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Monitoring Ovarian Function by Solid-Phase Chemiluminescence Immunoassay

J.B. Kim, P.S. Ku

Department of Obstetrics & Gynecology,
Korea University College of Medicine, Seoul, Korea

—국문초록—

Monitoring Ovarian Function by Solid-phase Chemiluminescence Immunoassay

高麗大學校 醫科大學 産婦人科學教室

김 종 배 · 구 병 삼

여성의 난소기능은 뇨중 Oestrone-3-glucuronide를 간편한 solid-phase의 화학발광성 면역학적 측정법 (Chemiluminescence Immunoassay(CIA)에 의하여 그 기능이 탐지될 수 있다. Oestrone-3-glucuronyl-6-bovine serum albumine에 대한 antiserum의 IgG fraction은 polystyrene시험관벽에 흡착시켰으며, 항원으로서는 estrone-3- glucuronyl-6-aminoethyl-ethyl-isoluminol을 항원 (antigen)에 label시킨 것이다. 시험 대상물인 뇨는 매일아침뇨 (early morning urine)을 희석(1:1000 V/V)한 후 100mcl를 취하여 이를 각기 이중분석액으로 택하였다.

시험관 내에서 결합반응 (1 hour at 4°C)이 일어난 후에는 시험관내의 액체를 전부 흡입 폐기시켰으며, 항체반응이 일어난 후 (antibody-bound fraction)에는 완충액 (400mcl)으로 한번 세척시켰다. 그후 염화수산화물 (2 N , 200 mcl)을 가하고 22°C에 60분간 방치 혼합케 한 후 효소 (microperoxidase)와 과산화수소를 가하면서 산화작용에서 발생되는 발광양을 10초 동안 측정하여 그 결과를 분석하였다.

위에 기술한 분석방법을 평가하면 다음과 같은 결론을 얻었다. Calibration curve sensitivity 3.12 ± 0.75 PG / tube (mean ± SD)였고, Intra-assay precision(CV%) 9.52 (20 replicates; 38.4 ± 3.66 nmol/1)와 8.81 (15 replicates; 102.4 ± 8.82nmol/1)였다. Inter-assay precision(CV%)은 11.9 (mean of 4 pools-7.03, 23.16, 52.11과 117.53 nmol/1)로 2개월 동안에 걸쳐 시행되었고, 평균 바이어스 (mean bias)는 -0.78 로 28에서 448 nmol 법위로서 매일아침 "뇨"의 차이분(different aliquots)은 좋은 결과를 얻었다.

건강한 여성으로부터 채취된 뇨중 Oestrone-3-glucuronide의 농도 (nmol/1)를 보면 월경주기의 여포기와 배난기 및 황체기에 있어서 각기 40.2 ± 9.9 , 102.3 ± 39.4와 84.3 ± 13.3

nmol/l였다.

이와같은 결과는 동일한 검사뇨를 방사면역학적 방법(RIA)으로 측정(6 menstrual cycle)한 결과와 유사한 측정치를 얻으므로서 간편하고 진보된 좋은 방법중의 하나라고 사료되는 바이다.

INTRODUCTION

Numerous methods have been devised to investigate ovarian function and to monitor or predict variations in potential fertility in the human female. These have been based on: (i) the secretion of pituitary and ovarian hormones (WHO, 1980); (ii) their biochemical or biophysical effect on responsive tissues (Moghissi, 1980); or (iii) the change in ovarian morphology as observed with ultrasound (Queenan et al, 1980). Consequently, a greater understanding of the interaction and temporal relationships of the many parameters associated with ovulation has been achieved.

The results from studies of ovarian function and steroid metabolism have suggested that the principal urinary metabolites of oestradiol and progesterone might be used as indices of the start and finish of the probable fertile period (Collins et al., 1981). Accordingly, methods based upon the principles of radioimmunoassay have been developed to measure the main oestrogen glucuronides and pregnanediol-3α-glucuronide in diluted urine (Samarajeewa et al., 1975; 1979). Subsequently, several investigators have undertaken the study of facets of this biochemical approach to the physiological problem of predicting and detecting ovulation and locating the fertile period (Stanczyk et al., 1980; Adlercreutz et al., 1980; Baker et al., 1980).

There is still the need, however, for simpler methodology. The increasing usefulness and availability of radioimmunoassay has raised several serious problems which include; (i) the radioactive half-life and radiolysis of the labelled reagent; (ii) health hazards associated with the use and disposal of radioactive compounds and the solvents necessary for liquid scintillation counting; (iii) the high cost and maintenance of equipment; and (iv) the dependence upon laboratory facilities and expertise. Consequently, radioimmunoassay procedures are unsuitable in the development of simple kits and instruments for potential clinic or homeuse to provide rapid and reliable information with regard to fertility. Several approaches have been suggested to overcome the disadvantages of radioimmunoassay while retaining the specificity of the antigen antibody reaction (Schall and Tenoso, 1981). In particular, the use of chemiluminescent markers in monitoring protein-binding reactions has been described (Schroeder et al., 1976; Simpson et al., 1978; Kohen et al., 1979).

Recently, chemiluminescent immunoassays have been developed for the measurement of urinary steroid metabolites (Barnard et al., 1981 (a); Barnard et al., 1981 (b); Eshhar et al., 1981) which have involved the use of a solid-phase separation system. The specific IgG is adsorbed onto the surface of polystyrene assay tubes and after the binding reaction the incubation mixture is aspirated. Subsequently, the tubes are rinsed with buffer to remove potentially interfering substances with the concomitant reduction in background chemiluminescence. These methods have been shown to be comparable in specificity and sensitivity to established radioimmunoassays.

Recent findings have suggested that the measurement of oestrone-3-glucuronide in early morning urine may the most useful parameter in the delineation of the fertile period (WHO, 1982). In this report a description is given of a

solid-phase chemiluminescence immunoassay for the measurement of this metabolite in daily samples of early morning urine. The method is assessed for specificity, sensitivity, accuracy and precision. In addition, a comparison is made between values of urinary oestrone-3-glucuronide as determined by chemiluminescence immunoassay (CIA) and a liquid-phase radioimmunoassay (RIA) using a tritiated antigen. The clinical and physiological value of the test is discussed.

MATERIALS AND METHODS

Reagents

Oestrone-3-glucuronide (E_1-3-G), oestradiol-3-glucuronide (E_2-3-G), oestradiol-17β-glucuronide (E_2-17β-G) and oestriol-3-glucuronide (E_3-3-G) were kindly donated by Dr. W. Coulson of the Courtauld Institute of Biochemistry, The Middlesex Hospital Medical School. All other steroids, microperoxidase (MP-11), bovine serum albumin (BSA Fraction V) and Sepharose-Protein A were purchased from Sigma London Chemical Co. Ltd., Poole, Dorset, U.K. The steroids were recrystallised in appropriate solvents before use. (6,7-^{3}H)-E_1-3-G (specific activity 38 Ci/mmol) and antiserum to E_1-3-G-6-bovine serum albumin were kindly donated by Dr. P. Samarajeewa of the Courtauld Institute of Biochemistry, The Middlesex Hospital Medical School. All other reagents were obtained from Hopkin and Williams Ltd., Romford, Essex, U.K. Anti-oestrone-3-glucuronide IgG was prepared by a procedure already reported (Kohen et al., 1980).

Buffers

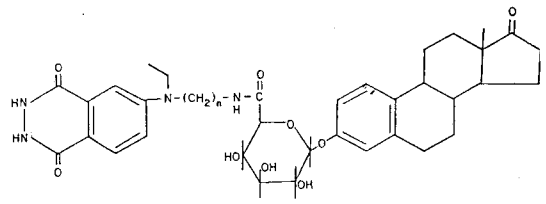
Two buffers were used: (i) a coating buffer (0.07M barbitol buffer: 14.4g sodium barbitol dissolved in one litre of double distilled water (pH 9.6); and (ii) an assay buffer (0.1M phosphate buffer): 2.5g sodium dihydrogen orthophosphate. $2H_2O$ plus 11.9g disodium hydrogen orthophosphate dissolved in one litre of double distilled water containing 0.1% BSA and 0.9% sodium chloride (pH 7.5).

Preparation of chemiluminescent marker conjugate

6-[N-(4-aminoethyl)-N-ethyl] amino-2, 3-dihydrophthalazine-1, 4-dione (AEEI) and 6-[N-(4-aminobutyl)-N-ethyl] amino-2, 3-dihydrophthalazine-1, 4-dione (ABEI) were synthesised according to a method previously reported (Schroeder et al., 1978). The conjugation of AEEI and ABEI to E_1-3-G and the purification of the products is similar to the methods described previously for oestriol-16α-glucuronide-ABEI conjugate (Kohen et al., 1980a). The proposed structures of E_1-3-G-AEEI and E_1-3-G-ABEI are shown in Fig. 1.

Antibody-coated tubes

Anti-oestrone-3-glucuronide IgG was suitably diluted in the coating buffer. Two hundred (200)μl were added to each polystyrene assay tube (Luckham Ltd., Victoria Gardens, Burgess Hill, Sussex, U.K.). After an overnight incubation at 4°C, the coating buffer was aspirated to waste and 300 μl of 0.3% BSA in saline was added to each tube. After incubation for 30min. at 22°C the solution was aspirated to waste and the tube stored at 4°C until required.



OESTRONE-3-GLUCURONIDE-AEEI (n=2)
OESTRONE-3-GLUCURONIDE-ABEI (n=4)

Fig. 1. The proposed structures of oestrone-3-glucuronide-AEEI and oestrone-3-glucuronide-ABEI

Sample collection and dilution

Daily samples of early morning urine (EMU) were collected from healthy, non-pregnant female volunteers throughout their complete menstrual cycle. Prior to analysis, 10μl of urine were diluted to one ml by the addition of 990μl of assay buffer.

Immunoassay procedure

(i) Radioimmunoassay (RIA)

Oestrone-3-glucuronide was measured by radioimmunoassay according to the method of Collins et al. (1979). The antibody-binding reaction was completed in the liquid-phase at 4°C and the antibody-bound and free fractions were separated with Dextran-coated charcoal, followed by centrifugation. The supernatent, containing the antibody-bound fraction, was decanted into scintillation fluid (Scintillation Cocktail T) and the radioactivity determined on an LKB Rackbeta 1216 (LKB Instruments Ltd., 232 Addington Road, South Croydon, Surrey, U.K.).

(ii) Chemiluminescence immunoassay (CIA)

One hundred (100)μl of diluted urine or 100μl of standard (range 1.56 to 400pg/100μl of immunoassay buffer) were added in duplicate to the antibody-coated tubes. Subsequently, 100 μl of E_1-3-G-6-AEEI (50pg/100μl assay buffer) were added and the mixture was incubated at 4°C for 1 hour and the contents of the tube were then removed by aspiration. Four hundred (400)μl of assay buffer were added to each tube and subsequently removed by aspiration.

Two hundred (200)μl of 2N sodium hydroxide were added to each tube and the contents incubated at 22°C for 60min. Microperoxidase was dissolved in assay buffer and the stock solution (1mg/ml) was stored at 4°C. The working solution was 20μg/ml (1:50, V/V). The oxidant solution was prepared by adding 100μl hydrogen peroxide to 10ml of double distilled water. One hundred (100)μl of the microperoxidase solution were added to the assay tube, which was placed in the luminometer. The chemiluminescence reaction was initiated by the rapid injection of 100μl of diluted hydrogen peroxide.

The light emitted was measured with an LKB Luminometer Model 1250 (kindly provided by LKB Instruments Ltd., South Croydon, Surrey, U.K.) in association with an LKB 1223 Databox i.e. an on-line luminescence analyser with conversational teleprinter control and a flat bed recorder (LKB 2210). The curette holder was fitted with an adaptor in order to accommodate the assay tube. An automatic dispenser was used to inject the solution of hydrogen peroxide (Hook and Tucker Instruments Ltd., Croydon, Surrey). The light emitted was monitored with the chart recorder and the signal intergrated over 10 seconds.

Calculation of results and statistical analyses

The unknown values were derived from the calibration curves (B/B^0, % vs concentration of E_1-3-G, pg) and multiplied by 2.242 to obtain the results in nmol/l (MW 446). The concentrations of E_1-3-G in EMU collected from different phases of the menstrual cycle were defined by the arithmetic means, geometric means and limits.

RESULTS

Detection limit of chemiluminescent conjugates

The results of an experiment to determine the detection limit of the two chemiluminescent conjugates (E_1-3-G-6-AEEI and E_1-3-G-6-ABEI) are shown in Fig. 2. It was calculated that the detection limits, defined as twice mean the background chemiluminescent signal, were 126fg (AEEI conjugate) and 218fg (ABEI conjugate).

Dilution of antibodies (IgG fraction)

The optimum dilution of anti-E_1-3-G IgG was determined by coating the polystyrene tubes with serial dilutions of the buffer (range 1:2000,

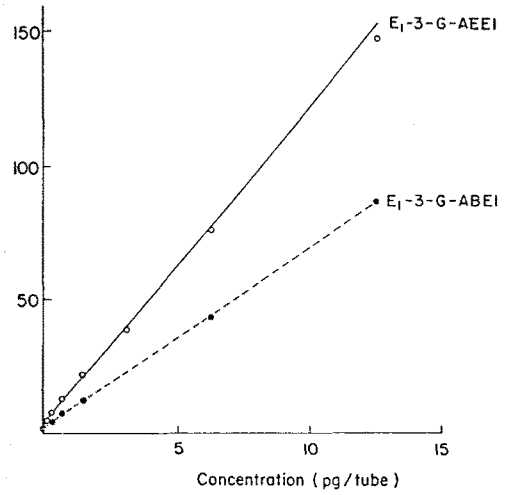


Fig. 2. The detection limit of two chemiluminescent conjugates of oestrone-3-glucuronide

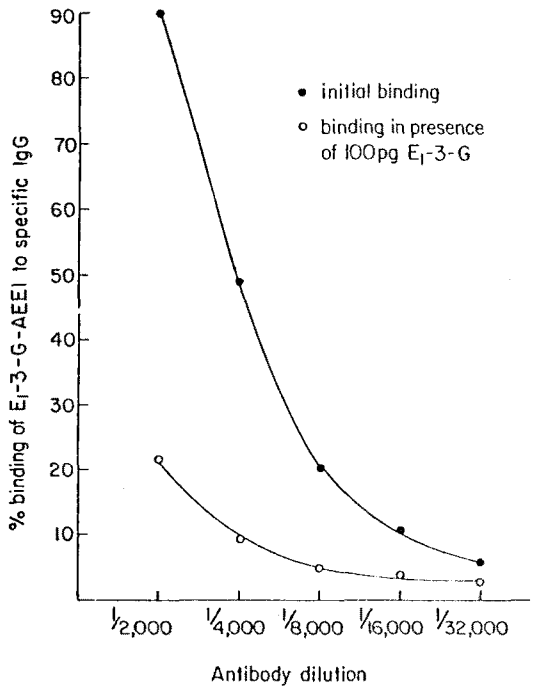


Fig. 3. Antibody dilution curve in the presence and absence of authentic E_1-3-G (100 pg).

V/V to 1:32000, V/V). One hundred (100)μl of E_1-3-G-6-AEEI (50pg) were incubated in the presence and absence of 100pg of authentic E_1-3-G. The results are shown in Fig. 3 and it was concluded that the optimum dilution was 1:4000 (V/V). A calibration curve (mean ± S.D.; n = 3) at this dilution is shown in Fig. 4.

Effect of sodium hydroxide on chemiluminescence

The amount of light emitted after incubating the antibody-bound fraction in the presence of sodium hydroxide (200μl; 2N) for different times (10 to 240min) at room temperature (22°C) was investigated. The results are shown in Fig. 5. It was concluded that, in the case of the AEEI conjugate, an incubation time of 60min at 22°C with 2N sodium hydroxide was adequate to ensure maximum light enhancement and reproducibility. E_1-3-G-6-AEEI (50pg) was used in all subsequent experiments.

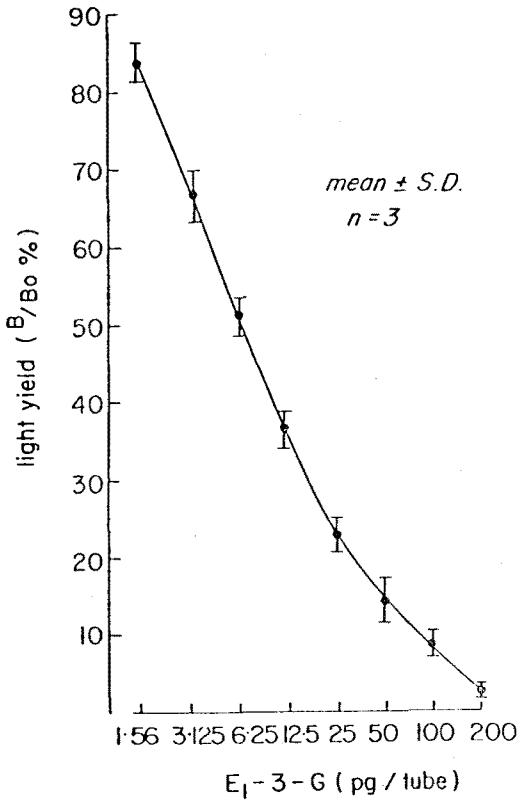


Fig. 4. A calibration curve.

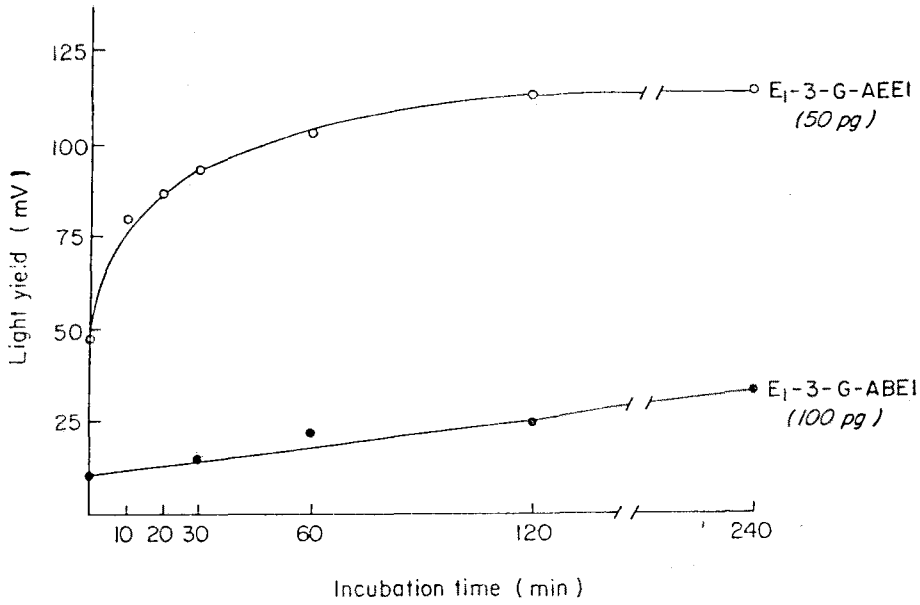


Fig. 5. The effect of sodium hydroxide on the chemiluminescence of two conjugates of E_1-3-G.

Characterisation of antibodies (IgG fraction)

Anti-E_1-3-G IgG was characterised according to the methods of Thorneycroft et al. (1970). The percentage cross-reactivity of related compounds was determined by a solid-phase RIA and CIA and the results are compared in Table 1.

Sensitivity

The minimum concentration of E_1-3-G that could be significantly distinguished from zero (Mean - 2SD; n = 6) was calculated from 3 calibration curves. The value (mean ± S.D.) was 1.45 ± 0.35pg/tube which is equivalent to 3.25 ± 0.78 nmol/l urine.

Accuracy

Increasing amounts of authentic E_1-3-G were added to urine from a two year old child which had been analysed previously. The results are shown in Table 2.

Precision

An estimate of the intra-assay variation was obtained by analysing replicate samples from two EMU within a single assay. The correspond-

Table 1. The cross reactions of selected compounds, expressed as % molar concentrations giving 50% inhibition of labelled antigen bound to anti-E_1-3-G IgG in 2 immunoassay systems.

Compound	CIA	RIA
Oestrone-3-glucuronide	100.00	100.00
Oestrone	21.20	37.00
Oestradiol	< 0.01	16.00
Oestriol	< 0.01	0.10
Oestrone-3-sulphate	11.20	21.00
Oestradiol-3-glucuronide	1.00	20.00
Oestradiol-17β-glucuronide	< 0.01	< 0.01
Oestriol-3-glucuronide	1.10	0.10
Oestrone-3-G-AEEI	–	22.00

Table 2. The measurement by CIA of E_1-3-G in EMU* containing additive amounts of analyte.

Amount added pg/100μl diluted urine (1/100; v/v)	Observed value pg/tube	Expected value* pg/tube	Recovery (%)	Bias (%)
200.0	212.4	202.3	104.9	+4.9
100.0	97.3	102.3	95.1	-4.9
50.0	48.5	52.3	92.7	-7.3
25.0	26.4	27.3	96.7	-3.3
12.5	15.8	14.8	106.7	+6.7
Mean			99.22	-0.78

* urine sample contained 2.3 pg.

ing value for inter-assay variation was obtained from the measurement of E_1-3-G in four urine samples used for internal quality control over a period of two months. The results are shown in Table 3.

Parallelism

The concentration of E_1-3-G was determined by CIA in 4 different aliquots of urine (equivalent to 4, 2, 1 and 0.5μl) from 3 samples of EMU. The corrected concentrations (mean ± SD) were 53.1 ± 5.4 nmol/l; 119.0 ± 4.6 nmol/l and 268.3 ± 21.5 nmol/l. The coefficients of variation (%) were 10.1, 3.9 and 8.0 respectively.

Correlation with RIA

Samples of EMU were collected daily throughout the menstrual cycle from six healthy female volunteers. The concentration of E_1-3-G was determined by both CIA and RIA. The results (mean ± SEM) are shown in Fig. 6. A typical chart recorder tracing of a standard curve and samples collected throughout one complete menstrual cycle are shown in Fig. 7. The concentration of E_1-3-G has been determined by CIA and RIA in 87 samples of EMU from healthy women and the results are shown in Fig. 8. The correlation coefficient 'r' between the two methods

Table 3. Intra and inter-assay variation of urinary oestrone-3-glucuronide as determined by CIA.

Sample	No. of determinations	nmol/l (mean ± SD)	Coefficient of variation (%)
(i) Intra-assay			
Sample 1	20	38.44 ± 3.66	9.52
Sample 2	15	102.40 ± 8.82	8.61
(ii) Inter-assay			
Pool 1	6	7.03 ± 1.16	16.50
Pool 2	6	23.16 ± 3.55	15.32
Pool 3	6	52.11 ± 3.82	7.33
Pool 4	6	117.53 ±10.02	8.52

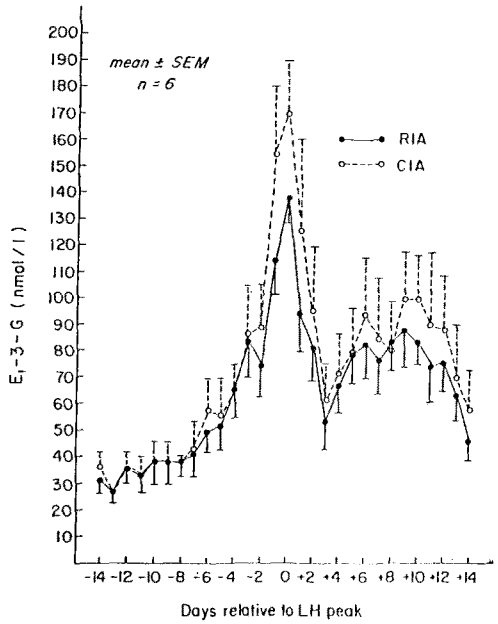


Fig. 6. The concentration of E_1-3-G in samples of EMU throughout six normal cycles.

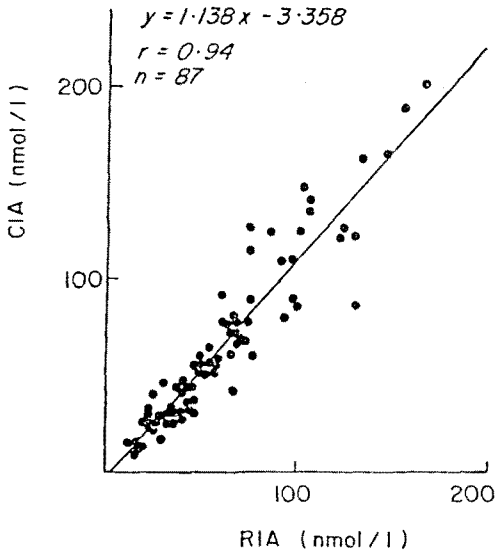


Fig. 7. The correlation between RIA and CIA.

was 0.94 (y = 1.138x + 3.358), where y is the value obtained by CIA and x the value determined by RIA.

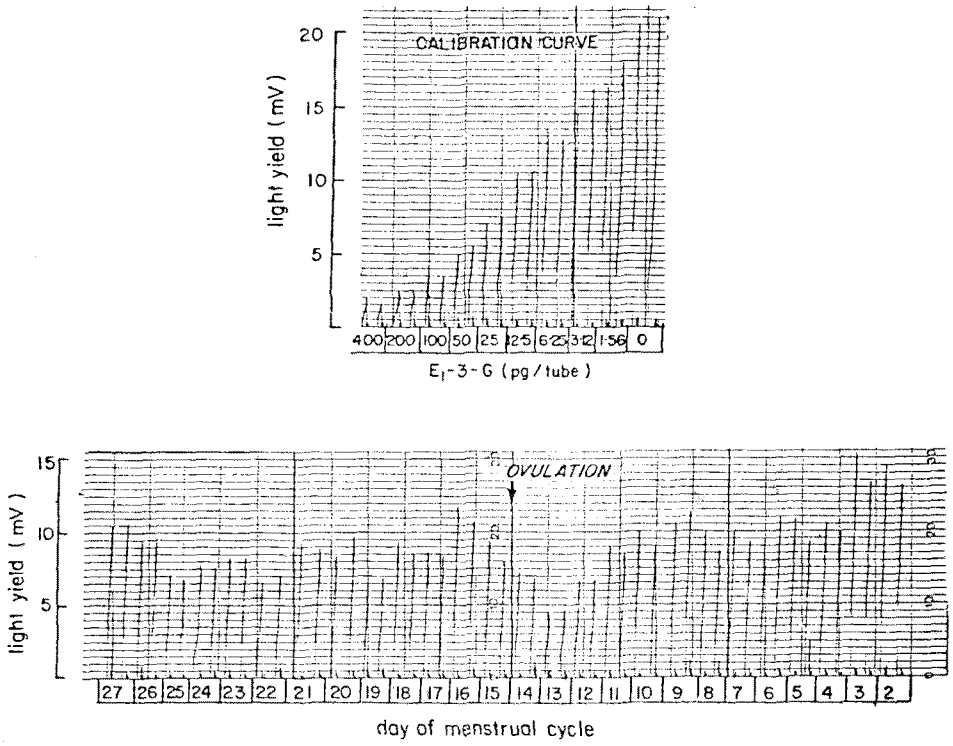


Fig. 8. A recorder trace of standard curve and samples throught one complete menstrual cycle.

Table 4. The concentration (nmol) of E_1-3-G by RIA and CIA during different phases of the menstrual cycle.

Days of cycle relative to LH peak (Day 0)	No. of samples	Arithmetic Mean ± SD	Geometric Mean	Range
(i) RIA				
-14 to - 5	10	38.4 ± 7.6	37.7	21.7 - 51.8
- 4 to +4	9	86.0 ± 26.7	82.7	53.0 -138.5
+ 5 to +14	10	75.4 ± 12.4	74.3	46.1 - 88.3
(ii) CIA				
-14 to - 5	10	40.2 ± 9.9	39.1	25.6 - 58.1
- 4 to +4	9	102.3 ± 39.4	96.1	60.6 -170.0
+ 5 to +14	10	84.3 ± 13.3	83.3	57.9 -100.05.

Normal ranges

The concentration of E_1-3-G in EMU collected during the early follicular, periovulatory and luteal phases of the menstrual cycle is shown in Table 4.

DISCUSSION

It is now well established that the measurement of selected steroid glucuronides in daily samples of early morning urine provide a simple, non-invasive approach to the monitoring of ovarian function in women (Collins et al., 1981). Moreover, the data obtained suggests that the concentration of E_1-3-G in EMU gives comparable information on follicular development to that obtained by the measurement of oestradiol in peripheral venous plasma. Indeed recent findings have indicated that the measurement of this metabolite alone may be the most reliable biochemical index for the determination of the start and finish of the fertile period (WHO, 1982). Consequently, there is increasing effort to simplify the methodology with the ultimate objective of producing a test that could be performed in the home so that the woman might obtain rapid and reliable information as to her own fertility.

In recent years there has been much controversy over the use of alternative, non-isotopic labels in immunoassay (Schall and Tenoso, 1981). Of the many methods developed and applied to biological samples, only a few have attained the high degree of specificity and sensitivity, equivalent to that of antigen-labelled RIA or the potentially more sensitive immunoradiometric assay (IRMA) using labelled antibodies.

The use of chemiluminescence to monitor specific binding reactions was first proposed by Schroeder et al. (1976). Since that time several homogeneous and heterogeneous immunoassays have been reported for the measurement of steroids and their urinary metabolites using isoluminol conjugates (Kohen et al., 1979; 1980a; 1980b; 1981; Barnard et al., 1981a; 1981b; Eshhar et al., 1981; Kim et al., 1981). The labelled antigens have been shown to be very stable over two years and the detection systems are relatively inexpensive and reliable.

The results of the present study have shown that it is possible to develop a simple non-isotopic immunoassay to monitor ovarian function. Furthermore, developments to the reported methodology are envisaged. For example, equivalent sensitivity to that obtainable by conventional RIA has already been achieved with isoluminol

labelled antigens which have relatively low quantum efficiency. It is anticipated that with the development of new, more efficient labels (e.g. acridinium compounds) and simpler oxidation systems, the possibility of obtaining sensitivities many orders of magnitude greater than those so far achieved will become a reality. In addition, the availability of extremely reliable yet relatively cheap and simple monitors, together with an appropriate reagent presentation (e.g. lyophilised pellets or reagent strip format) will facilitate the acceptability of luminescence techniques and will provide the opportunity for effective home or clinic monitoring of ovarian function with the concomitant use in family planning techniques and the treatment of infertility.

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