An evaluation of the automated assay of urinary oestrogens in pregnant women

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SYNOPSIS An automated assay suitable for estimating urinary oestrogens in pregnant women has been investigated. Fluorimetry was found to have considerable advantages over colorimetry. The fluorimetric assay was simpler, more precise, more sensitive, and eliminated the need for correction for non-specific chromogens; in the assay of oestriol in pregnant women there was no need for correction for non-specific fluorescence. Spectrofluorimetric and photometric analyses, recoveries, and reproducibility show that the method offers a robust means of providing values for urinary oestrogen in pregnant women on a scale of up to 100 tests a day, the time of the assay being one and a half hours.

Conaill and Muir (1967 and 1968) developed an automated assay for total oestrogens in pregnancy in which the Allen correction for non-specific chromogens was replaced by that of Fournier, Shields, Neil, Hayes, and Papineau-Couture (1966). Spectral studies of the effluent obtained during steady-state analysis suggested that this correction was justified. Brown, Mcnaughtan, Smith, and Smyth (1968) published a study of the estimation of oestrogens by the Kober colour reaction (Kober, 1931) coupled with the Ittrich extraction (Ittrich, 1958) using both colorimetry and fluorimetry. These workers found that at 120°C the Kober colour complex could be developed in five minutes and that a simple factor could be used to correct for non-specific fluorescence. We have repeated our original spectral studies and carried out spectrofluorimetric studies based on Brown's original observations. It was hoped that the use of fluorimetry would eliminate the need for two colorimeters and simplify the automated system.

Methods

Figure 1 shows the manifold used to compare

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colorimetry and fluorimetry; for either the fluorimetric or colorimetric methods the other instruments were bypassed. Where necessary urine samples were made up to a 24-hour volume of 1,500 ml. Five ml of diluted urine was hydrolysed with 5 ml of 50% hydrochloric acid at 100°C for one hour. After hydrolysis, the samples were placed in 8 ml AutoAnalyzer cups and sampled at 20 samples per hour, with a sample:wash ratio of 1:3. The samples were segmented with ether, passed through two double mixing coils, after which the phases were separated. The ether phase was then segmented with saturated quinol in 50% sulphuric acid and entered the digester; during its passage down the digester the ether was evaporated off and the Kober colour was developed. The first heater was set at 100° and the second heater at 180°; the motor gears were set at 6 rpm. At the end of the digester coil, the Kober complex was diluted with water and then segmented with paranitrophenol in chloroform. After the extraction in a pair of double mixing coils the phases were separated and the chloroform phase was passed into the flow cells of the two colorimeters and thence to the fluorimeter. The 540 mμ colorimeter had a light path of 15 mm while the 420 mμ had a light path of 8 mm. A mercury arc with a 535 mμ Balzar interference filter, provided the primary light source, and the monochromator

Fig. 1 The manifold used for urinary oestrogen assays by ether and/or colorimetry.

on the secondary side was set to read at 555 μ . The voltage control of the fluorimeter was set at half voltage with the course control set at 3 o'clock. The instrument was more stable when the day-to-day adjustments were made with the Iris diaphragm, which was adjusted so that an oestriol standard of 15 mg/l gave an 80% deflection on the recorder. The ether and chloroform reagents were pumped by a displacement bottle technique.

Reagents

All reagents were of Analar grade unless stated otherwise.

DIETHYL ETHER

Colour reagent

This was saturated quinol in 50% sulphuric acid.

To 15 g of quinol in a 2-litre flask, 500 ml distilled water was added. The flask was cooled in a water bath and the contents were stirred continuously while 500 ml of concentrated sulphuric acid was added. The solution was then stood overnight and any crystals formed were removed by filtration the following day. The reagent was stored in a dark bottle.

Colour extractant

This consisted of 2% (w/v) paranitrophenol in chloroform.

Stock standard

Oestriol in ethanol, 1 mg per ml.

Working standards

Of the stock solution, 0.25, 0.5, 0.75, 1.0, 1.25, and 1.5 ml solution were diluted to 100 ml with distilled water. These represented oestriol concentrations of 2.5, 5.0, 7.5, 10.0, 12.5, and 15.0 mg of oestriol per litre.

Fig. 2 Extinction spectra obtained on the products of the automated Kober reaction. Pools of various urines were assayed at steady state. Urine pools from males —; urine pools from non-pregnant females; urine pools from pregnant women — — —; urine pools from males to which oestriol had been added to concentration of 20 mg per litre — — — —; pure oestriol standard — — — — —.

Equipment

Standard Technicon equipment was used throughout, with the exception of the modification to the digester to alter the direction of the air flow and trap the evaporated ether (Conaill and Muir, 1968). A Locarte fluorimeter (model number MK 5) with a flow cell attachment and a recorder box was used.

Results

STUDIES ON THE COLORIMETRIC METHOD

Specificity

Several pools of urine from pregnant women were hydrolysed and each was sampled continuously until the recorder indicated that a steady state had been obtained. The products were then collected and 3 ml was placed in a 10 mm light path silica cuvette. The extinction spectra of the

Fig. 3 The colorimetric standard curve showing the mean value plus or minus 1 standard deviation.

products were rapidly scanned at 10-m μ intervals from 400 m μ to 600 m μ , using the Hilger Gilford spectrophotometer. The spectra of pure standards pooled urines from non-pregnant women, pooled male urines, and pooled male urines to which standard had been added are shown in Figure 2. The extinction spectra from pooled urines from pregnant women and that from pure standard added to male urine were similar.

The Fournier correction assumes that in ether extract of a urine, not containing oestrogens, gives a spectrum due to non-specific chromagens which decreases linearly from 400 m μ to 600 m μ and that the extinction at 540 m μ is half that found at 420 m μ . We were able to confirm that in pools of male and female urine the decreases in extinction were linear. In the automated assay the 15 mm and 8 mm flow cells are used and the optical extinction of a solution of haemoglobin in the 8 mm flow cell was half that found in the 15 mm flow cell.

Linearity

The working standards were processed in batches of ten. Figure 3 gives the mean extinctions and the standard deviation plotted against oestriol concentrations. The curve appears to be linear up to 10 mg per litre.

Precision

The repeatability in a single batch was determined by measuring 10 aliquots of three different urine samples from a pregnant woman. At the

Fig. 4 Fluorescent emission spectra of the automated Kober complexes obtained at steady state analysis, with the fluorimeter set at the higher sensitivity. Diluted urine pools from pregnant women — — — — — ; oestriol standard 500 μ g per litre — — — — — ; reagent solvent mixture — — — — — ; urine pools from non-pregnant women — — — — — ; urine pools from males

mean value of 7.3 mg per litre the standard deviation was 0.2 mg, at 14.0 mg per litre it was 0.9 mg, and at 24.6 mg per litre it was 2.0 mg. Reproducibility between batches was determined. A 24-hour specimen was split into small aliquots which were deep frozen, and were used as controls over a period of three months. The mean concentration was 26.4 mg per litre and the standard deviation 1.6 mg per litre. Over a similar period of time, 83 specimens were estimated in duplicate; the mean result was 15.1 mg per litre and the standard deviation 0.86 mg per litre.

Correlation with the Manual Ittrich Method

A series of 83 urines was estimated by the auto-

mated and manual Ittrich method as modified by Cartledge, Spencer, Swyer, and Woolf (1961). The coefficient of correlation was 0.83 which was significant at the 1% level.

Studies on the Fluorimetric Method

In the automated fluorimetric method, an aspirate of water, extraction solvents, and colour reagents provided the recorder base line. This base line was read continuously between the peaks, and this ensured that each sample was read against a reagent solvent blank.

Studies on the Specificity of the Fluorimetric Method

Spectrofluorimetric studies similar to those of Brown *et al* (1968) were made of the Kober colour. The apparatus was set to sample continuously from pooled hydrolysed urines, standards, and water. The primary beam was set at 535 $m\mu$ and the monochromator moved through the spectrum from 450 $m\mu$ to 600 $m\mu$. After each change of wavelength the recorder was allowed to settle before proceeding to the next. Emission spectra were obtained for pure standard solutions, reagent solvent blanks, and different pools of urine. By altering the iris diaphragm the fluorimeter sensitivity was adjusted so that the 500 μ g oestriol per litre gave an 80% deflection on the recorder. The resulting spectra obtained at the higher sensitivity are given in Figure 4. At this sensitivity setting and under normal wavelengths pools of urine from males and non-pregnant females gave the same deflection as the reagent blank; thus the use of the reagent solvent blank is a sufficient correction for non-specific fluorescence at this level of sensitivity.

The blank spectra were made by following the blank reading on either side of the 555 $m\mu$ wavelength until there was no reading on the recorder. At the higher sensitivity the reagent solvent blank had a peak at 550 $m\mu$ as distinct from the oestrogen peak at 555 $m\mu$. This appeared to move the oestriol peak to 550 $m\mu$, but in the difference spectra in the Table the peak was at 555 $m\mu$.

Correlation with the Colorimetric Method

In Fig. 5 the upper tracings show the colorimetric results on a two-pen recorder, while the lower tracing shows the simultaneous fluorimetric results recorded on a single-pen recorder. Each set of results shows a standard curve followed by total oestrogen assays on urines from pregnant women, a carryover experiment, the results in

Fig. 5 The 540 m μ and 420 m μ colorimeter output are in the upper recorder chart while the lower demonstrates the fluorimeter output. These charts demonstrate serially six standards from 2.5 to 15 mg oestriol per litre, assay peaks from urines from pregnant women, a carryover experiment in which the first and third samples are the same, assays on urine from non-pregnant women, and finally the assay run at continuous sampling.

Wavelength Standard (μ m)	Solution	Urine from Pregnant Women	Urine from Non- pregnant Women	Urine from Males	Blank	Difference Spectra	
						Pregnant Urine Reading Minus Blank Reading	Standard Reading Minus Blank Reading
500	—	—	—	—	—	—	—
510	—	—	—	—	—	—	—
520	—	—	—	—	—	—	—
525	8	9	—	—	—	9	8
530	25	18.5	—	—	—	18.5	25
535	37	33	3.0	3.5	4	29.0	33
540	55	55	8.5	9	9.5	44.5	44.5
545	75	81.5	14	14	15	60	60
550	85	96.5	15	15	16	79.5	69
555	75	93	11	12	13	80	62
560	50	75	6	6.5	6	69	44
565	30	57	0.5	1.0	1	56	29
570	9	45	—	—	—	45	9
580	6	23.5	—	—	—	23.5	6
590	—	7.5	—	—	—	7.5	—
600	—	—	—	—	—	—	—

Table Results of spectrofluorimetric studies of the Kober chromogen developed using the automated procedure¹
¹Readings in arbitrary fluorescence units

Fig. 6 100 urine samples from pregnant women estimated by colorimetry and fluorimetry.

Fig. 7 Standard curve for fluorimetric method. The mean of six estimations plotted for each point with the 1 standard deviation range.

urines from non-pregnant women, and finally the results of continuous sampling. The carry-over from a high to a low sample was calculated to be 5.5% of the high peak for both the colorimetric and fluorimetric systems. Urines from non-pregnant women gave no deflection. In Fig. 6 is shown a plot of 100 samples estimated by both systems. The coefficient of correlation for this set of results was 0.98.

Recovery of Added Oestriol

The mean recovery of oestriol added before hydrolysis was found to be 75% with a standard deviation of 5.8% ($n = 15$), and that of oestriol added after hydrolysis was 95.3% with a standard deviation of 4.6% ($n = 15$). These recoveries were used as routine quality controls.

Linearity of the Method

The standards were estimated in batches of six; in Fig. 7, mean values and their standard deviations are plotted against oestriol concentrations. The standard curve is linear to 10 mg per litre at the sensitivity used in the routine assay.

Reproducibility of the Fluorimetric Method

The repeatability within the batch was assayed at three levels, namely, 4.3 mg per litre, 9.0 mg per litre, 27.5 mg per litre; 12 assays were done at

each level. At these mean values the standard deviations were 0.21 mg, 0.22 mg, and 0.79 mg. The reproducibility between batches was estimated on 15 occasions, and the mean result was 9.5 mg per litre with a standard deviation of 0.41 mg. One hundred estimations were carried out in duplicate; the mean result was 11.18 mg per litre and the standard deviation ± 0.54 mg.

Discussion

Any new method of assay must fulfil criteria of validity before it can be adopted in a laboratory, and applies particularly to a method which brings a difficult hormone assay within the reach of area laboratories. The precision of the method was estimated at three levels and expressed as the coefficient of variation; for the colorimetric method it was 2.7%, 6.4%, and 8.0%, while for the fluorimetric method it was 4.9%, 2.4%, and 2.9%. As the oestriol concentration increased, the colorimetric method lost precision, but this was not so with the fluorimetric method. At the lowest level, 4.3 mg per litre, the fluorimetric method appeared to lose precision. With the sensitivity setting of the fluorimeter used in the routine assay, it is not possible to read oestriol concentrations to an accuracy greater than 0.2 mg per litre, which would explain this apparent loss of precision. The colorimetric method had a coefficient of variation between batches of 6.1% at 26.4 mg per litre, while the coefficient of variation for the fluorimetric method was 4.3% at 9.5 mg per litre, which agrees well with the coefficient of variation of 4.8% by the method of duplicate analysis. Our coefficient of variation for the automated ureas using diacetyl monoxime within the batch was 1.6%, while between the batches by the method of duplicate analyses it was 2.8%. As this is a relatively simple analysis compared with the oestrogen analysis, we feel that the coefficients of variation for the fluorimetric analysis are good and offer an improvement over the use of colorimetry.

The sensitivity of fluorimetry is greater than that of colorimetry as it was possible to detect less than 500 μg per litre but, with colorimetry, levels of less than 1 mg per litre could not be reliably detected. The enhanced sensitivity of the fluorimetric methods is well established. Van Kessel, Seitzinger, Schreurs, and Versteg (1969) have used the increased sensitivity of the fluorimetric methods to automate an aqueous Kober reaction with a final Ittrich extraction. In their method non-specific fluorescence and the interference of glucose have been overcome by the very great dilution permitted by fluorimetry.

The question of specificity and accuracy in oestriol assays is difficult, as the most reliable methods are those involving gas chromatography coupled with thin-layer chromatography and

isotopically labelled internal standards, which are obviously beyond the scope of an area laboratory. In this case we have compared the results obtained with the manual Ittrich procedure as modified by Cartlidge *et al* (1961). The coefficient of correlation was 0.83 which was significant at the 1% level. The evidence which we have presented as to specificity is the similarity between the spectrophotometric and spectrofluorimetric analysis of pure standards, standards added to urine, and pregnancy urines containing large amounts of oestrogens. Støa and Thorsen (1962), although somewhat critical of spectrophotometric analysis as a criterion of chemical specificity, expressed the view that spectrofluorimetric analysis offered a useful method of chemical characterization. It is our view that the combination of results of spectral analyses and the recoveries of pure oestriol added to urine samples before and after hydrolysis offer some guide as to the specificity of the method.

Unlike van Kessel *et al* (1969), we have retained the ether extraction. The use of ether rather than air segmentation, suggested by Child and Caisey (1966), provides an efficient extraction procedure with recoveries of $95.3\% \pm 4.6\%$, nor does it cause any increased carryover between samples. The use of a fluorimeter rather than two colorimeters has more to recommend it than simple convenience. Not only is the method more precise, but it does not depend on the assumption that behaviour of non-specific chromogens is the same in every urine sample. The choice of 535 m μ as the primary beam follows the studies of Scholler, Leymarie, Heron, and Jayle (1966) on the excitation of spectra of oestriol in chloroform. We have departed from their recommendations to read at 550 m μ , as on our instrument the emission maxima were at 555 m μ . Brown *et al* (1968) recommended a gap of 19 to 20 m μ between the primary and secondary wavelengths in order to eliminate exciting light, which this setting has enabled us to do. Strickler, Wilson, and Grauer (1961) found that 75% of the fluorescence of oestriol in chloroform was contained in the wavelengths 545-559 m μ .

Like Brown *et al* (1968) we have found that the use of a higher temperature speeds the development of the Kober colour; a sample takes 3.5 minutes to traverse the digestor. Strickler *et al* (1961) and Brown *et al* (1968) point out that the Kober complexes deteriorate at room temperature, and that the time between developing the complex and the reading must be kept to a minimum. In continuous flow analysis the time interval between the development and dilution of the Kober complex is always constant and the samples can be read continually against the reagent solvent mixture.

Another aspect which must be considered in the assessment of a new method is its practicality and the cost per assay. This method is a piece of chemical engineering which has made a tedious and difficult assay within the reach of any area laboratory, provided that the capital, about £3,000, is available, and any technician, familiar with continuous flow analysis, can perform oestrogen estimations at a rate of over 100 per day and provide an individual result within one and a half hours. The cost of the individual assay is approximately 1s 8d, including chemicals and technician's time, but excluding capital cost.

In conclusion, we regard the automated and fluorimetric Ittrich assay as a robust assay which can estimate oestriol on a large scale in most area laboratories.

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