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The Direct Radioimmunoassay of Oestrogen Glucuronides in Human Female Urine

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Radioimmunoassays for five oestrogen metabolites in urine are described; they are oestrone 3-glucuronide, oestradiol 3-glucuronide, oestradiol 17β-glucuronide, oestriol 3-glucuronide and oestriol 16α-glucuronide. These assays have proved accurate and reliable and can be performed rapidly; they have been carried out directly in diluted menstrual-cycle urine and pregnancy urine. No sample pretreatment was required. Preliminary results suggest that clinically useful information can be obtained by performing these assays on random urine specimens.

The production of antisera against steroid–protein complexes has encouraged the measurement of steroid hormones and metabolites in blood, and this tendency has to some extent replaced more traditional methods of urine assay (Buster & Abraham, 1975). However, in some clinical situations the integrated measurement of steroid production, such as is given by urine assay, is of greater significance. The rapid clearance of steroid glucuronides by the kidney (Smith, 1966) makes the measurement of these conjugates in urine a useful clinical parameter.

In the past analysis of steroids in urine has required the hydrolysis of conjugates before determination, a process that destroys the identity of the conjugate and can result in procedural loss. The immunoassay of steroid glucuronides not only avoids the hydrolysis step but also eliminates the subsequent extraction and purification before measurement. Moreover, in this form of assay the specificity is improved in other ways. The extra immunochemical determinant that the glucuronide substituent provides enables antibodies to discriminate better between similar conjugates. In forming the steroid glucuronide–protein immunogenic complex there is no need to introduce an artificial bridge, as a linkage to the protein carrier can be made through the existing carboxyl group of the glucuronyl moiety. In the radioimmunoassay of free steroids the use of an artificial bridge, e.g. hemisuccinate or carboxymethyl oxime, has given rise to strong cross-reactions with similar steroids differing at the position of conjugation (Bolton & Rutherford, 1976). Finally, the availability of ^{3}H-labelled steroid glucuronides makes it possible to use a labelled ligand that closely resembles the complete hapten conjugate. Jeffcoate *et al.* (1975) have shown that close resemblance between the hapten and bridge and the labelled ligand has advantages over radioimmunoassay systems where the labelled ligand resembles the hapten only.

For these reasons the radioimmunoassay of steroid glucuronides in urine has considerable practical advantage. Urines are simply diluted extensively before assay, a step that greatly minimizes interference from cross-reacting compounds. Antisera against five different steroid glucuronides, oestrone 3-glucuronide, oestradiol 3-glucuronide, oestriol 3-glucuronide, oestradiol 17β-glucuronide and oestriol 16α-glucuronide have been prepared in this laboratory together with their corresponding ^{3}H-labelled steroid glucuronides (Kellie, 1975). The present paper describes the validation and application of the five glucuronide assays to determinations on human female urine both throughout the menstrual cycle and during pregnancy.

An important factor contributing to the preference of measurements of steroids in blood to those in urine has been the inconvenience and inaccuracy associated with the collection of 24h urine samples. The paper describes ways of obtaining useful information from random urine samples.

Experimental

Materials

Unless otherwise stated, reagents were purchased from Fisons Scientific Apparatus, Loughborough, Leics., U.K.

Steroid glucuronides, used as assay standards and for preparing immunogens, were available in the Department, having been previously synthesized (Foggitt & Kellie, 1964; Elce *et al.*, 1967). Other

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steroids were purchased from Diosynth, Morden, Surrey SM4 5DZ, U.K. The following trivial names have been used: oestrone 3-glucuronide [3-hydroxyoestra-1,3,5(10)-triene-17-one 3-β-D-glucupyranosiduronic acid]; oestradiol 3-glucuronide [oestra-1,3,5(10)-triene-3,17β-diol 3-β-D-glucupyranosiduronic acid]; oestradiol 17β-glucuronide [oestra-1,3,5-(10)-triene-3,17β-diol 17β-β-D-glucupyranosiduronic acid]; oestriol 3-glucuronide [oestra-1,3,5(10)-triene-3,16α,17β-triol 3-β-D-glucupyranosiduronic acid]; oestriol 16α-glucuronide [oestra-1,3,5(10)-triene-3,16α-17β-triol 16α-β-D-glucupyranosiduronic acid]; pregnanediol 3-glucuronide [5β-pregnane-3α, 20α-diol 3α-glucupyranosiduronic acid].

Labelled ligands, namely [6,7-^{3}H]oestrone 3-glucuronide (38Ci/mmol), [6,7-^{3}H]oestradiol 3-glucuronide (40Ci/mmol), and [2,4-^{3}H]oestriol 3-glucuronide (37Ci/mmol), had been previously synthesized (Samarajeewa & Kellie, 1975) and were available in the Department. [6,7-^{3}H]Oestradiol 3-glucuronide (40Ci/mmol) was purchased from New England Nuclear, G.m.b.H., Frankfurt, Germany, and [6,9-^{3}H]oestriol 16-glucuronide (30Ci/mmol) from The Radiochemical Centre, Amersham, Bucks., U.K.

Steroid antisera. Oestrogen glucuronides conjugated to bovine serum albumin were prepared by the method of Erlanger *et al.* (1959). Antisera against these immunogens were available in the Department and had been raised as described by Samarajeewa & Kellie (1975). The dilutions of antisera used in the assays were as follows: anti-(oestrone 3-glucuronide), anti-(oestradiol 3-glucuronide), anti-(oestradiol 17β-glucuronide) and anti-(oestriol 3-glucuronide) were initially diluted with assay buffer by a factor of 50000 and anti-(oestriol 16α-glucuronide) was diluted by a factor of 13000. The assay buffer was phosphate buffer (9.57g of Na_2HPO_4 and 5.08g of $NaH_2PO_4,2H_2O$ per litre) containing 0.9% NaCl, 0.1% NaN_3 and 0.1% gelatine and made up in deionized water to pH7.0. Dextran/charcoal suspension, consisting of 1% Norit SX1 charcoal and 0.15% Dextran T40 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) in assay buffer, was made up fresh on the day of use, but at least 30min before required. The suspension was continuously stirred on ice during dispensing. The scintillation fluid used contained 0.5% 2,5-diphenyloxazole (Intertechnique, Uxbridge, Middx., U.K.) and 0.01% 1,4-bis-(5-phenyloxazol-2-yl)benzene in a toluene/Triton X-100 mixture (3:1, v/v).

All glassware was initially pretreated with a 1% solution of dichlorodimethylsilane in toluene, then rinsed successively in toluene, methanol and water. Thereafter cleaning was performed by soaking in a 1% solution of detergent (Divolab; Diversey Ltd., Barnet, Herts. EN4 0BU, U.K.) overnight, then rinsing in tap water and water, and finally drying in an oven at 60°C.

Urines. Successive daily urine collections were made throughout a complete menstrual cycle by 11 women volunteers with apparently normal menstrual cycles. Early-morning specimens were collected separately from those of the remainder of each 24h period. Urine volumes and times of collection were recorded, the specimens were preserved with 0.1% NaN_3 and stored at −15°C. One subject failed to complete all collections, another became pregnant during the cycle and has since given birth to a healthy baby. Two pregnant women (gestation over 16 weeks) volunteered to provide 24h urine collections at 2–6 week intervals during the course of pregnancy.

Methods

Before assay, urine samples were simply diluted with assay buffer. For menstrual-cycle urines 100-fold dilutions were necessary for oestrone 3-glucuronide and oestriol 3-glucuronide assays and 20-fold dilutions were necessary for the oestradiol 3-glucuronide, oestradiol 17β-glucuronide and oestriol 16α-glucuronide assays. Pregnancy urines were diluted either 1000- or 10000-fold. Standard solutions of steroid glucuronides were prepared in assay buffer over the range 20–20000nM and stored at 4°C. For a chosen urine dilution, steroid standards were selected over an appropriate range and diluted by the same factor with assay buffer.

A similar assay procedure was followed for each of the five conjugates. Suitably diluted urine or standard (100μl) was transferred in duplicate to 75mm × 12mm glass test tubes and 100μl (5nCi) of a solution of the labelled ligand in assay buffer and 100μl of diluted antiserum were added. The tubes were vortex-mixed, capped with Parafilm and incubated at 37°C for 1h, then placed in an ice bath for 30min. Dextran/charcoal suspension (200μl) was added, and the tubes were left for 15min on ice before centrifugation (7500*g*-min) at 4°C. Supernatant (250μl) was removed from each tube and counted for radioactivity in 2.5ml of scintillation fluid. Calibration graphs were plotted as the log of the amount of non-radioactive conjugate against the percentage inhibition of labelled ligand binding.

Determination of oestrogen glucuronide standards by a chemical method. Standard solutions (20–2000nM) of oestrone 3-glucuronide, oestriol 16α-glucuronide and oestriol 3-glucuronide were analysed for total oestrogen by the method of Brown *et al.* (1968). Results are not corrected for procedural loss.

Plasma lutropin assay. Plasma lutropin was determined by radioimmunoassay on successive days, in five subjects, over the mid-cycle period during the time of urine collection.

Creatinine determination. Creatinines were determined by the automated method of Chasson *et al.* (1961).

Results

Assay characteristics are given in Table 1. Sensitivity is defined as the dose of steroid that can be distinguished from zero dose of unlabelled steroid at the 95% level of confidence. Intra-assay coefficients of variation were calculated from duplicate determinations on urine samples (Cekan, 1975). Inter-assay coefficients of variation were calculated from successive determinations on a quality-control urine.

Accuracy of the methods was established by both internal and external recovery experiments. Determinations made on pooled urine over a range of dilutions resulted in a constant recovery of steroid (within the error limits shown in Table 1), provided that the unknowns were within the working range of the standard curve (i.e. between 20 and 80% inhibition of labelled-ligand binding). There was a linear relationship between the volume of urine assayed and measured steroid, as shown by the internal recovery correlation coefficients. Sample blanks performed over the same range of urine dilutions were not significantly different ($P<0.05$) from buffer blanks provided that urine was diluted 20-fold or greater.

External recovery experiments were performed by assaying increasing amounts of standard steroid added to the appropriate dilution of both charcoal-treated urine and pooled urine. Regression analysis (Table 2) indicated a linear relationship between added and recovered steroid. Recovery ranged from 92 to 123%.

Specificity of the various antisera was examined to assess the degree to which similar steroids would interfere with the assay. Cross-reactions are given in Table 3 and are calculated on a molar basis, as suggested by Davis & Loriaux (1975). They were assessed

Table 1. *Assay characteristics*

Sensitivity is the dose of steroid that can be distinguished from zero dose at the 95% level of confidence. Intra-assay precision was determined as the mean coefficient of variation of 60 duplicate samples. Inter-assay precision was determined as the coefficient of variation of successive estimations of a quality control urine (number of assays in parentheses). The internal recovery correlation coefficient tests proportionality between the dilution of urine assayed and the measured steroid. The recovery coefficient of variation is the precision of estimating steroid concentration over a range of urine dilutions.

Conjugate	Sensitivity (fmol/tube)	Intra-assay coefficient of variation (%)	Inter-assay coefficient of variation (%)	Internal correlation coefficient ($n=12$)	Recovery coefficient of variation (%)
Oestrone 3-glucuronide	24	13.9	16.0 (78)	0.993	14
Oestradiol 3-glucuronide	64	13.1	—	0.995	9
Oestradiol 17β-glucuronide	60	9.7	—	0.994	13
Oestriol 3-glucuronide	29	9.9	16.6 (10)	0.851	19
Oestriol 16α-glucuronide	78	13.2	13.2 (8)	0.991	8

Table 2. *Recovery of added oestrogen glucuronide from urine*

Regression analysis, $y=bx+a$, relates measured steroid to steroid added to urine samples, where y = measured steroid, x = added steroid, b = the slope of the regression line and a = the endogenous steroid present in the urine samples; n = the number of samples assayed and r = the correlation coefficient.

Conjugate	Urine sample	Urine dilution (factor)	Regression analysis			
			n	r	b	a(nM)
Oestrone 3-glucuronide	Charcoal-treated	×100	64	0.989	1.04	11
	Pooled	×100	48	0.978	1.12	183
Oestradiol 3-glucuronide	Charcoal-treated	×20	47	0.981	1.17	−28
	Pooled	×20	54	0.991	1.15	75
Oestradiol 17β-glucuronide	Charcoal-treated	×20	36	0.996	0.92	1
	Pooled	×20	27	0.998	1.08	23
Oestriol 3-glucuronide	Pooled	×100	14	0.97	1.09	284
Oestriol 16α-glucuronide	Charcoal-treated	×20	46	0.987	1.23	−6
	Pooled	×20	53	0.996	1.17	409

Table 3. *Specificity of oestrogen glucuronide antisera*

B_0 is the percentage binding of labelled ligand at zero dose of unlabelled steroid. Values given are cross-reactions expressed as percentage molar ratios; they have been assessed at two levels: at 20 and 50% inhibition of binding of labelled ligand relative to B_0.

Antiserum ...	Oestrone 3-glucuronide		Oestradiol 3-glucuronide		Oestradiol 17β-glucuronide		Oestriol 3-glucuronide		Oestriol 16α-glucuronide	
B_0 value ...	54		82		34		20		46	
Level of assessment ...	20%	50%	20%	50%	20%	50%	20%	50%	20%	50%
Cross-reacting steroid										
Oestrone 3-glucuronide	100	100	9.8	3.7	0.04	0.013	0.2	—	0.06	0.05
Oestradiol 3-glucuronide	4.8	4.7	100	100	0.067	0.063	2.1	1.0	0.06	0.06
Oestradiol 17β-glucuronide	0.1	—	0.2	—	100	100	0.2	—	19.1	6.4
Oestriol 3-glucuronide	0.1	—	0.3	—	0.04	0.007	100	100	0.28	0.12
Oestriol 16α-glucuronide	0.1	—	0.2	—	0.004	—	6.7	3.7	100	100
Oestrone	1.3	7.6	—	1.7	19	5.5	—	0.5	44	10
Oestradiol	—	0.02	—	19	29	8.9	—	0.5	13.5	8.3
Oestriol	0.53	0.06	—	0.50	0.57	0.32	—	58	14.7	12.8

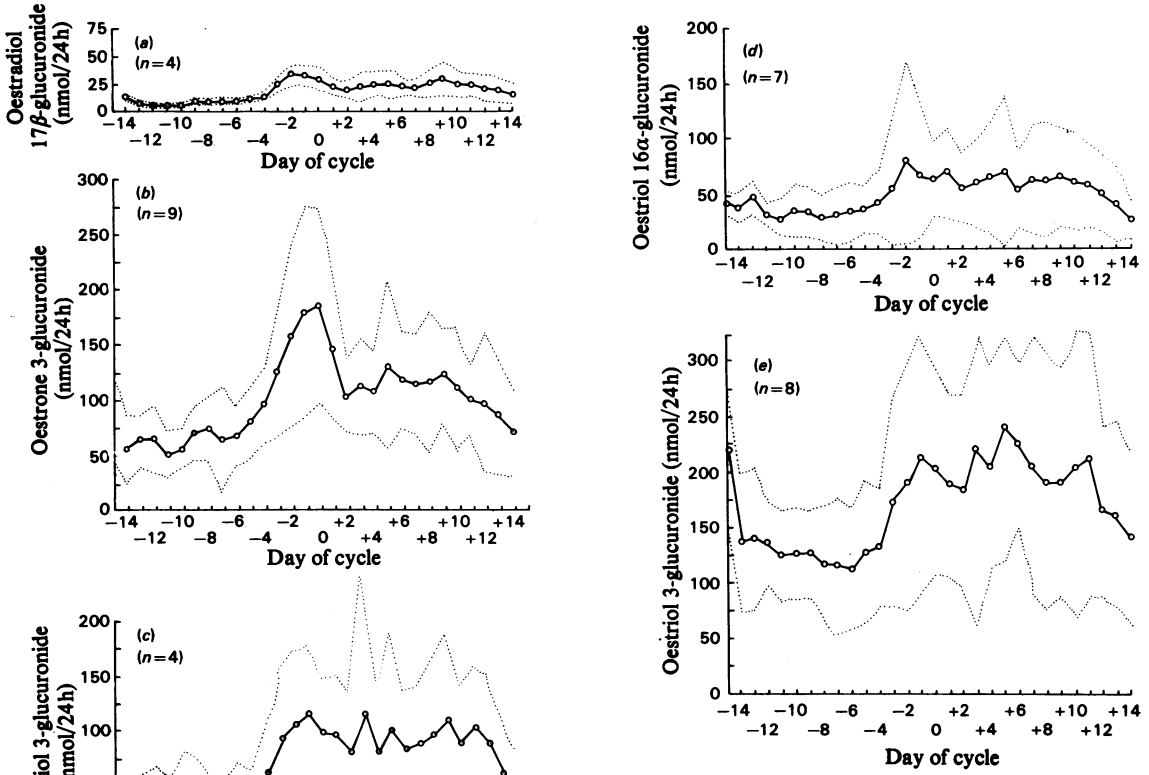

Fig. 1 *Mean mass excreted of oestradiol 17β-glucuronide (a), oestrone 3-glucuronide (b), oestradiol 3-glucuronide (c), oestriol 16α-glucuronide (d) and oestriol 3-glucuronide (e) in sequential 24 h urines throughout the menstrual cycle*
Results are means for *n* subjects; dotted lines indicate ±1 S.D.

at 20 and 50% inhibition of labelled-ligand binding, owing to non-parellelism between dose–response curves of different steroids (Baker, 1977).

Excretion of oestrogen glucuronides throughout the menstrual cycle

The patterns of excretion of oestrone 3-glucuronide, oestriol 3-glucuronide and oestriol 16α-glucuronide were assessed on a daily basis throughout a menstrual cycle in nine subjects. Similarly oestradiol 3-glucuronide and oestradiol 17β-glucuronide were assessed in five subjects. The results are expressed in Figs. 1(*b*), 1(*e*), 1(*d*), 1(*c*) and 1(*a*) respectively. As each daily collection consisted of a daytime and an early-morning specimen, the two measurements were totalled to give mass excreted for each day of the cycle. Unfortunately this daily period of collection was only approx. 24h (S.D.±1.4h). For the nine subjects cycle length ranged from 27 to 31 days, and where possible days of the cycle between different individuals were aligned relative to the plasma lutropin peak. In five women the day on which the lutropin peak occurred was designated day 0, the menses started on day 0+14; for the remaining women day 0 was determined by counting back 15 days from the start of the menses. The values given in Fig. 1 are mean values calculated on each day of the cycle for all the subjects investigated; standard deviations about the mean are also given.

The daily urinary excretion of pregnanediol 3-glucuronide was also measured in the nine women by direct radioimmunoassay (Baker *et al.*, 1976). In each case a value of 9μmol/24h was exceeded during the luteal phase, indicating adequate corpus-luteum function. Mean values±S.D. are shown in Fig. 2 for the mass of pregnanediol 3-glucuronide excreted per 24h.

The pattern of excretion of the five oestrogen conjugates was characterized by a low fairly constant output during the mid-follicular phase between days −12 and −5. Excretion then rose to a pre-ovulatory maximum between days −2 and 0 followed by a fall over the next 2 or 3 days. During the luteal phase mass excreted was variable but was higher than during the follicular phase; only with oestriol 3-glucuronide did it rise higher than the mid-cycle peak. Excretion of the five conjugates consistently fell over days +12 to +14; the fall continued over the first 2 days at the beginning of the cycle.

For each subject, daily values for all measured conjugates were totalled. 'Totalled oestrogen' ranges are shown for three stages of the menstrual cycle in Table 4, together with published total oestrogen values for a chemical method of assay. 'Totalled oestrogen' values ranged up to three times the total oestrogen values, yet contributions of minor oestrogen metabolites, such as bis-conjugates, sulphates

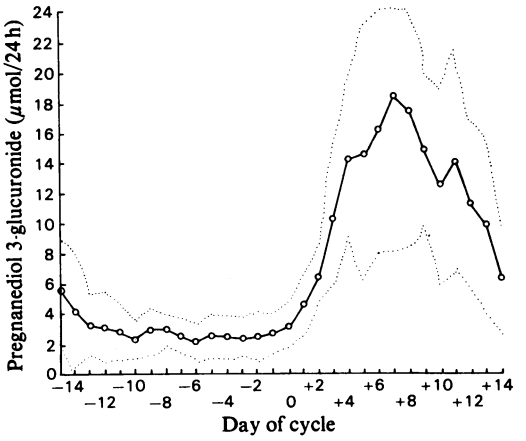

Fig. 2. *Mean mass of pregnanediol 3-glucuronide excreted in sequential 24h urines throughout the menstrual cycle* Results are means for nine subjects; dotted lines indicate ±1S.D.

Table 4. *Totalled oestrogen glucuronide values for eight subjects*

Subject or reference	Follicular range (nmol/24h)	Pre-ovulatory peak (nmol/24h)	Luteal range (nmol/24h)
JCW	160–200	420	250–430
KJ	130	500	100–370
POR	260–320	850	530–870
CSF	310–420	1040	490–1030
JG*	110–157	250	180–436
MT*	—	490	110–320
CMC*	260–446	850	390–655
PMB	110–200	410	190–658
Brown *et al.* (1968)	24–78	156–323	76–361

* Values do not include oestradiol 3-glucuronide and oestradiol 17β-glucuronide.

Table 5. *Measurement of total oestrogens (Kober–Ittrich method) in oestrogen glucuronide standards*
Regression analysis, $y = bx+a$, relates measured total oestrogen (y) to oestrogen glucuronide standard (x), where b is the slope and a is the intercept of the regression line; r is the correlation coefficient and n is the number of samples.

Steroid standard (50–2000nM)	Regression analysis			
	n	*r*	*b*	*a*(nM)
Oestrone 3-glucuronide	10	0.998	0.625	9
Oestriol 16α-glucuronide	12	0.995	0.510	−12
Oestriol 3-glucuronide	12	0.994	0.140	9

and unconjugated oestrogens, are not included in the 'totalled oestrogen' values. To investigate this discrepancy the oestrogen glucuronide standards used in the assays were analysed by a fluorimetric method. Total oestrogen values determined in this way on standard solutions (50–2000nM) showed good correlation between measured steroid and actual steroid, but with rather low recoveries (Table 5).

There was a large range of values about the mean daily excretion for each metabolite, particularly during the late follicular and the luteal phase. This reflects variation between different subjects rather than experimental error or day-to-day variation for one subject. Table 6 expresses each oestrogen conjugate as a percentage of 'totalled oestrogen' for eight women. The values given are mean percentages, averaged over one menstrual cycle, together with their respective standard deviations. The percentage of each oestrogen glucuronide excreted remains fairly constant over the menstrual cycle for a given individual (mean coefficient of variation = 23%), whereas up to a fivefold difference can exist for the excretion of a particular metabolite between the eight women (mean coefficient of variation = 40%). Since there is only limited variation in the proportion of any one conjugate throughout the menstrual cycle, the measurement of only one conjugate out of the five should be adequate to monitor the mid-cycle surge of oestrogen. To establish which would be the most useful conjugate to measure for this purpose, the ratio of the mid-cycle peak height to the follicular-phase baseline was determined for each subject by dividing the mean value on days −2, −1 and 0 by the mean value on days −12 to −6 inclusive. The ratios were then averaged for all subjects, and the results are shown in Table 7, together with their standard deviations, for each of the five metabolites. In addition, the day of the cycle on which the mid-cycle peak occurred was also recorded for each oestrogen conjugate.

This study was further extended by examining the ability to detect the mid-cycle rise. The mean and standard deviations of the follicular-phase baseline values (days −12 to −6) were calculated for each subject and each metabolite. A significant rise in the excretion of each conjugate was judged to be when the measured value exceeded two standard deviations above the mean of the baseline value. Table 8 records

Table 6. *Proportions of each oestrogen glucuronide averaged over menstrual cycle and expressed as percentage of 'totalled oestrogen'*
For details see the text.

	Percentage of 'totalled oestrogen glucuronide'				
Subject	Oestrone 3-glucuronide	Oestradiol 3-glucuronide	Oestradiol 17β-glucuronide	Oestriol 3-glucuronide	Oestriol 16α-glucuronide
CSF	16.6±3.5	19.8±6.0	3.7±1.3	41.8±8.2	17.4±4.2
KJ	38.6±5.3	30.8±3.5	5.6±1.1	20.6±6.8	4.3±1.1
POR	23.6±4.9	14.1±2.6	4.0±1.2	47.2±6.1	11.2±2.3
JCW	31.5±4.6	5.8±2.1	5.9±1.7	45.3±6.9	11.5±2.2
JG	26.5±6.5	—	—	52.5±6.6	20.5±5.3
MT	39.5±5.1	—	—	52.0±6.4	8.5±3.4
CMC	38.1±4.9	—	—	49.8±5.3	12.1±3.7
PMB	26.2±3.7	6.6±1.8	7.5±1.9	50.0±4.1	10.1±2.7

Table 7. *Detection of the pre-ovulatory oestrogen peak*
For details see the text.

		Numbers of subjects for which oestrogen excretion reaches a peak on given day of cycle					
Oestrogen glucuronide	Mean ratio of peak-to-baseline values	Day of cycle ... −4	−3	−2	−1	0	+1
Oestradiol 17β-glucuronide	3.4±0.8	—	1	3	1	—	—
Oestrone 3-glucuronide	2.8±1.1	—	1	3	3	3	—
Oestradiol 3-glucuronide	2.7±0.6	—	—	2	2	1	—
Oestriol 16α-glucuronide	2.3±0.6	—	2	2	2	2	—
Oestriol 3-glucuronide	1.7±0.3	—	—	1	3	4	1
[Oestrone 3-glucuronide]/[pregnanediol 3-glucuronide]	—	1	2	3	3	—	—

Table 8. *Detection of the pre-ovulatory oestrogen surge*
For details see the text.

Oestrogen glucuronide	Numbers of subjects for which steroid excretion on given day of cycle, exceeds 2 × S.D. of mean baseline values				
Day of cycle ...	−5	−4	−3	−2	−1
Oestradiol 17β-glucuronide	1	3	1	—	—
Oestrone 3-glucuronide	2	2	3	2	—
Oestradiol 3-glucuronide	—	2	2	1	—
Oestriol 16α-glucuronide	—	2	3	1	1
Oestriol 3-glucuronide	1	1	3	4	—
[Oestrone 3-glucuronide]/[pregnanediol 3-glucuronide]	3	3	1	2	—

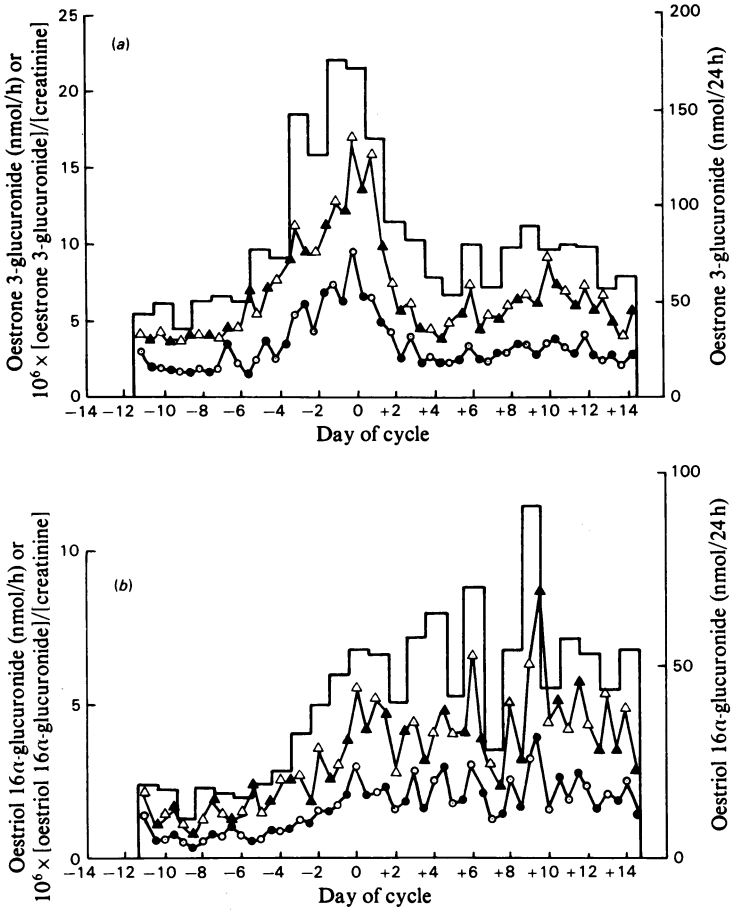

Fig. 3. *Comparison of the rate of excretion (mass/h) with the steroid/creatinine molar ratio for oestrone 3-glucuronide (a) and oestriol 16α-glucuronide (b) in a typical menstrual cycle (subject JW)*
Closed symbols represent early-morning urine specimens, and open symbols represent combined daytime specimens. Triangles represent steroid/creatinine values, circles represent excretion (nmol/h) and bars represent excretion (nmol/24h).

the day of the cycle and the number of subjects for which a significant increase in oestrogen excretion takes place.

The relative magnitude of the oestrogen glucuronide peak compared with the baseline, the day on which the peak occurs and the time at which a significant surge occurs, follow a similar pattern, dependent on the nature of the metabolite. The

successive events are: oestradiol 17β-glucuronide, oestrone 3-glucuronide and oestradiol 3-glucuronide together, oestriol 16α-glucuronide, then oestriol 3-glucuronide.

Inconsistencies in the timing of '24h' urine collections, together with a desire to obtain useful information from random urine samples, led to an alternative means of expressing excretion of oestrogen glucuronides. The integrated rate of excretion was determined in a typical cycle by dividing the mass of metabolite in each daytime and early-morning urine specimen by the exact time period of collection, thus giving a result in nmol/h. The results are shown in Figs. 3(*a*) and 3(*b*) for oestrone 3-glucuronide and oestriol 16α-glucuronide respectively. There is no obvious diurnal variation between daytime and early-morning collections. However, there is a suggestion of episodic excretion of oestriol 16α-glucuronide during the luteal phase, which is partially disguised by expressing results as excretion per 24h.

An alternative means of avoiding 24h urine collections is to express results as steroid/creatinine ratios. Figs. 3(*a*) and 3(*b*) compare the excretion rates (nmol/h) of oestrone 3-glucuronide and oestriol 3-glucuronide in one cycle with the steroid/creatinine ratio; correlation coefficients were respectively 0.929 ($n = 52$) and 0.949 ($n = 52$).

Oestrogen glucuronide/pregnanediol 3-glucuronide ratios, being independent of urine volume, may provide a means of assessing the mid-cycle oestrogen surge on random urine samples. Mean and range [oestrone 3-glucuronide]/[pregnanediol 3-glucuronide] values across the menstrual cycle are plotted for nine women (Fig. 4). The assay was not designed to measure follicular-phase pregnanediol 3-glucuronide with great precision, therefore measured pregnanediol 3-glucuronide concentrations of less than 1 μM were set equal to 1 μM in calculating the ratios. (A concentration of 1 μM could be distinguished from zero at the 99% level of confidence.) In all nine subjects (Table 8) a significant increase ($P<0.05$) in the ratio was detected 48–120h before the lutropin peak.

Excretion of oestrogen glucuronide during pregnancy

Fig. 5 shows the pattern of oestrogen glucuronides excreted in a subject who became pregnant during investigation. The notable difference from a non-pregnant subject is the increasing excretion of each conjugate during days +12, +13 and +14.

The excretion patterns of oestrone 3-glucuronide, oestriol 3-glucuronide and oestriol 16α-glucuronide during two normal pregnancies are depicted in Figs. 6(*a*) and 6(*b*). The output of oestriol 16α-glucuronide rises steadily after 20 weeks gestation and reaches a peak at, or just before, term; at this time it is some 2000-fold greater than normal luteal-phase excretion. Oestriol 3-glucuronide shows a smaller increase over

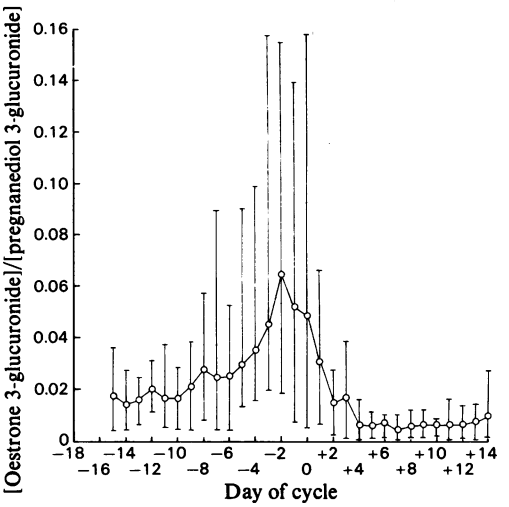

Fig. 4. *Mean molar ratio of oestrone 3-glucuronide to pregnanediol 3-glucuronide excreted throughout a normal menstrual cycle*

Results are means for nine subjects: bars indicate range values.

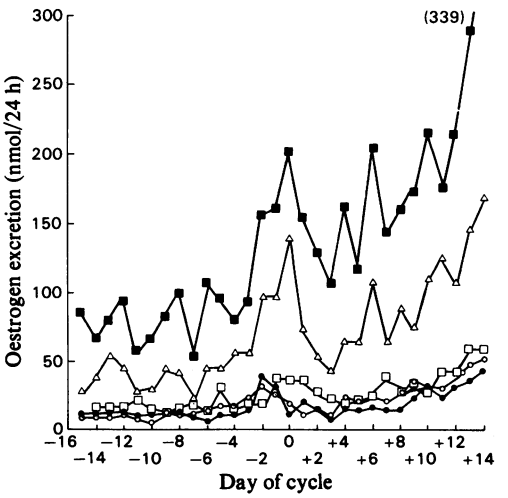

Fig. 5. *Mass of five oestrogen glucuronides excreted in sequential 24h urines throughout a conceptual cycle*

Key: △, oestrone 3-glucuronide; ●, oestradiol 3-glucuronide; ○, oestradiol 17β-glucuronide; ■, oestriol 3-glucuronide; □, oestriol 16α-glucuronide.

the same period to reach values approx. 200 times those of the luteal phase. Oestrone 3-glucuronide shows little change over the last 20 or so weeks of gestation, apart from a slight increase near term. Excretion is some 50–100 times that during the luteal phase.

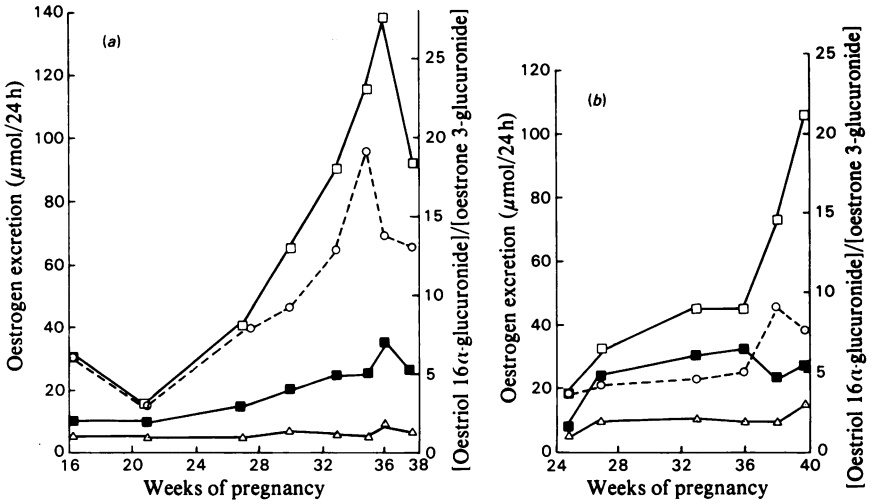

Fig. 6. *Mass of oestrone 3-glucuronide, oestriol 3-glucuronide and oestriol 16α-glucuronide excreted in 24h urines during the second and third trimesters of pregnancy, for two subjects: (a) JC and (b) AH*

Key: △, oestrone 3-glucuronide; ■, oestriol 3-glucuronide; □, oestriol 16α-glucuronide; ○, [oestriol 16α-glucuronide]/[oestrone 3-glucuronide].

The striking difference between the pattern of metabolites in a normal cycle and in the second and third trimesters of pregnancy is the change from oestriol 3-glucuronide to oestriol 16α-glucuronide as the major urinary oestrogen. Figs. 6(*a*) and 6(*b*) also demonstrate a tendency for the ratio of oestriol 16α-glucuronide to oestrone 3-glucuronide to increase throughout most of pregnancy.

Discussion

Cross-reactions of related oestrogen glucuronides were sufficiently low to have minimal effect on the accuracy of the five oestrogen assays. Unconjugated oestrogens, although showing rather higher cross-reactions, are present in urine only as about 1% of the total oestrogens (Kunzig & Geiger, 1976), and therefore probably do not interfere to a significant extent. A constant recovery of measured steroid over a range of urine dilutions was taken as further evidence that interference from cross-reacting steroids was minimal. Provided that urine was assayed at a dilution of at least 20-fold, urine blanks were not significantly different from buffer blanks.

External recovery experiments did not reveal a bias of scale or of zero bias in measured steroid. It therefore appeared that the five oestrogen assays would give valid results when applied directly to diluted urine.

Radioimmunoassay of oestrogen glucuronides in menstrual-cycle urine resulted in values for 'totalled oestrogens' which were considerably higher than total oestrogen values obtained by chemical methods (Brown *et al.*, 1968). Possibly there is a discrepancy in the radioimmunoassay method due to interference from cross-reacting steroids or a positive blank effect from the urine sample which has not been revealed by the specificity or recovery studies. However, the interference would have to be very great indeed to account for the radioimmunoassay result being up to 3 times that of chemical methods. Similar discrepancies have been reported by Adlercreutz *et al.* (1976) for the direct radioimmunoassay of oestriol 16α-glucuronide in pregnancy urine.

A further explanation could be that the radioimmunoassay values represent the true values; lower values by the chemical method arise from procedural losses associated with hydrolysis of the conjugate and solvent extraction. When total oestrogen determinations, by a chemical method, were performed on standard oestrogen glucuronides, the results were as low as 15% of their expected values. The recovery appears to be dependent on the nature of the conjugate. Perhaps procedural loss in chemical methods occurs to a different extent with oestrogen glucuronides compared with free oestrogen.

The pattern of excretion of oestrogen glucuronides during the menstrual cycle is similar to that determined by a chemical method (Johansson *et al.*, 1971), the peak of total oestrogen usually occurring on the same day or the day before that of the lutropin peak. The present work indicates that certain oestrogen

glucuronides give an earlier warning of impending ovulation than others. Also that the temporal order of excretion of the various conjugates reflects different metabolism. For instance, enterohepatic circulation of oestriol results in the later appearance of oestriol 3-glucuronide in urine than other conjugates (Goebelsmann *et al.*, 1966).

In the clinical application of the techniques described it is more convenient, in practical terms, to measure one conjugate rather than all five. Oestrone 3-glucuronide is probably the best conjugate to measure, since next to oestradiol 17β-glucuronide it has the highest mid-cycle peak-to-baseline ratio, yet it is excreted in 5 times the amount of the latter and can be detected in 100-fold-diluted urine. Also an increase in oestrone 3-glucuronide tends to occur sooner than most other conjugates, and its excretion shows a comparatively low degree of variation between individuals.

The assays described can be carried out within 3h; this would be of little advantage to the clinician if 24h urine specimens had to be collected. The present work shows that valid results can be obtained from random urine specimens by expressing results either as hourly rates of excretion or as oestrogen/creatinine ratios. Already an oestrogen/creatinine ratio has proved to be a satisfactory parameter for monitoring pregnancy urine (Metcalf & Hunt, 1976; Rao, 1977). In addition, the oestrone 3-glucuronide/pregnanediol 3-glucuronide ratio offers a simple alternative for the prediction of ovulation.

The results on oestrogen glucuronide excretion during pregnancy are of a preliminary nature. As with menstrual-cycle urine, the mass of oestrogen glucuronides in pregnancy 24h urines determined by radioimmunoassay showed higher values than those reported for chemical methods. In term urine, the values found for oestrone 3-glucuronide and oestriol 16α-glucuronide range from 53 to 128% greater than those reported by Ahmed & Kellie (1972) and 8 to 41% greater than those reported by Tikkanen (1972). However, the relative proportions of oestrone 3-glucuronide, oestriol 3-glucuronide and oestriol 16α-glucuronide at term (8, 18 and 74% respectively) were very similar to those determined by Ahmed & Kellie (1972) (5, 20 and 75% respectively). The marked increase in the excretion of oestriol 16α-glucuronide reflects increased production of oestriol by the foetoplacental unit (for review see Kunzig & Geiger, 1976). On the other hand the relatively constant mass of oestrone 3-glucuronide in pregnancy 24h urines may relate to the fact that the source of approximately half the oestrone precursors is the maternal adrenals (Siiteri & MacDonald, 1966). Oestrone 3-glucuronide excretion should therefore be less dependent on the state of the foetus than oestriol 16α-glucuronide excretion. If this is the case, then the ratio of oestriol 16α-glucuronide/oestrone 3-glucuronide should be a useful parameter for the monitoring of foetoplacental function.

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