We should like to thank Dr R. Y. Thomson and Mr R. Logan for their assistance in some of these experiments, Mr A. J. Taylor for technical assistance and Dr J. A. Roper of the Department of Genetics in this University for help in the radiation experiments. We are also indebted to Dr A. C. White of the Wellcome Physiology Research Laboratories for assistance in the supply of venom. The expenses of this work were defrayed from a grant to one of us (J. N. D.) from the Medical Research Council, and by a grant from the Rankin Fund of the University of Glasgow.

REFERENCES


A Chemical Method for the Determination of Oestriol, Oestrone and Oestradiol in Human Urine

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In connexion with a research programme to study the clinical significance of variations in the urinary excretion and metabolism of the oestrogens, an accurate method, suitable for day-to-day routine use, was required for the separate determination of oestriol, oestrone and oestradiol-17β in the low concentrations found in the urine of men and non-pregnant women. Since all available methods lacked the desired accuracy, sensitivity, specificity and convenience, it was decided to attempt to develop a more suitable procedure. The project was organized jointly with the Department of Biochemistry, and papers dealing with one aspect of the problem (Brown, 1952; Bauld, 1954) have already been published. The present paper describes in detail one complete procedure which appears to be more satisfactory in many respects than any that have hitherto been described, and has been used on a routine basis in clinical investigations for the past 18 months. A preliminary communication in which the method was briefly described has already been presented (Brown, 1955).

The method was evolved from the one described by Clayton (1949) and incorporates a new phase-change purification step involving methylation of the phenolic fraction. The methylated oestrogens are then further purified and separated from one another by chromatography on alumina columns and are estimated colorimetrically using an improved modification of the Kober colour reaction.
(Brown, 1952; Bauld, 1954). Interfering background colours are corrected for by the spectrophotometric method of Allen (1950).

**MATERIALS**

*Reagents.* Ether (J. F. Macfarlan and Co. Ltd., Edinburgh, A.R.) was washed with saturated FeSO₄ solution and water, distilled, stored in dark bottles, and used within a fortnight of purification. Ether recovered during the procedure was simply redistilled before using again. Light petroleum (b.p. 40–60°) and benzene (British Drug Houses Ltd., A.R.) were redistilled and saturated with water. Recovered benzene was washed thoroughly with water and distilled through a fractionating column. Ethanol (absolute) was refluxed with NaOH pellets to remove aldehydes and twice distilled. (Errors may arise through impurities in solvents (Bauld, 1955) but those specified here are quite satisfactory provided they are used in connexion with urine extracts.) H₂BO₃ powder was B.P. grade. Dimethyl sulphate was redistilled. NaOH and NaHCO₃ solutions were prepared on a w/v basis using A.R. chemicals. Concentrated carbonate solution, of pH 10-5 (approximately), was prepared by adding 20% NaOH (150 ml.) to 8% NaHCO₃ solution (1 l.). Alumina (Savory and Moore Ltd., London) of mesh size 100/150 and activity II–III (Brookmann & Schodder, 1941) was deactivated with 9–10% of water to the activity specified later.

*Colour reagents.* The oestriol colour reagent was prepared by dissolving quinol (30 g.) in 76% (v/v) H₂SO₄ (1 l.), the oestrone reagent by dissolving quinol (20 g.) in 66% (v/v) H₂SO₄ (1 l.) and the oestriadiol reagent by dissolving quinol (20 g.) in 60% (v/v) H₂SO₄ (1 l.). The quinol was laboratory grade, and the H₂SO₄ was AnalaR reagent, both manufactured by British Drug Houses Ltd. Solution was hastened by heating. Reagents were kept at least 24 hr. before use, the oestriol reagent was usually a light yellow, the oestrone was light brown to pink, and the oestriadiol reagent was light pink in colour. They were stable almost indefinitely at room temp. in the dark.

*Standard oestrogen solutions.* Standard solutions of pure crystalline oestrogens and their methyl ethers were prepared in ethanol (5 mg./100 ml.). Solutions were stored at 4° and were stable indefinitely.

*Apparatus.* Glassware was rinsed after use with tap, and then distilled water unless visibly dirty, when it was cleaned by (a) steeping in a chromic acid–sulphuric acid mixture; (b) washing with tap water; (c) soaking in an acid sulphite.

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**Urine hydrolysed with HCl**

extracted with ether

**Ether extract**

Washed with (1) satd. Na₂CO₃ soln. pH 10-5

(2) NaOH; NaOH wash neutralized to pH 10 with

satd. NaHCO₃ and shaken again with the ether extracts

(3) NaHCO₃

(4) water

**Ether evaporated**

Residue dissolved in ethanol

Benzene and light petroleum (equal vols.) added

extracted with water

**Benzene–light petroleum**

extracted with NaOH

**NaOH extract (oestrone-oestriadiol)**

H₂BO₃ added

Methylated with dimethyl sulphate at 37°

NaOH and H₂O₄ added

extracted with light petroleum

**Light petroleum extract**

washed with water

chromatographed on alumina

**Oestrone methyl ether**

**Oestriadiol methyl ether**

**Water extract (oestriol)**

H₂BO₃ and NaOH added

Methylated with dimethyl sulphate at 37°

NaOH and H₂O₄ added

extracted with benzene

**Benzen extract**

washed with water

chromatographed on alumina

**Oestriol methyl ether**

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Fig. 1. Flow sheet for the separation of oestrone, oestriadiol and oestriol as the methyl ethers.
solution (Na₂SO₄, approx. 0.2% acidified with H₂SO₄) to destroy traces of chromic acid which would otherwise be a harmful contaminant in the method, and (d) rinsing thoroughly with tap water, then distilled water, before being dried.

Optical densities were measured in a Unicam S.P. 600 spectrophotometer using 1 cm. glass cells.

**EXPERIMENTAL**

The extraction and purification stages are given first in general, then in detail and are summarized in Fig. 1.

*Hydrolysis of conjugated oestrogens in urine*

The hydrolysis of conjugated oestrogens in urine is a complex problem which is still being investigated. At present, acid hydrolysis only has been studied since it fits more easily than enzymic hydrolysis into a routine method. Preliminary experiments to find conditions giving maximum yields of oestrogens from urine showed that the best results were obtained by the method recommended by Marrian & Baud (1951) namely, boiling the urine under reflux 1 hr. with 15 vol. conc. H₂SO₄/100 vol. urine. However, when oestriol, oestrone or oestradiol-17β is added to the urine before this acid treatment, from 10 to 20% of the added oestrogen is lost during the hydrolysis. Furthermore, it seems that similar losses of endogenous oestrogens also occur. These losses are not prevented by the addition of reducing agents as suggested by Rosenmund (1948) and van Bruggen (1948), and, at present, have to be accepted along with the other known losses occurring in the method.

**Extraction with ether and removal of the acid fraction**

 Ether seems to be the most suitable solvent for extracting the three oestrogens from aqueous solutions but even so thorough extraction is necessary to extract all of the oestradiol.

Many workers remove the acid fraction from the ether extract of hydrolysed urine by shaking with NaHCO₃ solutions (Clayton, 1949). Cohen & Marrian (1934) passed CO₂ into alkaline solutions containing the acid and phenol fractions from urine until the solutions were neutral to phenolphthalein (pH 9-0) and then extracted the oestrogens with ether. Engel, Slauwhite, Carter & Nathanson (1950), and Engel (1950) modified this procedure and showed that it is more efficient than any other described for removing the acid fraction. In this laboratory it was found that oestradiol is easily extracted with ether from aqueous solutions at a pH as high as 10-5, provided the aqueous solutions are saturated with Na₂CO₃ or NaHCO₃ at that pH. In fact, the partition coefficient of oestradiol between ether and concentrated carbonate solution of pH 10-5 was found to be practically the same as between ether and saturated NaHCO₃. Apparently, even at this pH, ionic concentration rather than pH determines the solubility of oestradiol in aqueous solutions. Concentrated carbonate solution of pH 10-5, which is much more effective as a washing agent than NaHCO₃, can therefore be used for removing the acid fraction from ether extracts without any appreciable loss of oestradiol.

Urine extracts contain substances which change to coloured products when dissolved in alkali and cannot then be re-extracted with ether at a pH above 7. These substances, which are effectively removed from the oestrogen fraction in the procedures of Cohen & Marrian (1934) and of Engel et al. (1950), are changed and removed in the present method by shaking the ether extract directly with NaOH solution after first washing out the acid fraction with conc. Na₂CO₃ solution of pH 10-5. The NaOH, which also extracts some of the oestrogens, is then partly neutralized to pH 10, and the carbonate-ion concentration increased, by adding saturated NaHCO₃ solution. Shaking again with the ether layer extracts the oestrogens back into the ether and leaves the coloured substances in the aqueous layer which is then discarded. The ether is washed with saturated NaHCO₃ to neutralize any alkali still present, and thus eliminate excessive loss of oestradiol in the subsequent water wash. The ether is then washed with a small amount of water to remove the bicarbonate.

The efficacy of this treatment is illustrated in the following experiments. When the oestrogens were subjected to the procedure, 97% of the oestradiol and 100% of the oestrone and oestradiol were recovered in the ether phase.

An ether extract of hydrolysed male urine was divided into four equal parts and extracted (1) and (2) with 8% sodium bicarbonate, (3) with conc. carbonate solution of pH 10-5, (4) by the new procedure described above. The ether was evaporated, the residue taken up in toluene and extracted with NaOH. The alkali extracts of (2) and (3) were partly neutralized to a pH of 9-9-5 and extracted with ether according to the procedure of Engel (1950), and those of (1) and (4) were acidified with HCl and extracted with ether. The ether extracts were evaporated and the residues were heated with the 76% H₂SO₄ 2% quinol reagent as for oestriol in the modified Kober colour reaction. Uncorrected optical densities at 516 mμ, of the colours produced by the urine contaminants still present were (1) simple NaHCO₃ wash 0-315; (2) Engel procedure 0-120; (3) modified Engel procedure 0-097; (4) the new procedure 0-099. The new procedure incorporating the carbonate and alkali washes is therefore even more efficient and more convenient for removing acidic and alkali-unstable substances from urine extracts than the method described by Engel.

**The separation of the phenolic fraction and of oestriol from oestrone and oestradiol**

It is possible to methylate the three oestrogens together and separate the methyl ethers on a single chromatogram. However, better purification is achieved by dividing into an oestriol fraction and an oestrone plus oestradiol fraction before methylation, and chromatographing these on two separate columns after methylation. This preliminary division is conveniently performed at the same time as the neutral and phenolic fractions are separated from each other. In this way, Clayton (personal communication) extracted oestriol from benzene with water and then extracted oestrone and oestradiol with NaOH. However, this method is tedious as thorough extractions are required for quantitative results. The ease with which the oestrogens are extracted from benzene with aqueous solvents is increased by diluting with light petroleum, a solvent in which the oestrogens are relatively insoluble (Doisy, Huffman, Thayer & Doisy, 1941). A mixture of equal parts of benzene and light petroleum was found to be completely satisfactory. Using this and the extraction procedure described in the method, 98% of the oestriol is recovered in the water extract, and 90% of the oestradiol and 97% of the oestrone.
are recovered in the NaOH. Some oestradiol (4%) is lost in the water extract. These figures agree closely with those reported for the same system by Finkelstein (1952).

**Methylation**

There is reason to believe that much of the material in the oestrogen fractions at this stage is not truly phenolic. A specific separation of the true phenols from this non-phenolic material should be possible by chemically modifying their phenolic groups in such a way that their solubilities are changed without similarly affecting the non-phenolic material. After a few preliminary experiments, it was found that methylation of the phenol group is a very convenient method for achieving this, especially as the oestrogen methyl ethers can still be estimated directly with the Kober colour method (Marlow, 1950). The complete change in solubilities following methylation is illustrated by the fact that oestrone and oestradiol can be quantitatively separated from their methyl ethers by one light petroleum–NaOH partition, and oestril from its methyl ether by one benzene–NaOH partition, the methyl ethers being recovered in each case in the organic solvent phase.

The oestrogen fractions, in alkaline solution, are conveniently methylated with dimethyl sulphate, which is effective under these conditions. The usual methods of methylating phenols with dimethyl sulphate are, however, unsatisfactory when applied to these oestrogen extracts. In strongly alkaline solutions, methylation is slow compared to the rate at which the dimethyl sulphate is destroyed by hydrolysis. It was then found that this destruction is considerably retarded without seriously decreasing the rate of methylation, by methylating in borate buffer solutions between pH 10 and 11.5. The reaction takes place slowly at room temperature but at 37°C it is complete in 15–30 min., and under the conditions adopted, about 95% of the oestril, 96% of the oestrone, and 98% of the oestradiol are converted into their monomethyl ethers.

**The partial destruction of contaminating substances by oxidation and the extraction of the methylated oestrogens**

The methylation mixture is next made strongly alkaline to take advantage of the alkali insolubility of the true methylated phenols. The methylated phenolic fraction at this stage contains substances which can be oxidized to alkali-soluble products and thereby removed. H₂O₂ in alkaline solution at room temp., was found to be the best oxidizing agent for achieving this, having no effect on the oestrogen methyl ethers themselves under these conditions.

Oestril methyl ether is then extracted with benzene and the oestrone and oestradiol methyl ethers are extracted with light petroleum. In each case the partition favours completely the organic solvent phases. The considerable purification of oestrogen extracts from urine obtained by the methylation procedure is shown by the fact that the benzene and light petroleum extracts are completely colourless and all the visible pigments are discarded, apparently unchanged, with the aqueous layer.

**Chromatography**

Stimmel (1946) was the first to apply adsorption chromatography on alumina columns to the further purification and partition of the oestrogen fractions, but he experienced difficulty in eluting oestril from the columns. This difficulty, however, does not arise with the oestrogen methyl ethers, for being less polar, they are more suited to adsorption chromatography procedures.

Mixtures of oestrone and oestradiol methyl ethers are resolved with difficulty on columns of very active alumina but are easily resolved on columns of less active alumina. A suitable activity is obtained by adding 9–10% of water to an alumina with an original activity equivalent to Brockmann no. II (see Stewart, 1949). The resolution of oestrone methyl ethers on columns of this deactivated alumina is such that relatively crude chromatograms can be used, and those described in the method were chosen mainly on the grounds of suitability to a robust and convenient routine procedure. Furthermore, the chromatographic properties of this alumina are practically unaffected by small changes in water content and solvents applied to it should be saturated with water. Both these factors contribute to a robust chromatography procedure.

Eluents were selected to elute the oestrogen fractions in volumes which could be evaporated directly from the tubes in which the colour was later developed.

**Evaporation of solvents**

For the most reproducible results, solutions of oestrogen methyl ethers should be evaporated in the absence of air to complete dryness. Evaporating down under jets of air or impure nitrogen may cause losses, especially of oestrone methyl ether when dissolved in light petroleum. These losses seem to be due to impurities in the solvents which partly oxidize during evaporation and interfere in the development of the Kober colour. For this reason also the last traces of solvents should be removed before colour development. A distilling apparatus to take 4–6 Kober tubes at a time has been designed which functions as follows. Solvents are distilled from the tubes by heating in a water bath and applying a slightly reduced pressure from a water pump to hasten distillation. The vapour column effectively displaces air from contact with the boiling liquid. When the solvents have evaporated completely the vacuum is turned full on, the tubes are removed from the boiling-water bath, and the vacuum is released by admitting N₂. The vacuum is again applied and released and the tubes are then removed from the apparatus and are ready for colour development.

**Colour development and colorimetry**

The colour method is a development of the one described previously (Brown, 1952). Bauld (1954) has re-investigated this earlier colour method in more detail and the present method incorporates some of his refinements and resembles closely the method which he finally adopted.

The following changes have been made to the earlier method. In order to increase the sensitivity, smaller volumes of reagents are used, and the colour is not diluted before measurement. Three colour reagents are employed, each optimum for the respective oestrogen, a 76% H₂SO₄–2% quinol reagent for oestriol, a 66% H₂SO₄–3% quinol reagent for oestrone, and a 60% H₂SO₄–2% quinol reagent for oestradiol-17β. AnalR H₂SO₄ supplied by British Drug Houses Ltd. is specified; reagents prepared with this acid do not need the addition of oxidizing agents (see Bauld, 1954). The conditions chosen for the second stage of colour
development are an H₂SO₄ concentration of about 55% and
and a heating time of 10 min., neither of which is critical.
Bauld's most important improvement to the colour method
is the addition of fresh quinol to the mature H₂SO₄–quinol
reagents immediately before colour development. This is
conveniently done by adding the quinol to the oestrogen
fractions before evaporating the solvents. As recommended
by Bauld, the Kober tubes are standardized to approxi-
mately the same diameters.

When the colour method is applied to pure oestrogens,
colours produced are stable for at least 12 hr. and absorb
light maximally at the following wavelengths (uncorrected):
oestriol and oestrone, 513 mµ; oestradiol-17β, 514 mµ;
oestriol and oestrone methyl ethers, 516 mµ; oestradiol-17β
methyl ether, 518 mµ. The molecular extinction coefficients
are very nearly the same for the free oestrogens as for their
methyl ethers but exact comparison is not possible owing to
the small differences in the absorption maxima.

Fig. 2. Relationships between corrected spectrophotom-
erometer readings and amounts of oestrogen methyl ethers
expressed in terms of the weight of the free oestrogens.

- - Oestrone; O--O, oestradiol-17β; x-x, oestriol.

Measuring the optical densities at three wavelengths and
correcting for non-specific absorption by the method of
Allen (1950), is necessary to correct for colours due to
oxidized quinol and contaminating substances from urine.
In fact, the validity of results obtained by the method
depends, to a considerable extent, on the effectiveness of this
density correction. Corrected optical densities obey Beer's
Law closely (Fig. 2). New calibration curves are constructed
using standard solutions of pure oestrogen methyl ethers
whenever new H₂SO₄–quinol reagents are prepared.

THE METHOD IN DETAIL

Two or three determinations, each in duplicate, are
usually performed at one time; 24 hr. specimens of urine
are collected without preservative and stored at 4°. If the
24 hr. volume is less than 1200 ml., the specimen is diluted
to this volume with distilled water.

Hydrolysis and extraction

Urine (200 ml.) is heated to boiling under a reflux
condenser. Conc. HCl (11 N, 30 ml.) is then added through
the condenser and the urine–HCl mixture is boiled for 60 min.
and then cooled rapidly under running tap water. The
cooled hydrolysed urine is extracted once with 200 ml. and
twice with 100 ml. volumes of ether. The ether is then
extracted with concentrated carbonate solution, of pH 10.5
(80 ml.) (which is rejected), and then shaken thoroughly
with NaOH (20 ml. of 8%). The NaOH layer is not dis-
carded but is partly neutralized by adding 8% NaHCO₃
solution (80 ml.) and shaken again with the ether layer. The
aqueous layer is then discarded. The ether is washed first
with 8% NaHCO₃ solution (20 ml.) and then with water
(10 ml.). The water is drained off as completely as possible.

Extraction of the phenol fractions
and methylation

The ether solution is poured into a flask and the ether is
distilled just to dryness on a water bath. The flask is removed
immediately from the water bath, ethanol (1 ml.) is added
to dissolve the residue, since oestriol is not easily soluble in
benzene, and the flask is allowed to cool. Its contents are
transferred with benzene (25 ml.) to a separating funnel
containing light petroleum (25 ml.). The benzene–light
petroleum solution is extracted with two 25 ml. volumes of
water and then with two 25 ml. volumes of 1–6 N NaOH.
The water extracts, which contain the oestradiol fraction,
are added to a 100 ml. stoppered conical flask containing
H₂BO₃ (0.9 g.) and 20% NaOH (4 ml.). The NaOH extracts,
which contain the oestrone and oestradiol fractions, are
added to a similar flask containing only H₂BO₃ (0.9 g.).
The two flasks are placed in a 37° water bath and dimethyl
sulphate (1 ml.) is added to each. The dimethyl sulphate
is handled cautiously, in a fume cupboard, and volumes are
measured by means of a safety pipette. The flasks are
shaken until the H₂BO₃ and dimethyl sulphate have dis-
solved and are kept at 37° for 10–30 min. longer. The
procedure is repeated by adding more dimethyl sulphate
(1 ml.) together with 20% NaOH (2 ml.) to replace that
neutralized by the first 1 ml. of dimethyl sulphate. The flasks
are shaken until the dimethyl sulphate has dissolved, and are
either kept at 37° for a further 20–30 min. and cooled, or
allowed to stand at room temp. overnight.

The destruction of contaminating substances by
oxidation and the extraction of the methylated
oestrogens

NaOH (10 ml. of 20%) and H₂O₂ (2-5 ml. of 30%) are
added to each flask, the contents of which are transferred
to separating funnels. The methylated oestradiol fraction
is extracted with benzene (25 ml.) and the methylated
oestrone and oestradiol fraction is extracted with light
petroleum (25 ml.), the solvents being used first to rinse the
corresponding methylation flasks. The benzene and the
light petroleum extracts are washed twice with two 5 ml.
volumes of water and the water is drained off as completely
as possible.
Chromatography

The glass chromatogram tubes have an internal diameter of 13 mm., a capacity of 40 ml. of solvent, a sealed-in sintered glass support (porosity no. 3) for the alumina column, and an interchangeable B19 cone for connexion with receiving flasks or tubes. Chromatograms are usually run in groups of four or six. The rate of flow of solvents is adjusted to approximately 1 drop/2 sec. by applying slight suction from a manifold through two-way stop cocks, by which means suction can be applied to or released from individual chromatogram tubes and their receiving flasks.

A column is prepared by partly filling the chromatogram tube with benzene or light petroleum and then adding the alumina (2 g.), standardized as described later, in a thin stream so that entangled air frees itself during the passage through the solvent. When the alumina has settled, its surface is levelled by tapping, and overlaid with about a quarter of an inch of dry acid-ethanol washed sand to protect it from disturbance during the addition of solvents. Solvents are sucked through to the level of the sand but no lower before another solvent is added.

The methylated oestriol fraction in benzene is applied to an alumina column prepared in benzene, taking care not to transfer any water to the column. The column is then eluted with (a) 12 ml. of a mixture of 1-4% ethanol in benzene; the effluent removes a urine pigment band and is discarded, and (b) 15 ml. of a mixture of 2-5% ethanol in benzene; the effluent, containing all of the oestriol methyl ether, is collected in a 6 x 1 in. Pyrex test tube with a standard B19 socket (Kober tube) in which the colour is later developed.

The methylated oestrone and oestadiol fractions in light petroleum are similarly applied to another alumina column prepared in light petroleum. The column is eluted with (a) 12 ml. of a mixture of 25% benzene in light petroleum, the effluent being discarded; (b) 15 ml. of a mixture of 40% benzene in light petroleum; the effluent is collected into a Kober tube and contains all of the oestrone methyl ether; (c) a further 12 ml. of the same mixture of 40% benzene in light petroleum; the effluent being discarded; and (d) 12 ml. of benzene; the effluent is collected into a Kober tube and contains all of the oestadiol methyl ether.

Preparation and standardization of the alumina

Particular care is required in the standardization of the alumina used in this method. It is possible to alter either the volumes and eluting powers of solvents for each new batch of deactivated alumina, but it is easier in practice to adjust the alumina to a predetermined activity and keep the other factors constant. Alumina is deactivated by adding water (approx. 9-5 ml per 100 g.), stirring to break down moist lumps, and shaking the mixture thoroughly until quite homogeneous. Perceptible heat is evolved, and after allowing to cool the activity is tested by preparing a 2 g. column in light petroleum and applying a solution of oestrone methyl ether (10 µg.) in light petroleum (25 ml.) which has been washed with water. The column is eluted with 25% benzene in light petroleum in fractions and the oestrone methyl ether content of each fraction is determined colorimetrically. When the activity of the alumina is correct, oestrone methyl ether begins to appear in the 16th-20th ml. of eluate. If the alumina is too active, more water is added, if not active enough, more active alumina is added until the required activity is obtained. The behaviours of the oestrogen methyl ethers on this alumina always conform closely to the elution pattern from which the chromatography procedure described in the method is derived. Small deviations from this pattern are sometimes found with different batches of alumina and it is then necessary to alter slightly the volumes of eluents used in the method in order to ensure adequate safety factors in the blind cutting of fractions. The following procedures are undertaken with each fresh batch of alumina to check this point.

Another column (2 g.) is prepared in light petroleum; a water-washed solution of oestrone and oestadiol methyl ethers (10 µg. of each) in light petroleum (25 ml.) is applied to the column which is then eluted, first with 25% benzene in light petroleum (12 ml.), and then fractionally with 40% benzene in light petroleum. Oestrone methyl ether should be completely eluted in the first 12 ml. of the 40% benzene in light petroleum and oestadiol methyl ether should begin to be eluted when a total of about 30 ml. of eluate has been collected.

Another column is prepared and oestadiol methyl ether similarly applied and eluted with (a) 12 ml. of 25% benzene in light petroleum, (b) 27 ml. of 40% benzene in light petroleum, and then (c) fractionally with benzene. The oestadiol methyl ether should be eluted in the first 8-10 ml. of benzene eluate. Likewise oestrone methyl ether in benzene (25 ml.) is washed with water and applied to a 2 g. column prepared in benzene. When this column is eluted with 1-4% ethanol in benzene, oestrone methyl ether should begin to be eluted in the 15th-17th ml. of eluate.

Another similar column is eluted, first with 12 ml. of 1-4% ethanol in benzene, and then with 2-5% ethanol in benzene the first 12 ml. of which should elute the oestriol methyl ether.

The standardized alumina is stored in air-tight containers. Caking sometimes occurs during storage but is easily broken down by shaking.

Evaporation of solvents

Quinol (4 mg.) in ethanolic solution (0-2 ml. of 2%, w/v) and a small piece of porous tile are added to each eluate in its Kober tube. Solutions are evaporated completely to dryness by heating in a water bath. Blank tubes containing quinol only are prepared at the same time.

Colour development and colorimetry

The appropriate quinol-H₂SO₄ reagent (3 ml.) for the particular oestrogen is added to the oestrogen fractions in Kober tubes which are then heated for 20 min. in a boiling-water bath. The tubes are shaken twice during the first 6 min. of heating. After heating, the tubes are cooled in a bath of cold water. Water (1 ml.) is added to each oestriol tube, 0-5 ml. to each oestrone tube and 0-2 ml. to each oestadiol tube; the tubes are shaken and reheated in the boiling-water bath for 10 min. They are then cooled again in cold water for about 10 min. and optical densities are measured against similarly treated reagent blanks in the spectrophotometer at the following wavelengths: oestriol and oestrone fractions 480, 516 and 552 mµ; oestadiol fraction 480, 516 and 556 mµ.

Optical density readings (D) are corrected by applying the following formulae which are derived from Allen's (1950) formula multiplied by 2: oestrone and oestriol corrected readings = 2D₁₅₋₃₈₄(D₁₈₀ + D₅₃₅), oestriol corrected reading = 2D₁₅₋₃₈₄(D₁₈₀ + D₅₃₅). The amount of oestrogen
methyl ether present in each tube is found by applying the corrected readings to the particular standard calibration curve prepared with the pure oestrogen methyl ether. This is then converted into the corresponding amount of free oestrogen by multiplying by the ratio of the molecular weights. The 24 hr. excretion is calculated from this and the 24 hr. urine volume.

RESULTS

Recoveries of oestrogens added to hydrolysed urine

To test the extraction, purification and colorimetric stages of the method a series of recovery experiments were performed in which known amounts of oestriol, oestrone and oestradiol-17β were added to portions of acid-hydrolysed 24 hr. male urines. Blank determinations were made at the same time on the same urine. The amounts of oestrogens added, calculated on the basis of a 24 hr. specimen together with the percentages of these recovered after subtracting the urine blank values, are shown in Table 1.

Results from urines

Typical figures, including spectrophotometer readings, obtained from the urine of a woman in the early proliferative phase of the menstrual cycle and from a normal man are shown in Table 2, to illustrate the order of results found in these cases.

The agreement obtained between duplicate determinations is illustrated by the following figures taken from 100 unselected consecutive duplicate analysis performed by one worker. The series includes results from three normal menstrual cycles and five recoveries from male urine where the oestrogen levels were between 0 and 40 μg./day. The average difference between duplicates in terms of 24 hr. excretion was 0.3 μg. in the case of oestrone

Table 1. Recoveries of oestrogens from acid-hydrolysed male urine

Results are shown as the mean percentage recovery ± the standard deviation and are corrected for the endogenous blank values. Figures in parentheses refer to number of determinations.

<table>
<thead>
<tr>
<th>Amounts added per 24 hr. urine (μg.)</th>
<th>4-7</th>
<th>25-35</th>
<th>36-60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oestriol</td>
<td>88±10-8 (12)</td>
<td>85±3-8 (11)</td>
<td>83±3-0 (12)</td>
</tr>
<tr>
<td>Oestrone</td>
<td>87±11-7 (11)</td>
<td>84±5-2 (12)</td>
<td>82±6-1 (10)</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>80±5-5 (12)</td>
<td>91±7-0 (11)</td>
<td>86±6-6 (11)</td>
</tr>
</tbody>
</table>

Table 2. Typical results from urines containing small amounts of oestrogens

Duplicate determinations.

<table>
<thead>
<tr>
<th>Optical densities measured</th>
<th>Corrected values</th>
<th>Calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>480 mμ.</td>
<td>516 or 518 mμ.</td>
<td>552 or 556 mμ.</td>
</tr>
<tr>
<td>Case I. Normal male (1/6 of a 24 hr. specimen)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oestriol</td>
<td>0-276 0-276</td>
<td>0-234 0-234</td>
</tr>
<tr>
<td>Oestrone</td>
<td>0-137 0-140</td>
<td>0-147 0-149</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>0-235 0-261</td>
<td>0-195 0-214</td>
</tr>
<tr>
<td>Case II. Normal female (early proliferative phase; 1/8-5 of a 24 hr. specimen)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oestriol</td>
<td>0-252 0-226</td>
<td>0-210 0-186</td>
</tr>
<tr>
<td>Oestrone</td>
<td>0-128 0-096</td>
<td>0-119 0-090</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>0-212 0-196</td>
<td>0-169 0-162</td>
</tr>
</tbody>
</table>

Table 1. Recoveries are between 80 and 90% even at levels corresponding to 4 μg. per day. Similar results with a greater scatter, but not recorded here, were obtained in a small series at 2.5 μg./day. The scatter of results is greatest at the lowest levels as would be expected, for the colour due to added oestrogen is then so slight that errors in instrumentation and in measuring the endogenous oestrogen blank, become large in comparison. These recovery experiments show that the method is reliable for measuring differences in oestrogen concentrations of the order of 4 μg./24 hr. urine specimen.

Typical figures, including spectrophotometer readings, obtained from the urine of a woman in the early proliferative phase of the menstrual cycle and from a normal man are shown in Table 2, to illustrate the order of results found in these cases.

The agreement obtained between duplicate determinations is illustrated by the following figures taken from 100 unselected consecutive duplicate analysis performed by one worker. The series includes results from three normal menstrual cycles and five recoveries from male urine where the oestrogen levels were between 0 and 40 μg./day. The average difference between duplicates in terms of 24 hr. excretion was 0.3 μg. in the case of oestrone and oestradiol or 0.4 μg. in the case of oestriol; 95% of the duplicate measurements did not differ by more than 1.0 μg. in the case of oestrone and oestradiol or 1.5 μg. in the case of oestriol.

The resolution into two components of the Kober colour produced by an oestrone fraction from urine is shown in Fig. 3.

The chromatographic behaviours of a number of oestrogen methyl ethers and of the methylated oestriol, oestrone and oestradiol fractions from
pooled luteal-phase urine are shown in Table 3. The oestrogen levels per litre of urine were: oestriol 14 μg.; oestrone 6 μg.; and oestradiol 1 μg.

**DISCUSSION**

The fractions obtained by applying the method to urine still contain impurities which interfere in the colorimetric measurement of the oestrogens and have a considerable effect on the specificity of the method. However, a considerable amount of evidence, none of which is completely conclusive, has been accumulated to show that the results are significant even when the urine contains only small amounts of oestrogens.

The red colour of the Kober reaction, with an absorption maximum about 515 μm, is highly specific for the natural oestrogens. However, many of the impurities present in urine extracts produce yellow colours in the Kober reaction and these may form a considerable portion of the total colour, especially when the oestrogen concentration is low (see Table 2). For instance, in the case of male urine, the optical densities at about 516 μm, contributed by the yellow colours as calculated by the correction formula are about 0.9, 0.67 and 0.92 of the total optical densities of the oestriol, oestrone and oestradiol fractions respectively. This is a serious objection, for it is necessary in these cases to rely very much on the spectrophotometric method for correcting for this interference. The spectrophotometric correction is based on the assumption that the contaminating colours have linear wavelength/absorption curves in the region of the absorption maximum of the oestrogen red colour. This is difficult to prove, but in all the cases where this point has been investigated colours produced with urine extracts have been resolvable into two components, one with the same absorption maximum as the oestrogen Kober red colour, and the other with a linear wavelength/absorption curve in this region of the spectrum (Fig. 3).

The chromatographic behaviours of the urinary Kober chromogens estimated as oestrogens after

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**Table 3. Elution of oestrogen methyl ethers and urine fractions from alumina* columns**

Results are expressed as the percentage present in each eluate and in the case of the urine fractions refer only to the Kober chromogens estimated as oestrogens after applying the colour correction.

<table>
<thead>
<tr>
<th>Eluate (ml.)</th>
<th>Benzene-light petroleum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25/75</td>
</tr>
<tr>
<td>Methyl ethers</td>
<td>12 4</td>
</tr>
<tr>
<td>Oestrone</td>
<td>- -</td>
</tr>
<tr>
<td>Oestradiol-17β</td>
<td>- 28</td>
</tr>
<tr>
<td>Equilin</td>
<td>- - 64</td>
</tr>
<tr>
<td>Equilenin</td>
<td>- 3 68</td>
</tr>
<tr>
<td>Oestradiol-17α</td>
<td>- 36</td>
</tr>
<tr>
<td>Urine fraction</td>
<td>- 9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Eluate (ml.) ethanol-benzene</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-5/97-5</td>
</tr>
<tr>
<td>1-4/98-6</td>
</tr>
<tr>
<td>Oestriol</td>
</tr>
<tr>
<td>Urine fraction</td>
</tr>
</tbody>
</table>

* Activity slightly greater than that specified in the method.  
† Very pigmented fraction
applying the colour correction provide further evidence that the method may be reasonably specific. These substances are eluted from the columns in exactly the same manner as the corresponding pure oestrogen methyl ethers (Table 3). The interfering chromogenic materials, however, do not usually behave as sharp bands on the columns but tend to trail through the oestrogen bands without affecting their elution patterns. The chromatographic behaviours of oestrone and oestradiol-17β methyl ethers are not specific for these methoxy steroids alone since equilenin and equilin methyl ethers behave like oestrone methyl ether and oestradiol-17α like its 17β isomer (Table 3).

Further evidence for the validity of the method comes from its practical application. Values found in normal and pathological states agree with those determined biologically by other workers, or expected on clinical grounds. This evidence has been discussed elsewhere (Brown, 1955). Other evidence comes from a comparison of results obtained by this method with those obtained by the very different method of Bauld (1955). Parallel estimations performed on the same urine sample with the two methods agreed very closely (Marrian, 1955). As the purification processes employed in the two methods differ considerably, it is unlikely that exactly the same contaminants would be present in the final extracts from both methods.

A measure of the accuracy of the method can be calculated from the recovery figures given in Table 1 and from the 10–20% losses which occur during acid hydrolysis. Assuming that hydrolysis of conjugated oestrogens is complete, these figures give an overall recovery for the whole method of between 60 and 75%, so that values obtained by the method should be this proportion of the true amounts of oestrogens present in the urine.

The reproducibility of the method is indicated by the agreement between duplicates which in 95% of determinations is better than 1.0 μg./24 hr. urine for oestrone and oestradiol and 1.5 μg./24 hr. urine for oestriol. These differences between duplicates are well within the instrumental errors of the method.

As shown in Table 1, the mean recovery is practically independent of oestrogen concentration between 4 and 60 μg./day, but the scatter of results becomes considerable at the lower levels. The sensitivity of the method therefore seems to depend on the sensitivity of the colour reaction and the accuracy of the colour measurements, not on the extraction procedure. Differences in oestrogen concentration of the order of 2.5 μg./24 hr. urine can be detected, without however, any accuracy. Levels of endogenous oestrogens lower than 5 μg./24 hr. urine probably have little quantitative or absolute significance except in showing daily differences in excretion in a series of urines from the one case.

Unfortunately these levels include most of the oestriol and oestradiol values found in male urine.

One person can conveniently do four to six complete estimations in 2 days and can maintain this output without assistance. The method has been in use in this laboratory for more than 18 months without once giving trouble.

**SUMMARY**

1. A new chemical method which appears satisfactory for the separate estimation of oestril, oestrone and oestradiol-17β in the urine of men and non-pregnant women is described.

2. The method involves acid hydrolysis, ether extraction, a new phase-change purification procedure for the phenolic fraction depending on methylation of the phenol group, separation of the oestrogen methyl ethers by chromatography on alumina columns, colorimetric measurement using an improved Kober colour method, and spectroscopic correction for interfering chromogenic material.

3. The method is discussed on the grounds of specificity, accuracy, reproducibility, sensitivity and convenience.

The author wishes to thank Professor G. F. Marrian, F.R.S., for his supervision and interest in the work, and also to acknowledge the valued interchange of ideas which occurred between himself and Dr W. S. Bauld who was also working on the same problem. Thanks are also due to Mr H. A. F. Blair and Miss Janet Mackie for their skilled assistance and for their technical experience, which helped considerably in designing a feasible method.

**REFERENCES**