The Radioimmunoassay of Steroid Glucuronides

THE OESTROGEN C-3 GLUCURONIDES AS HAPTENS

By PADMANANDA SAMARAJEEWA and ALEXANDER E. KELLIE

*Courtauld Institute of Biochemistry, Middlesex Hospital Medical School,
London W1P 5PR, U.K.*

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Antisera were prepared against three related oestrogen ring-A glucuronides, oestrone 3-glucuronide, oestradiol 3-glucuronide and oestriol 3-glucuronide. The corresponding 6,7-³H-labelled conjugates were synthesized as radioligands and the cross-reactions of the antisera against ring-A oestrogen glucuronides and other steroid conjugates were examined. The specificity of the antiserum against oestriol 3-glucuronide was compared with that raised against oestriol 16 α -glucuronide, and the measurement of the former conjugate in late-pregnancy urine is discussed.

Many steroids, whether secreted internally or administered, are excreted as urinary steroid glucuronides. Conventional assay methods do not measure these conjugates directly but use a hydrolysis, solvolysis or oxidation-fission step to release the steroid moiety for determination by a variety of methods. This step is undesirable, for not only does it destroy the identity of the conjugate (e.g. oestriol 3-glucuronide, oestriol 16 α -glucuronide and oestriol 3-sulphate 16 α -glucuronide in late-pregnancy urine are all assayed as oestriol), but it is impossible to determine accurately how much of the conjugate is destroyed during the fission. There is no valid reason why steroid glucuronides should not be measured directly by radioimmunoassay.

Steroids are not immunogenic but can be made so by covalent linkage to a macromolecular protein carrier such as bovine serum albumin. The steroid-protein complexes are immunogenic in heterologous species and the antibodies thus induced show a high specificity in binding the steroid used as a hapten and can be used as binding proteins for the sensitive immunoassay of steroids. No-one has yet succeeded in joining steroids directly to a protein carrier and virtually all steroid immunogens have been prepared by converting the steroid into a derivative which contained a carboxylic acid group, e.g. a hemisuccinate or an *O*-(carboxymethyl)-oxime. Such derivatives can be joined covalently to a protein carrier by the mixed acid anhydride reaction (Erlanger *et al.*, 1957) whereby a peptide bond is formed between the carboxylic acid group of the steroid derivative and ϵ -amino groups of lysine residues in the polypeptide chain of the carrier protein. With steroid glucuronides no derivative formation is necessary, for they can be linked covalently directly to the lysine residues and the resulting immunogenic complexes (Fig. 1) will induce, in suitable host animals, antibodies which bind

steroid glucuronides with high sensitivity and specificity (Kellie *et al.*, 1972). Of the many factors which are known to affect the specificity of the resultant antisera the position of attachment to the steroid nucleus is important (Midgley & Niswender, 1970). The present paper describes the preparation of antisera against three related oestrogen ring-A glucuronides, oestrone 3-glucuronide, oestradiol 3-glucuronide and oestriol 3-glucuronide, and examines the cross-reactions of these antisera with cognate compounds.

Experimental

Materials

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. Components of the liquid scintillant [xylene, dioxan, naphthalene and 2,5-diphenyloxazole (PPO)] were purchased from Fisons Scientific Apparatus, Loughborough, Leics. LE11 0RG, U.K., and were of scintillation quality. Unless otherwise indicated chemical reagents were of analytical grade and were obtained from BDH. 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 before use with hot concentrated HCl, water and

Fig. 1. Fragment of a steroid glucuronide-bovine serum albumin complex

The hapten is oestrone 3-glucuronide.

methanol before being dried at 60°C. Thin-layer chromatography was carried out on prepared silica-gel plates (Kieselgel 60F₂₅₄; Merck) supplied by Anderman and Co., East Molesey, Surrey KT8 0Q2, U.K. Sephadex G-25 was obtained from Pharmacia (G.B.) Ltd., Paramount House, London W5 5SS, U.K.

Bulk steroids were purchased from Diosynth, Morden, Surrey SM4 5DZ, U.K.

Synthesis of oestrogen 3-glucuronides (haptens)

Oestrone 3-glucuronide. This was prepared from oestrone by the method of Conrow & Bernstein (1971).

Oestradiol 3-glucuronide. This was obtained from the former conjugate by reduction with borohydride (Elce *et al.*, 1967).

Oestriol 3-glucuronide. This was also prepared by the method of Conrow & Bernstein (1971) commencing with 16 α ,17 β -diacetoxyoestriol to ensure conjugation at C-3 (Elce *et al.*, 1967). In each case the corresponding glucuronide triacetate methyl ester was isolated in a crystalline form and hydrolysed at 15°C with methanolic NaOH.

Other steroid conjugates used for testing antisera

for cross-reaction were available in the Department, having been previously synthesized (Foggitt & Kellie, 1964; Elce *et al.*, 1967).

Synthesis of labelled oestrogen 3-glucuronides (radio-ligands)

[6,7-³H]Oestrone 3-glucuronide. Δ^6 -Oestrone [3-hydroxyoestratetra-1,3,5(10),6-en-17-one] was converted into the corresponding unsaturated glucuronide triacetate methyl ester, m.p. 252–254°C (Conrow & Bernstein, 1971) and this compound was hydrolysed to yield Δ^6 -oestrone 3-glucuronide, m.p. 283–287°C decomp.). Catalytic reduction of the product with carrier-free ³H in the presence of 5% palladium on charcoal gave [6,7-³H]oestrone 3-glucuronide (specific radioactivity 37 Ci/mmol).

[6,7-³H]Oestradiol 3-glucuronide. Half of the yield of [6,7-³H]oestrone 3-glucuronide was treated in methanol solution with NaBH₄ and reduced to [6,7-³H]oestradiol 3-glucuronide (specific radioactivity 36 Ci/mmol). Both radioactive glucuronides were purified by partition chromatography on Celite in the system 2-methylpropan-2-ol-ethylene dichloride-acetic acid-water (6:44:15:35, by vol.), which separates the two compounds (Bush, 1957).

[2,4-³H]Oestriol 3-glucuronide. Considerable difficulty was experienced during attempts to prepare this conjugate by chemical synthesis. The 16 α ,17 β -diacetate of Δ^6 -oestriol (16 α ,17 β -diacetoxyoestra-tetra-1,3,5(10),6-en-3-ol) was not available, and attempts to introduce tritium into oestriol 3-glucuronide at C-6 and C-9 by exchange reaction (Block & Djerassi, 1973) were unsuccessful, although this approach has been used to prepare [6,9-³H]oestriol 16 α -glucuronide (V. E. M. Chambers, personal communication). It is possible that the presence of the glucuronide residue at C-3 affects the phenolic character of ring A and prevents activation of the hydrogen atoms on the adjacent carbon atoms.

The required conjugate was prepared biosynthetically by incubating guinea-pig liver homogenate with [2,4-³H]oestriol in the presence of UDP-glucuronic acid (Goebelsmann *et al.*, 1965). On the basis of the transfer of radioactivity, the formation of oestriol 3-glucuronide corresponded to 80% of the available labelled steroid, and partition chromatography of the product on a Celite column (2-methylpropan-2-ol-ethylene dichloride-acetic acid-water, 4:6:3:7, by vol.) confirmed that the C-3 glucuronide was the main product. The conjugate, after preparative t.l.c. on silica gel (ethyl acetate-ethanol-acetic acid, 3:1:1, by vol.; R_f 0.33) behaved as a homogeneous compound. The location of radioactivity on the silica-gel plate was made by using a spark-chamber radiochromatogram scanner (Hesselbo, 1969). Because of the practical difficulty of determining the small mass available it was not possible to measure the specific radioactivity of the product, which was assumed to be the same as that of the [2,4-³H]oestriol used (37 Ci/mmol).

Preparation of steroid glucuronide immunogens

The oestrogen glucuronides were covalently linked to bovine serum albumin by the mixed acid anhydride reaction, as described by Erlanger *et al.* (1957, 1959), in dimethylformamide. This reaction proceeds in two stages; during the first stage, when the mixed anhydride is formed, anhydrous conditions are essential. During the second stage, when the serum albumin is introduced, water must be present to dissolve the protein and hence only anhydrous solvents which are wholly miscible with water are suitable.

Oestrone 3-glucuronide-bovine serum albumin complex. Oestrone 3-glucuronide in the acid form (35.7 mg; 0.08 mmol) was dissolved in dimethylformamide (1.28 ml) and the clear solution was cooled at 10°C for 10 min (pH 4). To this cooled solution tributylamine (0.078 ml; 0.08 mmol) was added without change of pH value, and subsequently isobutyl chloroformate (0.018 ml; 0.08 mmol). On addition of the latter reagent, fumes were observed and the solution (pH 4.5) was further cooled to 4°C for 20 min while

being stirred magnetically. The formation of the mixed acid anhydride was complete after this period.

Bovine serum albumin (128 mg; 0.0015 mmol) was dissolved in water (3.3 ml) and to this solution dimethylformamide (3.3 ml) was added, producing a cloudy solution. To this, 1 M-NaOH was added dropwise (0.13 ml) until the solution became clear (pH 8.0). This solution was also cooled to 4°C and was added to the cold mixed acid anhydride solution. The mixture became cloudy (pH 7.5) and was stirred at -10°C for 4 h. After 1 h the pH had fallen (to 6.5) and 1 M-NaOH (0.05 ml) was added dropwise to give pH 7.5. These conditions were maintained over a period of 4 h.

The clear reaction mixture containing oestrone 3-glucuronide-bovine serum albumin complex was separated by gel filtration on a Sephadex G-25 column (50 cm $\times$ 2 cm internal diam.). The column was developed with water and fractions (10 ml) were collected. Portions of these fractions were diluted and scanned in the u.v. region (Pye-Unicam SP. 800 B). The oestrone 3-glucuronide incorporated into this complex was determined by the Kober reaction (Nocke, 1961). The determination of serum albumin in these fractions was originally made by u.v. absorbance at λ_{max} , 278 nm, but in later work this was replaced by the method of Lowry *et al.* (1951). In three or four fractions the molar ratio of oestrogen conjugate to protein remained constant, and these fractions were combined and freeze-dried to yield a white feathery powder which was stored under N₂ at 4°C.

In the oestrone 3-glucuronide complex the average molar ratio (steroid/protein) was 13.0, corresponding to 18.5% of the theoretical maximum. Corresponding values for the oestradiol 3-glucuronide-serum albumin complex were 9 mol/mol (15%) and for the oestriol 3-glucuronide complex 12 mol/mol (19.5%).

Preparation of antisera

A conventional immunization procedure was used. The freeze-dried immunogen (1 mg) was homogenized in water (1 ml) in the presence of Freund's complete adjuvant (1 ml) and small doses equivalent to approx. 20 μ g of steroid conjugate were injected intramuscularly and subcutaneously at multiple sites on the flanks of three New Zealand white rabbits. Booster doses of the same composition were given 2 weeks after the primary injection and subsequently at 1-month intervals, and test blood samples were withdrawn 10 days after the third and subsequent injections. Each test antiserum sample was examined for antibody titre, i.e. the reciprocal of the antiserum dilution binding 50% of the radioligand under standard 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 dilutions of 1/70000-1/700000 (Fig. 2).

Fig. 2. Antibody-titre response of New Zealand white rabbits immunized with oestrogen 3-glucuronide-bovine serum albumin immunogens

The ordinate units are the reciprocal of the antiserum dilution binding 50% of the radioligand under standard conditions (see the Experimental section). ■, Oestrone 3-glucuronide-protein complex (E_1 -3-g-BSA); ▲, oestradiol 3-glucuronide-protein complex (E_2 -3-g-BSA); ●, oestriol 3-glucuronide-protein complex (E_3 -3-g-BSA). Arrows denote times of immunizations.

Fig. 3. Antiserum dilution curve against oestradiol 3-glucuronide

The upper curve (●) represents incubation with the radioligand [6,7- 3 H]oestradiol 3-glucuronide, 60 pg [* E_2 -3-g (60 pg)]. The lower curve (○) represents incubation with the same radioligand (60 pg) in the presence of non-radiolabelled oestradiol 3-glucuronide (500 pg).

Sensitivity of antisera

The sensitivity of antisera samples was examined by constructing an antiserum-dilution curve in the presence and in the absence of non-radioactive steroid glucuronide, e.g. anti-(oestradiol 3-glucuronide) serum at serial dilutions (1/3000–1/300000) was incubated at 30°C for 0.5 h with [6,7- 3 H]oestradiol 3-glucuronide (0.13 pmol) in the absence and in the presence of non-radioactive oestradiol 3-glucuronide (1.12 pmol). Unbound radioligand was removed by adding dextran-charcoal suspension and the bound ligand was determined by β -scintillation

counting (Packard model nos. 3375 and 3385). It is noteworthy that most steroid glucuronides do not mix with toluene-based phosphors, but a dioxan-xylene-bound medium was satisfactory. The mixture used contained 2,5-diphenyloxazole (3.5 g) and naphthalene (100 g) in ethanol (230 ml), xylene (385 ml) and dioxan (385 ml), and gave a counting efficiency for 3 H of about 35%. The graphical presentation of this assay (Fig. 3) makes it possible to determine the change in percentage binding of the radioligand brought about by 1.12 pmol (500 pg) of non-radioactive conjugate at different dilutions of the antiserum.

Calibration and specificity of the antisera; the radioimmunoassay. Calibration graphs and cross-reactivity tests for the radioimmunoassay of oestrogen 3-glucuronides were prepared by incubating the diluted serum (1/72000; 100 μ l) in phosphate-gelatin buffer [0.1 M, adjusted with 0.1 M-NaOH to pH 7.0, containing 0.9% NaCl and 1% (w/v) gelatin] with the appropriate radioligands (0.13 pmol, 100 μ l) and a range of the non-radioactive steroid conjugate (0–1000 pg; 100 μ l) at 30°C for 0.5 h. Unbound conjugate was removed by adding dextran-charcoal suspension [200 μ l; Norit A (0.25 g), dextran A (0.025 g) in phosphate-gelatin buffer (100 ml)] and the bound radioactivity was determined by β -scintillation counting of a 200 μ l sample. Calibration graphs were constructed by plotting the log of the amount of non-radioactive conjugate against the percentage inhibition of radioligand binding (Weinstein *et al.*, 1972). Cross-reaction of other steroid conjugates against each antiserum 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).

Results

Cross-reactions of anti-(oestrogen 3-glucuronide) sera

The cross-reactions of the three oestrogen 3-glucuronide antisera against ring-A oestrogen glucuronides are summarized in Fig. 4. In greater detail the cross-reactions of these antisera against other steroid conjugates and against free steroids (Table 1) illustrate the relative specificity of these preparations.

The antiserum against oestradiol 3-glucuronide cross-reacts strongly with oestrone 3-glucuronide but not significantly with oestriol 3-glucuronide. The cross-reactivity of the anti-(oestrone 3-glucuronide) antiserum against oestradiol 3-glucuronide is less marked and against oestriol 3-glucuronide is negligible. In contrast the antiserum against oestriol 3-glucuronide shows little cross-reaction against oestrone 3-glucuronide or oestradiol-3-glucuronide and in this respect shows high specificity.

None of these antisera showed any cross-reactions with ring-D oestrogen glucuronides or with C₁₉ ring-A conjugates, whether glucuronide or sulphate.

Free steroids. It is not surprising to find that antisera induced against oestrogen 3-glucuronides also cross-react with free oestrogens (Fig. 5), for there is a close resemblance between the immunogens used in the present work ('steroid-glucuronyl serum albumin') and those used by other workers ('steroid-succinyl albumin') to raise antisera for the radioimmunoassay of free steroids. In the latter application the 'bridge' joining the steroid to the serum albumin is an awkward necessity, whereas in the present work it acts to some extent as an additional antigenic discriminant and it is logical to consider the steroid glucuronide as the true hapten (Hoffmann *et al.*, 1975).

The antiserum against oestriol 3-glucuronide reacts equally with oestriol and its C-3 glucuronide, but the other two antisera, against oestrone 3-glucuronide (Table 1) and oestradiol 3-glucuronide (Table 1), show stronger affinity for the corresponding glucuronide than for the free steroid. Moreover, comparing the magnitude of the reaction of the three antisera against the free oestrogens and their corresponding C-3 glucuronides, the same order is maintained.

Sensitivity of the radioimmunoassay

The antibody titre and to some extent the sensitivity of the assay depend on the dilution of the antiserum

Fig. 4. Cross-reactions of the three oestrogen 3-glucuronide antisera against ring-A oestrogen glucuronides

(a) Anti-(oestrone 3-glucuronide) serum (1/72000); (b) anti-(oestradiol 3-glucuronide) serum (1/72000); (c) anti-(oestriol 3-glucuronide) serum (1/150000). ●, Oestrone 3-glucuronide (E₁-3-g); ○, oestradiol 3-glucuronide (E₂-3-g); △, oestriol 3-glucuronide (E₃-3-g).

Table 1. Percentage cross-reactions of anti-(oestrogen 3-glucuronide) sera

The value 100 represents the reactivity between the antiserum and homologous antigen. Nil = <0.1.

Steroid conjugates	Anti-(oestrone 3-glucuronide)	Anti-(oestradiol 3-glucuronide)	Anti-(oestriol 3-glucuronide)
Oestrone 3-glucuronide	100	22	Nil
Oestradiol 3-glucuronide	5	100	1
Oestriol 3-glucuronide	Nil	Nil	100
16 α -Hydroxyoestrone 16 α -glucuronide	Nil	Nil	Nil
Oestradiol 17 β -glucuronide	Nil	Nil	Nil
Oestriol 16 α -glucuronide	Nil	Nil	Nil
Oestriol 17 β -glucuronide	Nil	Nil	Nil
17-Epioestriol 16 α -glucuronide	—	—	Nil
Androsterone 3 α -glucuronide	Nil	Nil	Nil
Aetiocholanolone 3 α -glucuronide	Nil	Nil	Nil
Dehydroepiandrosterone 3 β -glucuronide	—	Nil	Nil
3-Epiandrosterone 3 β -glucuronide	Nil	Nil	Nil
Oestrone 3-sulphate	—	—	Nil
Oestradiol 3-sulphate	—	—	Nil
Dehydroepiandrosterone 3 β -sulphate	—	—	Nil
Pregnenolone 3 β -sulphate	—	—	Nil
Free steroids			
Oestrone	37	3	Nil
Oestradiol	16	33	Nil
Oestriol	Nil	0.8	100
6-Oxo-oestradiol	—	1	Nil
6-Oxo-oestriol	—	Nil	20

Fig. 5. Effect of free oestrogens on the binding of [2,4-³H]-oestriol 3-glucuronide (60 pg) by rabbit anti-(oestriol 3-glucuronide) serum (dilution 1/150000)

●, Oestriol 3-glucuronide (E₃-3-g); ○, oestriol; ▲, oestradiol; △, oestrone.

Fig. 6. Effect of the concentration of the radioligand [2,4-³H]oestriol 3-glucuronide on the serum dilution curves of anti-(oestriol 3-glucuronide) serum

in relation to the concentration of the radioligand used. Fig. 6 illustrates with reference to anti-(oestriol 3-glucuronide) antiserum the effect of lowering the concentration of the radioligand [2,4-³H]oestriol 3-glucuronide on the antibody titre of the antiserum. At a radioligand concentration of 100 pg/ml (33 pg/300 μ l of incubation medium), 50% of the radioactivity is bound at a serum dilution of (1/2500000) and it is clear that this antiserum can be used at considerable dilution. A limit is, however, set by the practical difficulty of counting procedure, and a convenient dilution of 1/150000 covers a calibration range of 0–100 pg of oestriol 3-glucuronide.

Discussion

In raising antisera against free steroids it is widely accepted that the link between the steroid nucleus and the protein carrier should not involve the functional groups which frequently are associated with rings A and D (Niswender *et al.*, 1975; Abraham, 1975). For this reason the covalent link to the carrier is frequently made at C-6 in ring B or at C-11 α in ring C, points of attachment remote from the func-

tional groups of steroids such as oestradiol, testosterone and progesterone; in this way antisera of high specificity have been prepared. When raising antisera against steroid glucuronides the freedom of choice is more limited, and if the glucuronyl bridge is employed as the link to the protein carrier the choice must be that of a naturally occurring steroid glucuronide, e.g. oestriol 3-glucuronide (ring A) or oestriol 16 α -glucuronide (ring D). Experimental evidence also supports the view that high specificity is more dependent on antigenic discriminants remote from the point of linkage than on structural difference near this point (Abraham, 1975; Kohen *et al.*, 1975; Niswender *et al.*, 1975) and on this basis there is good reason to expect higher specificity with an antiserum against oestriol 3-glucuronide (D ring exposed) than with an antiserum against oestriol 16 α -glucuronide (A ring exposed) (Fig. 7). This follows from the fact that oestrone, oestradiol and oestriol have different ring-D functions, whereas the phenolic A ring is common to all oestrogens. This difference is illustrated in Table 2, which is based on results from the present work with reference to antiserum to oestriol 3-glucuronide and on data relating to the cross-reactions of antiserum to oestriol 16 α -glucuronide (Niswender *et al.*, 1975). From this it is clear that the former antiserum is specific to the unique ring-D structure of oestriol, whereas the latter responds to all the compounds with a free phenolic ring A.

With reference to the antisera against the C-3 glucuronides it is noteworthy that the anti-(oestrone 3-glucuronide) serum cross-reacts weakly with oestradiol and its C-3 glucuronide, although the ring-D structures have little in common. Similarly the antiserum against oestradiol 3-glucuronide reacts weakly with oestrone and its C-3 glucuronide, but not significantly with oestriol or its C-3 glucuronide, which do have structural features in ring D in common with the hapten (hydroxyl group at C-17 β). This anomaly is also apparent in the failure of the antiserum against oestriol 3-glucuronide to cross-react with oestrone, oestradiol and their respective C-3 glucuronides.

For some years the excretion of oestriol in late-pregnancy urine has been used as an indication of foetal welfare in the terminal stages of difficult pregnancies (Beling, 1963). This practice is based on the belief that the introduction of a hydroxyl group at C-16 α of the oestrogen nucleus takes place principally in the foetal adrenal and liver (Diczfalusy, 1969) and that a progressive increase in the excretion of oestriol is characteristic of normal pregnancy. The compound usually measured for this purpose is oestriol 16 α -glucuronide, which is the principal oestrogen conjugate of late-pregnancy urine, and the determination is based on hydrolysis of the conjugate and measurement of oestriol by colorimetric or fluorimetric methods. With reference to this practical application, the antiserum against oestriol 3-glucuronide shows

Fig. 7. Oestriol glucuronide immunogens

(a) Oestriol 16 α -glucuronide-bovine serum albumin, ring A exposed; (b) oestriol 3-glucuronide-bovine serum albumin, ring D exposed. BSA, bovine serum albumin.

Table 2. Percentage cross-reactions of antisera against oestriol 3-glucuronide and oestriol 16 α -glucuronide

The value 100 represents the reactivity between the antiserum and the homologous antigen. Nil = <0.1.

Compound tested	Antiserum against	
	Oestriol 3-glucuronide	Oestriol 16 α -glucuronide*
Oestriol 3-glucuronide	100	Nil
Oestriol 16 α -glucuronide	Nil	100
Oestrone	Nil	46
Oestradiol	Nil	100
Oestriol	100	53

* Results from Niswender *et al.* (1975).

considerable promise and could be used for the direct radioimmunoassay of oestriol and oestriol 3-glucuronide with little interference by related conjugates. The antiserum does not cross-react with oestriol 16 α -glucuronide, nor is it likely to measure the bis conjugate oestriol 3-sulphate 16 α -glucuronide, which is a minor component of late-pregnancy urine (Straw *et al.*, 1955).

A significant proportion (11–24%) of oestrogens excreted in maternal urine are present as ring-A oestrogen glucuronides, and the major components of this fraction are oestrone 3-glucuronide (0.13–1.77 mg/24 h), 16 α -hydroxyoestrone 3-glucuronide (0.77–3.21 mg/24 h) and oestriol 3-glucuronide (2.2–7.2 mg/24 h) (Ahmed & Kellie, 1972). On the assumption of a daily excretion of oestriol 3-glucuronide of 0.5 mg/litre (500 ng/ml) it should be possible to dilute urine by a factor of 1000 (50 pg/100 μ l) and still measure this conjugate directly by radioimmunoassay. To make similar immunoassay measurements of oestriol 16 α -glucuronide it would be necessary to destroy the phenolic character of ring A, possibly by

using 3-methoxy- or 3-benzyloxy-oestriol 16 α -glucuronide as a hapten.

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