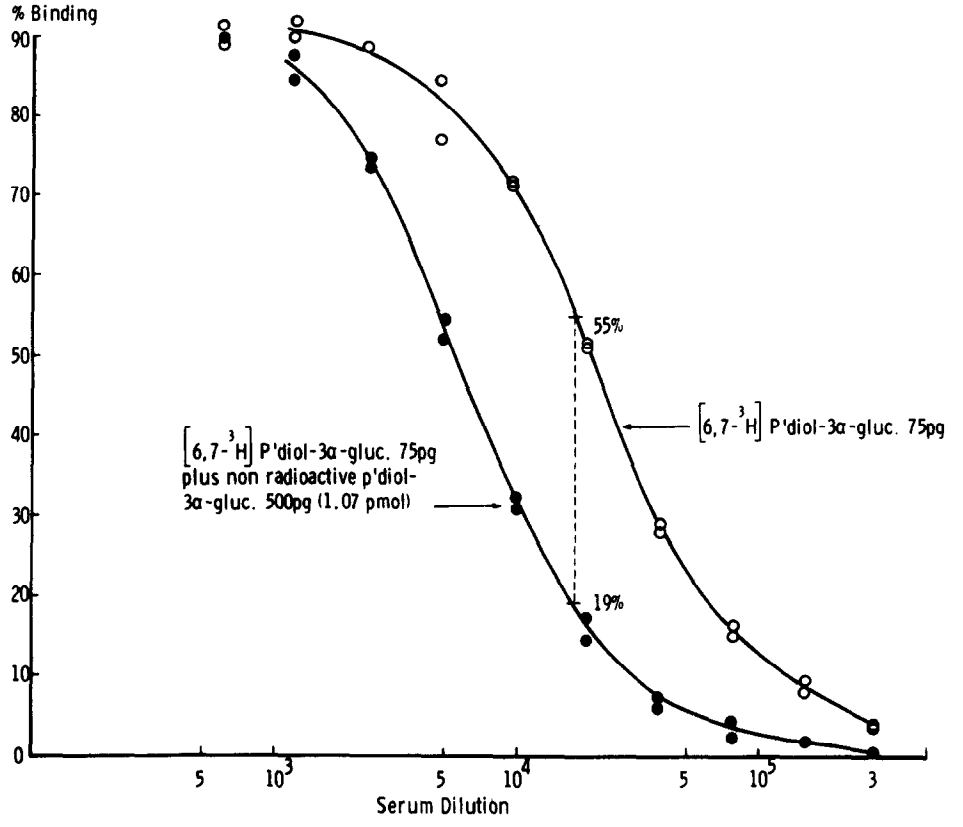

Fig. 4. Anti-5β-pregnanediol-3α-gluc. serum dilution curve.

is diluted. The lower curve relates to the change in the percentage binding of the radioligand brought about by the presence of the non-radioactive conjugate (500 pg) at different dilutions of the antiserum. At a final dilution of 1/18,000 the non-radioactive conjugate (500 pg) changed the binding from 55% to 19%.

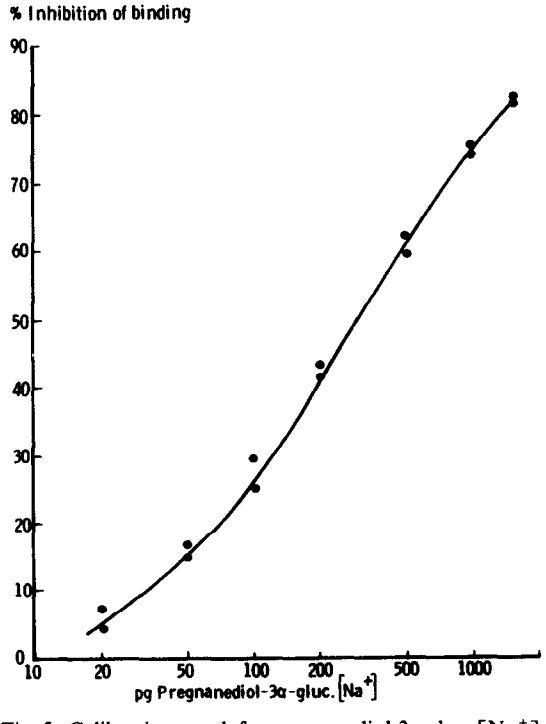

Fig. 5. Calibration graph for pregnanediol-3α-gluc. [Na^+]-0–1000 pg (0–2.13 pmol).

Calibration and specificity of the antiserum

Calibration graph and cross-reactivity tests for the radioimmunoassay of 5β-pregnane-20α-ol-3α-yl-glucuronide serum were prepared by incubating the diluted serum (1/18,000) in phosphate gelatine buffer with the radioligand [6,7-^{3}H]-5β-pregnane-20α-ol-3α-yl-glucuronide (~10 nCi) and a range of non-radioactive ligands (0–2 pmol) at 37°C for 1 h. Unbound conjugate was removed by adding dextran coated charcoal suspension [200 μl; Norit A (0.25 g) dextran (0.025 g) in phosphate gelatine buffer (100 ml)] and the bound radioactivity determined by β-scintillation counting of a 250 μl aliquot. Calibration graph (Fig. 5) was constructed by plotting the log of the amount of non-radioactive conjugate against the percentage inhibition of radioligand binding (Weinstein *et al.*, 1971)[15]. Cross reaction of other steroid conjugates and free steroids is defined as $100\,x/y$, where x is the mass of non-radioactive homologous steroid conjugate and y is the mass of heterologous conjugate required to produce 50% inhibition of the binding of the radioligand by the antiserum (Thorneycroft *et al.*, 1970)[16].

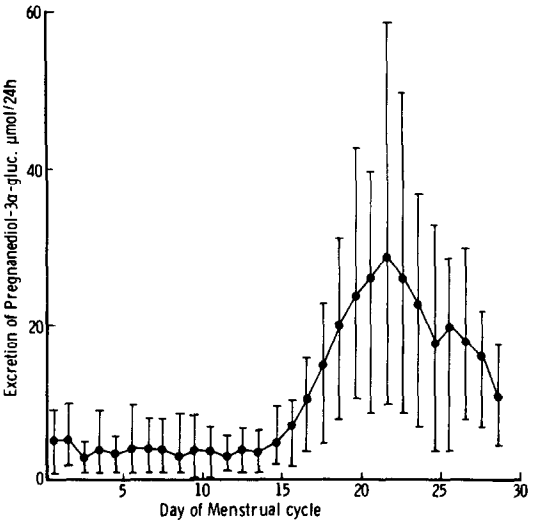

Fig. 6. Daily excretion of 5β-pregnanediol-3α-glucuronide throughout the menstrual cycle.

Cross-reaction of anti-5β-pregnane-20α-ol-3α-yl-glucuronide serum

The cross-reactions of anti-5β-pregnane-20α-ol-3α-yl-glucuronide serum against other steroid conjugates and free steroids are illustrated in Table 1. Specificity studies with other 3α-glucuronides available from the Department showed no cross reactions. However some degree of cross-reactions was recorded with free 5β-pregnane-3α,20α-diol "pregnanediol" (4.6%).

Radioimmunoassay

From the preliminary studies it is quite clear that substantial dilution of menstrual cycle urine is necessary before application of the assay procedure. In practice follicular phase urines were diluted 1000 fold and luteal phase urines 2000 fold and this means that inhibitors, competing glucuronides or free steroids present in the urine were effectively diluted out. Urine samples from seven women throughout the menstrual cycle were analysed by direct radioimmunoassay and expressed as μg/24 h. The mean values and ranges are illustrated in Fig. 6. The mean and range values for the follicular phase were 3.8 ± 0.67 (3.0–5.4) μmol/24 h. and for the luteal phase 17.7 ± 6.9 (6.9–29.0) μmol/24 h. (Application of the direct RIA of pregnanediol-3α-glucuronide to menstrual cycle urines will be reported in a subsequent publication).

DISCUSSION

The radioimmunoassay

Although the RIA of steroids and steroid metabolites [17] is now commonplace, the antisera used for these analyses are artificial in the sense that the steroid hapten used has, invariably, been joined to its protein carrier by a 'bridge' or linking derivative—e.g. as a hemisuccinate on an O-carboxy-methyloxime [18]. As a consequence throughout these applications the steroid hapten has never had precisely the same structure as the steroid radioligand. This artificial device has been found necessary because of the practical difficulty of joining steroids directly to proteins. No such difficulty arises when steroid glucuronides are used as haptens for the carboxylic acid group of the conjugate can be linked directly and covalently via the ε-amino groups of the lysine residues to the polypeptide chain of the carrier protein [19]. The steroid glucuronide is thus the sole antigenic discriminant [20] and the radioligand can be made to have the same structure as the hapten, i.e. 5β-pregnane-

Table 1

Cross-reactions of Anti-5β-pregnanediol-3α-glucuronide Serum against Steroid Glucuronides and Free Steroids

Compound	% Cross-reaction*
STEROID GLUCURONIDES	
1) 5β-Pregnane-3α,20α-diol-3α-glucuronide	100
2) 5β-Pregnane-3α,20β-diol-3α-glucuronide	Nil
3) 5β-Pregnan-20-one-3α-glucuronide	Nil
4) 5β-Pregnan-20-one-3β-glucuronide	Nil
5) 5-Pregnen-20-one-3β-glucuronide	Nil
6) 5α-Androstan-17-one-3α-glucuronide	Nil
7) 5β-Androstan-17-one-3α-glucuronide	Nil
FREE STEROIDS	
1) 5β-Pregnane-3α,20α-diol (Pregnanediol)	4.6
2) 5β-Pregnane-3α,20β-diol	Nil
3) 5β-Pregnane-3α,17,20α-triol	Nil
4) 5β-Pregnane-3α,17,20β-triol	Nil
5) 5α-Pregnane-3β,16α,20β-triol	Nil
6) 3α-Hydroxy-5β-pregnan-20-one	Nil
7) 3β-Hydroxy-5β-pregnan-20-one	Nil

Nil = <0.1%

* Defined according to Thorneycroft et al., (1970) as (x/y) x 100 where x is the mass of the unlabelled steroid conjugate and y is the mass of the heterologous compound required to produce 50% inhibition of the binding of labelled conjugate by antibody.

20α-ol-3α-yl-glucuronide and [6,7-^{3}H]-5β-pregnane-20α- ol-3α-yl-glucuronide.

From the onset, in order to favour the production of specific antisera, considerable effort was made to ensure the purity of the 5β-pregnane-20α-ol-3α-yl-glucuronide. Whether the "pregnanediol-3α-glucuronide" available from commercial sources comes from human or equine sources it is likely to be contaminated by cognate glucuronides which are present in late pregnancy urine and are notoriously difficult to separate. For these reasons the preparation of 5β-pregnane-20α-ol-3α-yl-glucuronide by an unambiguous chemical synthesis was undertaken. This synthesis followed classical lines and the major problem encountered was the physical separation of 5β-pregnane-3α,20α-diol and 5β-pregnane-3α-20β-diol. This enforced separation had the incidental advantage that it facilitated the separation of 5β-pregnane-3α,20β-diol-3α-glucuronide which was used in cross reactivity studies.

The formation of the 5β-pregnane 20α-ol-3α-yl-glucuronide-BSA complex which was accomplished by the mixed acid anhydride method [11, 12] led to the incorporation of 15 mol of pregnane-diol-3α-glucuronide per mole of BSA. This compares with 12 mol/mol incorporation when using oestrogen ring A-glucuronide 119]. Yet when the non-oestrogen steroid glucuronide-BSA complex was used to induce the formation of antibody in New Zealand white rabbits, lower antibody titres were achieved at plateau levels. Several batches of immunogen have been prepared and have been used in rabbits without achieving very high antibody titres e.g. antipregnanediol-3α-glucuronide serum—50% binding of the radioligand at dilutions of 1/18,000–1/30,000 compared with anti-oestrogen-3-glucuronide values ranging from 1/75,000–1/150,000. From these results it appears that pregnanediol-3α-glucuronide is less effective as an immunogen than steroid glucuronides previously used.

Specificity studies showed no cross reactions with the related 3α-glucuronides tested. However, some degree of cross-reaction was recorded with free 5β-pregnane-3α,20α-diol 'Pregnanediol' (4.6%), but it is unlikely that measureable amounts of free pregnanediol exist in fresh urine.

In retrospect, the synthetic route to Δ6-ene-5β-pregnanediol-3α-glucuronide via the borohydride derivative [10] is cumbersome and an alternative method based on the photolytic conversion of progesterone-3-methoxy ether to 6β-hydroxyprogesterone is now preferred [21, 22]. The catalytic reduction of 5β-pregn-6-en-20α-ol-3α-yl-glucuronide with carrier free tritium gave the required radioligand [6,7-^{3}H]-5β-pregn-20α-ol-3α-yl-glucuronide at a specific radioactivity of 42 Ci/mmol.

From the antiserum dilution curve Fig. 4, which is constructed under the conditions of assay, in the absence of competition from non-radioactive pregnanediol-3α-glucuronide 50% binding of the radioligand (~10 nCi) occurs at a final dilution of 1/18,000. In the presence of competing non-radioactive pregnanediol-3α-glucuronide (~1 pmol) binding is reduced to approximately 19%. A calibration graph (Fig. 5) using a final dilution of antiserum of 1/18,000 gives a useful linear response between 0.1 pmol–2 pmol.

Thus, we have reported the development of a specific radioimmunoassay procedure for the measurement of pregnanediol-3α-glucuronide in urine. This procedure is sensitive and specific and it will enable us to measure pregnanediol-3α-glucuronide throughout the menstrual cycle and during pregnancy. Preliminary studies carried out throughout the menstrual cycle from 7 women showed a significant rise in oestrone-3-glucuronide excretion some days before the maximum plasma LH is reached and a significant increase in pregnanediol-3α-glucuronide excretion after ovulation. It has been suggested that the ratio urinary oestrone-3-glucuronide/pregnanediol-3α-glucuronide might be used to predict impending ovulation and the duration of the fertile period (Baker *et al.*[23]). This is a ratio of molar concentration and as such would be independent of absolute volume of urine or rate of excretion. It might be applicable to random urine samples, thus avoiding 24 h collection, which is normally required for many routine urinary estimations. The levels of pregnanediol-3α-glucuronide during pregnancy enables us to follow the progressive development of placental function [24].

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