A Specific Method for the Determination of Millimicrogram Amounts of Urinary Oestrone

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1. A new combined radioactivity-fluorescence method is described for urinary oestrone, which involves acid hydrolysis, extraction and purification of the phenolic fraction, saponification, a Girard T separation and alumina chromatography of acetylated oestrone. 2. Sulphuric acid fluorescence is used for quantitation and specificity is achieved by the addition of tritiated oestrone to the urine hydrolysate. This radioactive tracer functions both as an internal corrector for purification losses and enables the demonstration of constant specific activity through the oestrone peaks to act as an index of specificity in each determination. 3. By using one-fifth of a 24 hr. urine sample, 1 μ g. of urinary oestrone/24 hr. can be determined with an accuracy of $\pm 4\%$. Fluorescence emission spectra from processed urine samples are identical with that of authentic oestrone acetate. 4. The advantages of the method are its high sensitivity and specificity, which is achieved with relative convenience.

A recent study that involved the search for small variations in the urinary excretion of oestrogen in the human male (Exley & Corker, 1965) demanded the use of an accurate, highly sensitive and specific routine method. Despite improved methodology in the chemical assay of urinary oestrogens introduced by Brown (1955), followed by Bauld (1956), Brown, Bulbrook & Greenwood (1957), Salokangas & Bulbrook (1961), Preedy & Aitken (1961) and Barlow (1963), it is still extremely difficult to determine accurately low-titre urine concentrations with respect to either sensitivity, specificity or ease of method. The Kober colour reaction utilized by the earliest workers lack sensitivity and its specificity is remarkably impaired by non-specific colour, which must be corrected by Allen's (1950) formula. Even with this correction, low-titre urines are impossible to assay because of non-oestrogenic interference. Modifications of the Kober reaction (Ittrich, 1958, 1960), though improving the specificity, still leave the problem of low sensitivity. Sulphuric acid fluorescence, on the other hand, offers great sensitivity, but low specificity. Many contaminants completely destroy the validity of fluorescence readings either by forming yellow colours or by quenching the fluorescence. To obtain high specificity a much greater purification is therefore required for a fluorimetric assay than for those methods that utilized the Kober colour reaction. Preedy & Aitken (1961) achieved this order

of specificity with a fluorimetric procedure by including two gradient-partition column-chromatographic steps. The method is very lengthy, an individual oestrogen determination requiring 30 fluorescence readings. Barlow (1963), using a combined radioactivity-fluorescence method, shortened the above procedure by the use of paper and column chromatography, but the method is still tedious, and unfortunately also lacked the high accuracy required for our investigation.

The ratio of the excretory urine concentrations of one classical oestrogen to another has been reported to vary surprisingly little in normal and diseased states (Migeon, 1953; Eberlein, Bongiovanni & Francis, 1958), except for pregnancy (Brown, 1956) and the neonatal state (Diczfalusy, Tillinger & Westman, 1957). A method suitable for detecting variations of one of the oestrogen concentrations is therefore just as reliable a parameter for correlating total change as those lengthy methods that measure all three oestrogens. It was therefore decided to measure oestrone, because this particular oestrogen is amenable to treatment with the highly efficient Girard T purification procedure.

The present paper describes a new accurate radioactive method for the determination of millimicrogram amounts of urinary oestrone that utilizes the high sensitivity of fluorescence for determination, the Bauld (1956) saponification step and the Girard T procedure to achieve high specificity.

EXPERIMENTAL

Methods

Materials

Ether (peroxide-free) was washed with saturated FeSO_4 solution and water and then redistilled. Ether recovered during the procedure was redistilled before being used again. Light petroleum (b.p. 40–60°) and benzene (A.R.; British Drug Houses Ltd., Poole, Dorset) were distilled and saturated with water. Ethanol was refluxed over CaH_2 for 1 hr. before distillation. Dichloromethane was redistilled.

The NaOH and NaHCO_3 solutions were prepared on a w/v basis by using A.R. chemicals. Concentrated carbonate solution of pH 10.5 (approx.) was prepared by adding 20% NaOH (150 ml.) to 8% NaHCO_3 solution (1 l.). The Girard T reagent was obtained from British Drug Houses Ltd. Acetic acid (A.R.) was purified by refluxing over CrO_3 for 1 hr. before distillation. The acetic anhydride used for acetylation was refluxed over calcium carbide for 4 hr. and distilled, the middle fraction at 139° being taken. Pyridine was refluxed over BaO for 4 hr. and distilled, the middle fraction at 115° being taken. The pyridine was stored over fresh BaO .

The choice of H_2SO_4 reagent for the fluorescence reaction is important: H_2SO_4 (A.R.; British Drug Houses Ltd.) had the lowest fluorescent background. The 90% (w/w) H_2SO_4 was prepared by adding 996 ml. of conc. H_2SO_4 to 200 ml. of distilled water. The 65% (w/w) H_2SO_4 was carefully prepared by adding 230 ml. of conc. H_2SO_4 to 200 ml. of distilled water. Fresh H_2SO_4 reagents were prepared every 2–3 weeks. Alumina (Savory and Moore Ltd., London, N.15) was deactivated with 11% of water.

Standard oestrone acetate solutions were prepared in ethanol and stored at 4°, and were stable indefinitely. $[6,7\text{-}^3\text{H}_2]\text{Oestrone}$ (specific activity 4.5 mc/mg.), purchased from The Radiochemical Centre, Amersham, Bucks., was found by thin-layer chromatography to be 99% radiochemically pure, and was used without further purification.

The phosphor solution used for scintillation counting was made up by adding 2,5-diphenyloxazole (3.0 g.) to 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.3 g.) in reagent-grade toluene (1 l.).

Apparatus

General glassware was rinsed after use with tap and distilled water, unless visibly dirty, when it was cleaned by a $\text{CrO}_3\text{--H}_2\text{SO}_4$ mixture. Tubes (B10; Quickfit and Quartz Ltd., Stone, Staffs.) used for collection of chromatographic fractions and subsequent fluorescence determinations were silicone-treated to prevent adsorption on the glass surfaces. The tubes were immersed in a solution of dichlorodimethylsilane in toluene (5%, v/v), then rinsed twice with toluene, before a final rinse with methanol and heating in an oven at 110° for 30 min.

Glass chromatography tubes for alumina columns had internal diam. 1 cm.; they had a sealed-in glass support (porosity no. 2) and the lower end had a stopcock.

An Aminco-Bowman spectrophotofluorimeter was used for the fluorescence measurements; slit arrangement no. 4 was used.

Radioactive counting was performed on a Packard Tri-Carb liquid-scintillation spectrometer (model 3314).

General outline of method. Hydrolysis, extraction and separation of the phenolic fraction is performed essentially as described by Brown (1955). After hydrolysis, a known amount of high-specific-activity tritiated oestrone is added to the urine, such that no measurable fluorescence is contributed by the added oestrone. The phenolic fraction containing oestrone and oestradiol is subjected to the saponification step of Bauld (1956) and then to a Girard T separation. The separated oestrone is acetylated and chromatographed on a small alumina column. The specific activity of each column fraction through the radioactive peak enables the calculation by isotope dilution of the amount of oestrone present in the urine hydrolysate.

Procedure

Sample collection. Total 24 hr. urine samples are collected without preservative and stored until use at -15° .

Hydrolysis. A suitable portion of a 24 hr. sample of urine (usually one-tenth to one-twentieth) is diluted to 100 or 200 ml. with distilled water. The urine is heated to boiling under a reflux condenser. Conc. HCl (10 N) (15 ml./100 ml. of diluted urine) is added through the condenser, and the mixture boiled for 60 min. and then cooled rapidly under running tap water.

Addition of tritiated oestrone. $[6,7\text{-}^3\text{H}_2]\text{Oestrone}$ of high specific activity is added to the cooled hydrolysate. Approx. 7500 counts/min. is added in ethanol (0.4 ml.). The fluorescence contribution of the oestrone of high specific activity is not detectable.

Extraction. The extraction and preliminary purification is performed essentially by the method of Brown (1955). The hydrolysate with added tritiated oestrone is extracted in separating funnels with ether (3 $\times$ 1 vol.). The acidic fraction is removed by shaking with concentrated carbonate solution (40 ml./100 ml. of diluted urine). The ether is then shaken thoroughly with 2 N- NaOH (10 ml./100 ml. of diluted urine); the NaOH is, however, not discarded, but is partially neutralized by 8% NaHCO_3 solution (40 ml./100 ml. of diluted urine) and shaken again with the ether. The aqueous phase is now discarded, and the ether washed with water (2 $\times$ 30 ml.), filtered over Na_2SO_4 and then evaporated to dryness on a water bath.

Partitioning of the phenolic fraction and saponification. The dried residue is dissolved in 25 ml. of benzene–light petroleum (1:1, v/v) and transferred to a small separating funnel. The benzene–light petroleum is washed with water (2 $\times$ 12.5 ml.) and then extracted with 0.4 N- NaOH (2 $\times$ 12.5 ml.). The organic phase is discarded and the aqueous layer transferred to small round-bottomed flasks. NaOH (0.6 g.) is added and the mixture boiled under reflux for 30 min. The contents are then transferred to a 50 ml. B24 (Quickfit and Quartz) tube, and NaHCO_3 (3 g.) added. The mixture is next extracted with dichloromethane (20 ml.), which is washed with water (2 $\times$ 4 ml.), filtered over Na_2SO_4 and evaporated to near dryness on a water bath, the last 0.5 ml. being evaporated at 40° under a jet of N_2 . The residue is dried in a vacuum desiccator for 30 min.

Girard T separation. Acetic acid (1 ml.) containing Girard T reagent (5%, w/v) is added to the dried residue, and the tube stoppered and placed in a boiling-water bath for

20 min. Alternatively, the reagents can be left at room temperature overnight. After cooling, chilled citrate-phosphate buffer, pH 7.0 (10 ml.), is added, and the aqueous phase is washed with ether (3 × 10 ml.). Conc. HCl (10N) (2.5 ml.) is then added and the solution left at room temperature for 1 hr. The mixture is next extracted with dichloromethane (15 ml.) and the aqueous layer discarded. The dichloromethane is washed with 0.1N-NaOH in 20% (w/v) NaCl (2 × 10 ml.), then with 20% (w/v) NaCl (10 ml.) and finally with water (2 × 10 ml.), before being filtered over N_2SO_4 and evaporated to near dryness on a water bath; the last 0.5 ml. is evaporated to dryness under N_2 at 40°. The residue is dried in a vacuum desiccator for 30 min. With unusually highly pigmented extracts, the Girard T stage is repeated.

Acetylation. Acetic anhydride (0.25 ml.) and pyridine (0.05 ml.) are added to the dried residue, and the tube is tightly stoppered. The acetylation is performed by heating on an aluminium block at 70° for 30 min. The tube is then cooled and the reagents blown off by a N_2 jet at 40°. The residue is dissolved in 1 ml. of benzene-light petroleum (1:1, v/v).

Alumina chromatography. The acetylated oestrone is chromatographed on a column containing 2 g. of alumina. The column is prepared by adding alumina to the tube, which is partially filled with water-saturated light petroleum (b.p. 40–60°). The alumina is gently packed and then overlaid with about $\frac{1}{4}$ in. of acid-washed sand. The sample in the benzene-light petroleum (1:1, v/v), (1 ml.) is carefully transferred to the chromatographic column, and the tube containing the sample washed by further amounts of benzene-light petroleum (1:1, v/v) (2 × 1 ml.); when the last portion reaches the sand surface another 7 ml. is added to the column. This 10 ml. of benzene-light petroleum (1:1, v/v) assists in removing any last traces of fluorimetric impurities, and is collected as a full 10 ml. fraction. The oestrone acetate is now eluted with water-saturated benzene-light petroleum (7:3, v/v) (20 ml.). Ten fractions (2 ml.) are collected in small silicone-treated B10 (Quickfit and Quartz) tubes by using a fraction collector.

Radioactive counting. A 0.5 ml. sample from the 10 ml. and each of the ten 2 ml. fractions is pipetted directly into 20 ml. scintillation-counting phials and evaporated to dryness under N_2 at 40°. Phosphor solution (5 ml.) is added to the phials and the samples are counted in a Tri-Carb liquid-scintillation counter. The counting efficiency for ^3H was 40%. Fractions eluted from the alumina column do not cause quenching of the ^3H counts.

Fluorimetry. The fluorimetric method used is a modification of that of Preedy & Aitken (1961). The remaining 1.5 ml. of each of the 2 ml. fractions in the silicone-treated B10 tube is evaporated to dryness under N_2 at 40°. Benzene-ethanol (19:1, v/v) (0.1 ml.) is added to each tube together with 90% (w/w) H_2SO_4 (0.2 ml.) and the mixture heated on an aluminium block at $80 \pm 1^\circ$ for 10 min. After cooling, 65% (w/w) H_2SO_4 (1.4 ml.) is added and the mixture shaken and allowed to stand for 1 hr. before reading. Two reagent blanks and three standards (40 $\mu\text{g.}$) of oestrone acetate are similarly prepared. Calibration graphs of 5–200 $\mu\text{g.}$ of oestrone showed a linear relationship between amount of oestrone and the fluorescence reading. Fluorimetry is carried out with an Aminco-Bowman spectrophotofluorimeter, with 457 $\text{m}\mu$ as exciting light, and reading the fluorescence emission at 475 $\text{m}\mu$ with slit arrangement no. 4.

Calculations. The fluorescence reading of any column fraction, and the number of radioactive counts/min. in that fraction, enables the specific activity in counts/min./ $\mu\text{g.}$ of the fraction to be determined. The alumina chromatography is arranged so that no radioactivity occurs in the first three fractions, and the radioactive peak occurs in column fraction 5 or 6. The mean fluorescence of the first three fractions represents the column reagent blank for the fluorescence readings of all the subsequent column fractions. Fluorescence standard readings and their respective blanks are determined at the same time. The specific activity of each fraction containing significant amounts of oestrone acetate is therefore:

$$\frac{\text{counts/min./fraction}}{\mu\text{g. of oestrone acetate/fraction}} \times 1000$$

Each oestrone determination is made by using the mean of at least three and usually four or five specific activities:

$$\mu\text{g. of oestrone/day} / \frac{1}{\text{day urine hydrolysate}}$$

$$= \frac{\text{counts/min. added} \times y}{\text{mean sp. activity}}$$

RESULTS

Accuracy. The ratio of specific activities obtained from urines to which radioactive oestrone was added before acid hydrolysis to that after was 0.95. This suggests that little free oestrone can be destroyed during this hydrolytic stage.

Although assessment of the accuracy by recovery methods is not necessary with a method that is internally corrected for recovered tritium, the sensitivity of the method depends on the overall recovery of all the various stages. The extraction of oestrone from aqueous solutions in preparation for the Girard T stage takes advantage of the partition coefficients reported by Engel, Slaunwhite, Carter & Nathanson (1950), and was found to be in the range 80–85%. The Girard T separation stage gave recoveries within the range 80–90%. The completeness of acetylation and the recovery of oestrone acetate from alumina columns was in the range 80–90%. The actual mean overall recovery of the method assessed as tritium counts/min. obtained in the total column fractions against tritium counts/min. added was 57.5% (n 80) (range 50–65%). When 290 and 145 $\mu\text{g.}$ amounts of authentic oestrone were added after acid hydrolysis to one-tenth of a 24 hr. urine sample from an adrenalectomized and oophorectomized patient the results suggested that 288.4 and 158 $\mu\text{g.}$ respectively had been added, i.e. an average recovery of 104%.

The accuracy of each single determination was assessed by calculating the standard deviation about the mean (100%) of three, four or five specific activities. The average accuracy of 80 separate determinations was $\pm 2.7\%$.

Precision. The precision, s , of the method was

Fig. 1. Alumina chromatography of oestrone acetate showing fluorescence (●) and radioactivity (×) measurements on individual column fractions. Urine was from: (a) normal male; (b) adrenalectomized and oophorectomized patient; (c) pseudohermaphrodite. The correspondence of points has not been drawn for (b), but the fluorescence curve drawn so that the small amount of oestrone acetate can be clearly seen. The specific activities (in counts/min./μg.) plotted above (○), however, show that at least two fluorescence points would be superimposed by the radioactive points.

calculated by the method of Snedecor (1952), from the differences between the two results of duplicate determinations. The s values, from eight duplicate determinations expressed in μg./24 hr., were 0.15 (range 1–10 μg.) and 0.05 (range 1–4 μg.).

Sensitivity. The qualitative sensitivity of the method is the least amount of substance that can be measured distinguishable from zero and is defined as $ts/\sqrt{N}$, where t is Student's t test, s is the precision as above and N is the number of duplicate determinations performed (Snedecor 1952). Calculations show that for $P=0.01$ this value is 0.10 μg./24 hr., and thus the minimum amount of oestrone distinguishable from zero for one-tenth of a 24 hr. sample is 10 mμg.

The quantitative sensitivity or the error of a determination can be calculated from s (Brown & Blair, 1960). Calculation shows that the minimum amount that can be estimated in one-fifth of a 24 hr. urine sample with an accuracy of $\pm 4\%$ is 1 μg./24 hr. An assay made on one-tenth of a 24 hr. sample from an adrenalectomized and oophorectomized patient produced three specific activities with S.D. $\pm 5\%$ from the mean when the value was as low as 0.24 μg./24 hr.

Specificity. Measurements obtained when the method was applied to a normal male urine are shown in Fig. 1(a). Fluorescence-intensity and radioactivity curves of the alumina column fractions have been plotted. Points at which the curves are superimposed contain oestrone of constant specific activity. The calculated specific activities of the column fractions are plotted above the elution curves. The results are typical in that there are always three or more fractions containing oestrone of near constant specific activity. The non-oestrogenic fluorescence is completely removed from the added tritiated oestrogen peak.

The fluorimetric and radioactivity findings for a bilaterally adrenalectomized and oophorectomized patient are shown in Fig. 1(b). The results suggest that there is a definite small amount of oestrone in this patient's urine. The highest fluorescence in the column fractions represented only 8 mμg. of oestrone acetate. Curves obtained from the urine of a suspected pseudohermaphrodite are also shown (Fig. 1c).

A further check of specificity was made by comparing the fluorescence emission spectra obtained from several column fractions of some of the urines with the fluorescence spectra of authentic oestrone acetate. Fig. 2 shows a typical comparison. These results confirm the high specificity of the method.

DISCUSSION

Evidence for the accuracy of the conditions for hydrolysis and the procedures for extraction and

Fig. 2. Comparison of fluorescence emission spectra of authentic oestrone acetate (A) with a typical column reagent blank (B), a typical normal male urine (C) and urine from a pseudohermaphrodite (D).

purification of the phenolic fraction has been presented by Diczfalussy & Lindkvist (1956), Brown *et al.* (1957), Brown & Blair (1960) and Salokangas & Bulbrook (1961).

Radioactive oestrone is added after acid hydrolysis and therefore there is no correction for losses during this hydrolytic step. The unavailability of radioactive oestrone conjugates means that it is not possible to determine accurately destructive losses during this acid hydrolysis, and attempts to do so by adding radioactive free oestrone before hydrolysis would be misleading. However, the results suggest that, although it is not possible to assess the true overall accuracy of the full method, the actual results obtained approximate to the true result. Brown & Blair (1960), using oestrone, reported a mean value of 0.95 for the acid hydrolysis/enzymic hydrolysis ratio, thus assuming that no destruction occurs enzymically; this suggests that only 5% was not recovered after acid hydrolysis, and agrees with our present finding.

The minimum amount of oestrone distinguishable from zero has been assessed statistically as $0.10 \mu\text{g./24 hr.}$; thus $10 \mu\text{g.}$ is just measurable in one-tenth of a 24 hr. urine sample. Since the mean recovery of the method is 57%, and of this the peak tube will contain only about 30%, and since only

three-quarters of each tube is taken for fluorimetric assay, the actual amount of oestrone being assayed at the limit of sensitivity is less than $2 \text{ m}\mu\text{g.}$

The most sensitive method for determining urinary oestrone by the Kober colour reaction is that of Brown & Blair (1960), who used the Girard T separation on one-half of 24 hr. urine samples and obtained a detection limit of $0.045 \mu\text{g./24 hr.}$ ($P = 0.01$). The recovery of this method was reported to be 90%, which means that $20 \text{ m}\mu\text{g.}$ was distinguishable from zero, compared with $6 \text{ m}\mu\text{g.}$ by the present method. The present method is therefore more than three times as sensitive as the above method, and, since these authors stated that their method was ten times as sensitive as the original Brown (1955) assay, this means the sensitivity of our method is at least 30 times as sensitive as the Brown (1955) technique. This increase is attributable to the very high sensitivity of fluorescence. Both Preedy & Aitken (1961), using a lengthy fluorescence method, and Barlow (1963), using a combined radioactivity-fluorescence method, reported that $0.2 \mu\text{g./24 hr.}$ (with one-tenth of 24 hr. urine samples) was the detection limit of their methods. The present method is thus twice as sensitive as these above fluorescence methods.

Conclusive evidence for the absolute specificity of a quantitative method can seldom be obtained, but often very convincing evidence can be presented. Finkelstein (1960) commented on the fact that the fluorescence spectrum of the final sample for fluorimetry ought to be compared with authentic oestrogen, to establish specificity. The present method produces column fractions in which correspondence between the radioactivity and fluorescence measurements for oestrone acetate nearly always occurs, and in which the fluorescence spectra are identical with that of authentic standard. The positive oestrone values found after ablative adrenal and ovarian surgery have also been found by Irvine, Aitken, Rendelman & Folca (1961), using the fluorescent technique of Preedy & Aitken (1961), who found oestrone present in the urine of this type of patient. Our results suggest that the fluorescence is not due to contamination.

Specificity is achieved by the use of the Bauld (1956) saponification procedure and by the efficient Girard T separation stage before the alumina chromatography. The ability to measure $1 \mu\text{g./24 hr.}$ for one-fifth of a 24 hr. sample with an accuracy of $\pm 4\%$ shows the accuracy and sensitivity of the method. The only other combined radioactivity-fluorescence procedure to date is that of Barlow (1963); this method included neither of the two short purification stages mentioned above, but instead used a more lengthy paper-chromatographic step, and in our hands did not reach the accuracy of the present method.

The advantages of the method are thus its high sensitivity, accuracy and specificity coupled with ease of performance. Internal compensation for unpredictable losses permits reliable and comparable assays by personnel with varying experience of the method, which takes only 2 days to complete. The method enables accurate quantitative determinations of oestrone for those low-titre urines for which the Kober colour methods have been considered unreliable. Over 100 satisfactory determinations have been carried out in the last year in this Laboratory.

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