A note on the chemical determination of 16-*epi*-oestriol

In the course of studies on the excretion and metabolism of the four epimeric oestriols[1,2] two different methods for the chemical assay of 16-*epi*-oestriol[3] have been developed in this laboratory. In *Method A* the separation of 16-*epi*-oestriol and oestriol is performed by methylating the aqueous fraction containing both these compounds followed by gradient elution chromatography of the two methyl ethers on an alumina column and colorimetry. Since the methylation technique described by BROWN[4] for oestradiol-17β, oestrone and oestriol failed to methylate 16-*epi*-oestriol quantitatively, *Method A* necessitated the development of a new methylation procedure. In *Method B* the separation of the two oestriols was obtained by converting 16-*epi*-oestriol into its acetonide[5], a reaction which is stereoselective for *cis*-glycols and therefore not undergone by oestriol as a *trans*-glycol. Following partition of the residues between chloroform and aqueous alkali the acetonide of 16-*epi*-oestriol is completely separated with the organic layer whereas oestriol as a strong phenol remains in the alkali phase. The oestriol fraction is methylated, chromatographed on alumina and colorimetrically assayed by means of the Kober reaction, whereas 16-*epi*-oestriol is chromatographed immediately as the acetonide followed by colorimetry. Both these methods may be incorporated into the BROWN[4] procedure thus making possible the routine determination of 16-*epi*-oestriol, oestriol, oestrone and oestradiol-17β from the same sample of urine.

EXPERIMENTAL

The steroid-containing residue is dissolved in 0.5 ml of ethanol, taken up with 25 ml of benzene/petroleum ether (1 : 1) and extracted with water (4 × 12.5 ml): 16-*epi*-oestriol/oestriol fraction (I). By this treatment 95.9% of 16-*epi*-oestriol (K = 0.41)[6] and 99.97% of oestriol (K = 0.075)[7] should pass into the aqueous layer. The addition of another 25 ml of benzene/petroleum ether to the organic layer is followed by extraction with 0.4 N sodium hydroxide (2 × 25 ml): oestrone/oestradiol fraction (II). Both fractions are made up to N sodium hydroxide and boiled under reflux for 30 min. After cooling the extracts are partly neutralized[8,9] with carbon dioxide to pH 9.3–9.5. Fraction (I) is extracted twice with ether (25 ml), the ether extract washed with water (5 ml) and taken to dryness. Fraction (II) is extracted with benzene (25 ml) and treated exactly as described by BROWN[4] and BROWN *et al.*[10] up to colorimetry of oestrone and oestradiol-17β as their methyl ethers (Table I).

Method A

Determination of 16-epi-oestriol as the methyl ether. The ether residue of (I) is dissolved in anhydrous acetone (10 ml). Ethylene glycol* saturated with potassium carbonate (0.5 ml) and methyl iodide (1 ml) are added and the mixture is boiled under reflux (30 min). After cooling, acetone, ethylene glycol–potassium carbonate and methyl iodide are added in amounts as above. After refluxing again for 30 min, acetone and methyl iodide are evaporated *in vacuo* at 30–40° in a stream of nitrogen. The residue is dissolved in N sodium hydroxide (50 ml), shaken with hydrogen peroxide (2.5 ml of 30%) and extracted with benzene (25 ml). After washing with

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TABLE I

OPTIMUM REACTION CONDITIONS AND RESULTS OF THE MODIFIED KOBER COLOUR REACTION

Steroid	*Kober reagent (ml)*	H_2SO_4 *concentration (% v/v)*	*Time of reheating (min)*	*Wavelength for measuring log I_0/I (mμ)* E_1	E_2 ($E_{\lambda max}$)	E_3	*Ecorr/10 μg ± S.D.*	$E^{1\%}_{1cm}$
3-methoxy-oestrone	2.1	66	6	482	519	556	0.729 ± 0.012 (11)	1660
3-methoxy-oestradiol-17β	2.8	55	10	488	522	556	0.584 ± 0.003 (9)	1390
3-methoxy-oestriol	2.1	76	14	478	517	556	0.540 ± 0.006 (9)	1250
3-methoxy-16-*epi*-oestriol	2.4	66	5	478	517	556	0.680 ± 0.009 (9)	1530
16-*epi*-oestriol-acetonide	2.4	66	5	472	514	556	0.559 ± 0.006 (8)	1190

The Kober reagents were prepared by dissolving 2% w/v of quinol (*puriss.*, Merck, Darmstadt) in aqueous solutions of H_2SO_4 (d = 1.84), *p. a.* Merck). – Colour development: *(1)* addition of the appropriate colour reagent to the tubes containing the dry residues of the column fractions and 4 mg quinol, *(2)* heating of the mixtures on a boiling water bath for 20 min and shaking of the tubes after 2 and 5 min of heating, *(3)* cooling of the tubes in a bath of ice water (5 min), *(4)* addition of water to make up the reaction mixtures to a final volume of 3.2 ml, *(5)* shaking and reheating of the tubes (see table). Extinction coefficients (log I_0/I) were measured in a Beckman spectrophotometer DU against similarly treated reagent blanks using 10 mm glass cells. $E_{corr} = 2E_2 - (E_1 + E_3)$. For more details of the colour reaction, see ref. [11].

water (2 × 5 ml) the benzene residue is transferred to an alumina column (10 × 45 mm; approx. 5 g Al_2O_3 Merck, Darmstadt, deactivated by adding 14% (v/w) of water) with benzene (5 ml). The donor vessel (25 mm int. diameter) of the gradient apparatus is filled with 3% ethanol–benzene (55 ml). The recipient vessel (25 mm int. diameter) containing benzene (55 ml) is connected to the donor by a capillary siphon and fitted with a stirrer. The percolation rate is adjusted to approx. 15 ml/h. 16-*epi*-oestriol methyl ether is eluted in the 25–36 ml portion, and oestriol methyl ether in the 39–60 ml portion of effluent (Fig. 1). The column fractions are evaporated and colorimetrically assayed by the modified Kober reaction described in Table I.

Method B

Determination of 16-epi-oestriol as the acetonide. The ether residue of (I) is dissolved in anhydrous acetone (10 ml) containing 1% (w/v) anhydrous hydrochloric acid and shaken for 30 min at room temperature. The acid is neutralized with N sodium hydroxide (2.74 ml) and acetone is evaporated *in vacuo* at 30–40° in a stream of nitrogen. The residue is partitioned between 0.4 N sodium hydroxide (25 ml) and chloroform (25 ml). The aqueous layer is reextracted twice with chloroform (25 ml). After this treatment 97.7% of 16-*epi*-oestriol acetonide (K = 2.54) is recovered in the organic phase, whereas oestriol (K = 0.01) [13] is left in the aqueous alkali phase. The combined chloroform extracts (*cis*-glycol fraction) are washed with water (2 × 5 ml) and evaporated to dryness. The residue is transferred to a column (10 mm int. diameter) containing 2 g of alumina (Merck, Darmstadt, exposed to an atmosphere saturated with water vapour at room temperature for 2 weeks) with benzene (5 ml). A prewash of 0.8% (v/v) ethanol/benzene (12 ml) is discarded. The acetonide is eluted with 3% (v/v) ethanol/benzene (25 ml) (Fig. 2) and the dry residue is colorimetrically

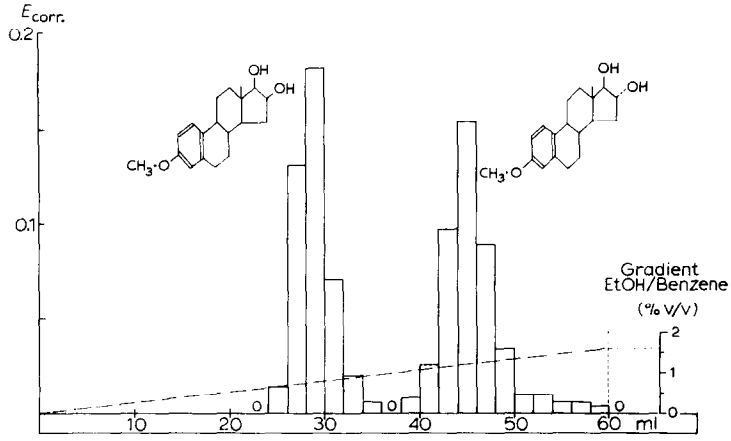

Fig. 1. Separation of the methyl ethers of 16-*epi*-oestriol and oestriol by gradient elution chromatography on alumina. The gradient was calculated according to

$$C = C_0 \left[1 - \left(\frac{a}{a + bt} \right)^{1/b} \right]$$

C_0 = 3% (v/v) ethanol–benzene, V_0 = 55 ml (benzene), R_2 = 0.250 ml/min, R_1 = 0.125 ml/min
a = 440, b = —1 (*cf.* ref. [12]).

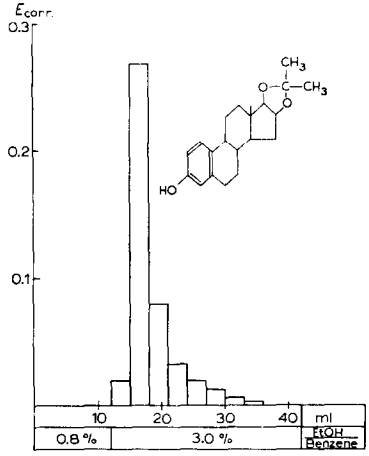

Fig. 2. Absorption chromatography of 16-*epi*-oestriol-acetonide on alumina.

assayed with the 16-*epi*-oestriol colour reagent (Table I). The 0.4 *N* sodium hydroxide layer (*trans*-glycol fraction) is adjusted to pH 9.3–9.5 by dissolving carbon dioxide, extracted with ether and methylated as described in *Method A*. The methylated fraction is chromatographed on alumina according to BROWN[4] and oestriol methyl ether is assayed by the modified Kober reaction (Table I).

RESULTS

In some recovery experiments the accuracy of both these methods has been checked. Pure crystalline 16-*epi*-oestriol when added to 15% (v/v) aqueous hydrochloric acid in 10 μg amounts was recovered by *Method A* combined with the extraction procedure of BROWN[4] with an average of 78% (5 Expts.). When using *Method B* under these conditions the overall recovery was 76% (4 Expts.).

Application of both methods to human urine is in progress and will be published in full detail elsewhere.

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