Automation and limitation of 17 β oestradiol dehydrogenase in monitoring ovarian function

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SUMMARY An automated enzymatic assay which measures combined concentrations of urinary oestrone and oestradiol within 15 minutes of hydrolysis has been developed using placental 17 β oestradiol dehydrogenase. This assay is comparable to radioimmunoassay in its precision and sensitivity and is quicker, more convenient, and more economical. The 17 β oestradiol dehydrogenase assay was used to monitor ovarian response in infertile women being treated through stimulated ovarian cycles or in vitro fertilisation programmes. Because the assay did not respond to oestriol it was less reliable than total urinary oestrogens in monitoring ovarian responses, particularly during treatment with drugs which enhanced oestriol excretion. In natural ovulatory cycles rising 17 β oestradiol dehydrogenase values preceded peak luteotropin concentrations by up to 18 hours and improved detection of ovulation. The 17 β oestradiol dehydrogenase assay processes large numbers of urine samples quickly and economically and enables accurate monitoring of oocyte maturity and ovulation.

Serial urinary oestrogen concentrations are an important measure of ovarian response and are particularly useful in clinical programmes treating infertile women through stimulated ovarian cycles.¹ In vitro fertilisation programmes also require intensive monitoring of oestrogen concentrations to evaluate oocyte maturity before laparoscopy.² Laboratories performing assays for these programmes are required to process large numbers of samples in a single working day at a reasonable cost. To meet such requirements the well established fluorimetric assay for total urinary oestrogen has been modified to a three to four hour procedure.³ This assay may be unsuitable for many laboratories because it is labour intensive and requires high levels of technical skill. Recently developed rapid radioimmunoassays have served as a successful alternative procedure, whereby up to 40 serum or urine samples can be analysed for oestrogens in four hours. These assays, however, are associated with problems of specificity, expensive labile reagents, and variable non-specific method blanks.⁴

In this report we describe an enzymatic method⁵ which measures combined concentrations of urinary oestradiol and oestrone using placental 17 β oestradiol dehydrogenase (EDH; EC 1.1.1.62). This assay was automated using a COBAS-BIO centrifugal analyser and the results obtained were used

to monitor ovarian function. In many patients who ovulated naturally the results of the rapid enzyme assay compared with those of a reference fluorometric method for measuring total urinary oestrogens. In several infertile women being treated in a stimulated ovarian cycle programme, however, problems were encountered which occasionally limited the reliability of the EDH assay.

Patients and methods

A total of 981 urine samples were collected from 42 patients. Twenty seven patients collected serial 24 hour urine samples ($n = 405$) throughout their menstrual cycles. Of this group, 15 patients showed normal menstrual cycles and 12 patients were treated in a stimulated ovarian cycle programme. Another group of 15 patients from an in vitro fertilisation programme collected serial three hourly urine samples ($n = 576$) from days 11 to 15 of their menstrual cycles. In this second group, eight patients had spontaneous luteinising hormone surges while seven patients had ovulation induced with 3000 IU of human chorionic gonadotrophin. The regimen of treatment used in the stimulated ovarian programme has been described elsewhere.⁶

The reagents for the EDH assay were supplied by Bio Merieux, France, and included two enzymes, EDH and glucose-6-phosphate dehydrogenase, and

substrates. To evaluate the assay performance purified 17 β oestradiol (cat no E8750), oestrone (E9750), 17 β oestradiol glucuronide (E1127), oestrone β -D glucuronide (E1752), and β glucuronidase (G1758) were purchased from Sigma Chemicals, St Louis, United States. Urinary luteinising hormone concentrations were measured in a rapid two hour radioimmunoassay.⁷ Oestradiol concentrations were also measured in a commercial radioimmunoassay procedure supplied by Amer-sham International, Buckinghamshire, England.

In the automated enzyme procedure the following reagents were mixed together and placed in the reagent tray of the COBAS-BIO centrifugal analyser: nicotinamide dinucleotide (NAD) (1 mmol/l), nicotinamide adenine dinucleotide phosphate (NADP) (1 μ mol/l), glucose-6-phosphate (1 mmol/l), glucose-6-phosphate dehydrogenase (50 U/l) in 20 ml of 0.05M Tris buffer. These reagents were mixed with hydrolysed urine samples for three minutes and EDH (20 U/l) was then automatically added. Absorbance readings at 340 nm were monitored at 30 second intervals to measure the production rate of the reduced form of NAD, NADH. The results obtained for unknown urine samples were compared with three serially diluted oestradiol standards (40, 80, and 160 μ g/l). The Table lists the variables entered into the programme of the COBAS-BIO centrifugal analyser.

With appropriate assay conditions a linear relation exists between EDH activity and the total concentrations of oestradiol and oestrone in urine. The EDH enzyme rapidly interconverts oestrone and oestradiol to generate a hydrogen atom with every cycle, which is then transferred to NAD to form increasing quantities of NADH. The rate of appearance of NADH reflects EDH activity. Each EDH cycle relies on a hydrogen atom from the cofactor NADPH, which is continuously formed by a regenerating cycle involving glucose-6-phosphate dehydrogenase. The concentration of NADPH is maintained at a value 1000-fold less than initial NAD concentrations and does not contribute

appreciably to the absorbance reading at 340 nm.

The following hydrolysis procedure was adopted. Urine samples were initially adjusted to pH 6.5 with 2M acetate buffer and a 1 ml aliquot of urine was hydrolysed by incubation with 50 μ l of glucuronidase for 30 minutes at 37°C. Salts were precipitated by adding 1 ml of ethanol to 1 ml of hydrolysed urine and incubating the mixture at 4°C for 30 minutes. The mixture was centrifuged at 3000 g for 15 minutes and the supernatant (100 μ l) was diluted fourfold with 500 μ l of Tris buffer (0.05 M, pH 7.2) containing glycerol (3M) and bovine albumin (1 g/l).

Results

ASSAY VARIABLES

The specificity of the EDH assay has been studied in detail and reported elsewhere.⁵ As the EDH assay reacts with both oestrone and oestradiol, it is difficult to compare patient data obtained by the EDH assay with reference radioimmunoassay procedures, which measure only a particular oestrogen. Instead, a series of recovery experiments was performed to evaluate the efficiency of the EDH procedure. Also the EDH assay was compared with a commercial oestradiol radioimmunoassay using a randomised series of purified oestradiol preparations in assay buffer. When oestradiol preparations (50, 85, 150 μ g/l) were assayed ($n = 10$) the respective EDH results were within $91 \pm 4\%$, $87 \pm 6\%$, and $85 \pm 9\%$ of expected values. When a randomised series of oestradiol preparations ($n = 20$) in the range of 5 to 150 μ g/l was assayed by both radioimmunoassay (y) and the EDH assay (x), the regression analysis was $y = 1.6x - 5$, with a correlation coefficient (r) of 0.92.

The automated EDH assay evaluated 24 hydrolysed urine samples within 15 minutes of hydrolysis. The sensitivity of the EDH assay for oestradiol, defined as the minimal value distinguishable from zero, was 12 pg in a 30 μ l sample (4 nmol/l). Similarly, the sensitivity for oestrone was 15 pg in a 30 μ l sample (5 nmol/l). The within batch precision expressed as a percentage of the coefficient of variation (CV%) was 4.4% and 4.9% for oestradiol concentrations of 30 and 190 μ g/l, respectively on 50 samples. The between assay variation (CV%) as measured in 40 separate assays was 7.5%, 6.5%, and 5.8% for oestradiol concentrations of 45, 74, and 181 μ g/l respectively. Similar levels of precision were obtained for oestrone preparations.

The efficiency of the hydrolysis step was evaluated with preparations of oestradiol and oestrone glucuronide in the range of 1, 5, 20 and 40 μ g added to 10 ml of assay buffer. The results obtained by the EDH assay after enzyme hydrolysis were $76 \pm 8\%$

Variables entered into COAS-BIO

Temperature	37.0
Analysis type	6
Wave length	340
Sample volume	20
Diluent volume	30
Reagent time	250
Incubation time	180
Start reagent volume	10
Time of first reading	120
Time interval	30
Number of readings	15
Printout mode	1

of expected values for both oestradiol and oestrone. Although the recovery of oestrogens by glucuronidase hydrolysis was not high, the precision (CV%) of this step was acceptable at $10.5 \pm 1.5\%$. Attempts to automate hydrolysis were unsuccessful. Including glucuronidase in the initial reagent mixture resulted in variable sensitivity and poor assay precision. Using any level of glucuronidase between 5 and 100 U/l resulted in decreasing within assay precision to $18 \pm 7\%$, decreasing between assay CV to $26 \pm 12\%$, and increasing sensitivity to $8 \pm 3 \mu\text{g/l}$.

CLINICAL APPLICATION

Fig. 1 shows the range of EDH assay results from the ovulatory cycles of 15 normal women. Daily serum luteinising hormone concentrations were also measured, and most patients (9/15) showed peak urinary oestrogen values 24 hours before the luteinising hormone surge. Two patients showed earlier oestrogen peaks at 45 and 42 hours, while four patients peaked on the same day as the luteinising hormone surge.

Fig. 2 shows the individual results for the EDH assay performed on urine samples collected every three hours from patients undergoing in vitro fertilisation. In patients who ovulated naturally, peak urinary oestrogen concentrations were signalled by a rapid two to fivefold rise from baseline values. The time of peak oestrogen concentrations in relation to luteinising hormone surge varied between individuals. In some patients significantly rising oestrogen concentrations were evident 18 hours before the increase in luteinising hormone, while other patients showed rising oestrogen at the same time as rising

Fig. 1 Range of oestrone and oestradiol concentrations measured in 15 normal fertile women by the automated oestradiol dehydrogenase assay showing mean values with two standard deviations. Patients collected 24 hour urine samples, and results were calculated with day zero as the day of peak serum luteinising hormone concentrations.

Fig. 2 Individual profiles of oestradiol dehydrogenase assay in patients undergoing in vitro fertilisation with natural and induced ovulation.

luteinising hormone concentrations. In patients in whom ovulation was induced, rising urinary oestrogen concentration followed the administration of human chorionic gonadotrophin, which confirms that human chorionic gonadotrophin induces steroidogenesis as well as inducing ovulation. Compared with the rises seen in natural cycles, these patients had lower oestrogen peaks which were only one and a half to threefold greater than baseline values. Correcting urinary EDH assay results for urinary volume differences between individuals had no significant effect on the timing of the oestrogen rise or the oestrogen peak.

Total urinary oestrogen concentrations measured by a fluorimetric assay were compared with EDH results for 24 hour urine collections. Fig. 3 shows the results for four patients from a stimulated ovarian programme; these highlight the complications in monitoring ovarian function by the EDH assay. In most patients (10/12) similar times for peak urinary oestrogen concentrations were seen when urine samples were assayed by either the EDH procedure or the fluorimetric assay. EDH assay results, however, were roughly half total oestrogen values. Also in the luteal phase of four patients, small sharp rises in total oestrogen concentrations after administration of human chorionic gonadotrophin were seen,

Fig. 3 Individual profiles of four patients with total urinary oestrogen assay compared with oestradiol dehydrogenase concentrations in 24 hour urine collections. These patients show ovulatory cycle (a), conception cycle (b), drug treatment (c), and non-ovulatory cycles (d).

which were not evident in the EDH assay (Fig. 3a). Generally, the results from the EDH assay paralleled those of the total oestrogen assay, but in two patients total oestrogen results continued to rise after EDH results had started to fall (Fig. 3b). In these patients peak EDH assay values preceded total oestrogen peak concentrations by six to eight hours. In one patient drug treatment had an adverse effect on results of the EDH assay. An epileptic woman undergoing anticonvulsant treatment during her treatment cycle had EDH assay results which were one tenth of the total oestrogen concentrations, and so it was difficult to determine increasing EDH results in this patient (Fig. 3c). Finally, when patients failed to ovulate during their treatment cycles EDH assay results were often less than 20 $\mu\text{g/l}$, and small changes in EDH assay values were difficult to determine accurately (Fig. 3d).

Discussion

Since the discovery of high oestradiol 17 β dehydrogenase activity in the human placenta by Ryan and Engel in 1953,⁸ there has been extensive research into its kinetics, specificity, and structure.^{9,10} Despite the potential of EDH to measure oestrogen concentrations, there have been few clinical reports of its application in monitoring ovarian

function. Perhaps earlier manual methods for EDH were considered laborious and associated with variable precision and sensitivity. Consequently, the enzymatic EDH oestrogen assay was overshadowed by the more specific and reliable radioimmunoassay procedures. The recent development of automatic centrifugal analysers to measure enzymes and their appearance in routine clinical laboratories opens new opportunities for the EDH procedure. Automated spectrophotometric analysers enable simultaneous absorbance readings of groups of patients to be made, while an additional data processing unit calculates small changes in the rate kinetics of enzymes. The manual EDH method required up to two hours before sufficient NADH had accumulated for measurement. The automated EDH described in this report monitored NADH production with increased sensitivity and precision and enabled urinary oestrone and oestradiol concentrations to be measured within 15 minutes. It was not possible to automate the hydrolysis step using a COBAS BIO centrifugal analyser.

In the present study the EDH assay was less sensitive than total oestrogen estimation in monitoring ovarian function. This was due to the inability of EDH to react with oestriol. The importance of urinary oestriol measurement in monitoring ovarian function is evident in the following brief review of

the biosynthesis of oestrogens. The major oestrogen secreted by the ovary is 17 β oestradiol, which is rapidly interconverted into oestrone. Both steroids are excreted into the urine as glucuronides and sulphates. A substantial proportion of oestrone is also metabolised in the liver into oestriol, and oestriol glucuronide represents up to half of total urinary oestrogens.¹¹ Consequently, EDH values were less than half of the total oestrogen concentrations, and small changes in total oestrogen output were not detectable in the EDH assay. Moreover, the percentage of oestriol glucuronide increased during treatment with anticonvulsant drugs, analgesics, and antibiotics such as phenobarbitone, ampicpyrnyne, and rifampicin, respectively. These drugs induce microsomal hepatic enzymes, which in turn increase the conversion of oestrone into oestriol,¹² as was dramatically illustrated in a patient from this study.

The problems encountered with the EDH assay are also common to specific immunoassays that measure only a proportion of oestrogens in urine. All urinary assays, apart from total urinary oestrogen estimations, are thus vulnerable to changes in the relative proportions of excreted oestrogens, which may vary between individuals and during treatment with drugs.

In monitoring oocyte maturity in normal ovulatory women the rapid EDH assay was a valuable complementary test to routine urinary monitoring of luteinising hormone. By considering rising EDH assay values in urine samples taken every three hours in this study, clinicians were able to determine accurately the optimum time for laparoscopy or insemination. In most patients peak values in the EDH assay preceded rises in luteinising hormone concentrations by 24 hours and allowed clinicians to eliminate false positive results found in routine haemagglutination assays for luteinising hormone.¹³ It is noteworthy that in this study two patients showed earlier peak values with the EDH assay than with the total oestrogen assay. This may be due to a slight delay in the metabolism of oestrone into oestriol and suggests that the EDH assay may sometimes signal oocyte maturity before total oestrogen assays.

In clinical programmes treating infertility with in vitro fertilisation or artificial insemination, serum oestradiol estimations by radioimmunoassay are often used to determine oocyte maturity.² To many patients, repeated blood sampling is inconvenient and serial urine collections may be preferred. Moreover, serial three hourly urine collections may give a more accurate guide to the stages of follicular development than that supplied by daily blood sampling. In assaying large numbers of urine samples

the automated EDH assay described in this report has comparable sensitivity and precision with radioimmunoassay procedures. Furthermore, it has advantages over radioimmunoassay procedures in its improved speed, convenience, and economy.

We are grateful to Professor JB Brown, Dr M Martin, and Dr MA Smith of the department of obstetrics and gynaecology, University of Melbourne, for their valued advice and access to material from their stimulated ovarian cycle programme. Also we thank Dr WIH Johnston, Director, Reproductive Biology Unit, for access to material from his in vitro fertilisation programme.

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