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* Information available on application to Ministry of Supply, London.

The Determination of Dienoestrol

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(Received 8 October 1946)

Since the clinical and veterinary use of synthetic oestrogens has become so general it is important to devise a simple routine method for the determination of these substances, particularly in biological samples. It would also be an advantage to possess a method applicable at the same time to pharmacological preparations.

Many attempts have been made in this direction, references to which are given by Malpress (1945) but it is probably fair to state that none is applicable to samples of synthetic oestrogens containing biological contaminants. The colouring matter in urines for example, interferes with colorimetric methods, e.g. the Dingemanse (1939) antimony pentachloride method (Warren & Goulding, 1945) and the presence of other phenols interferes with methods dependent on nitration (Malpress, 1945). No method has yet been evolved which will overcome these difficulties by quantitative separation of the synthetic oestrogen or a derivative.

The present communication describes a method of separating a derivative of dienoestrol in about 70 % efficiency from most other contaminants likely to be found in biological samples. It makes use of the facts that dienoestrol is a diene and reacts with maleic anhydride and that the resultant adduct is soluble in aqueous sodium bicarbonate solution,

(Wilder Smith, 1946). The method may be represented diagrammatically as follows:

The only other reactions likely to occur besides the dienoestrol-maleic anhydride addition, are (1) reaction with any other suitable conjugated dienes present, and (2) reaction with alcohols to form half esters. Both (1) and (2) would give new bicarbonate soluble substances. From the fact that the method does give a small blank result with urines containing no dienoestrol, it appears that either (1) and/or (2) do in fact take place to some extent. Indeed, if the urine extract-maleic anhydride reaction mixture is heated at 100° for 30 min. a blank of several mg./l. of normal urine can be obtained. If, however, the reaction is carried out at room temperature, the blank formed amounts to approximately 1 mg./l. normal urine extract. It has been found possible to remove from this blank, by a short steam distillation, most substances interfering with the colour reaction described later. The dienoestrol-maleic anhydride adduct, after hydrolysis to the dicarboxylic acid, is non-volatile in steam. This hydrolysis occurs during the sodium bicarbonate extraction.

METHOD

For the preliminary work, dienoestrol in varying amounts was added to a benzene extract corresponding to 1 l. of hydrolyzed normal male urine, thus evading the time-consuming continuous benzene extraction for each determination. In all the later experiments anisole was added to the benzene extract and the benzene removed by distillation, leaving an anisole solution of the extracted material, though results can be obtained by working directly on the concentrated benzene extract, provided the dienoestrol concentration is not high (over 10 mg./l. urine).

(a) For concentrations of dienoestrol not below 2-5 mg./l. urine

(1) *Initial bicarbonate extractions.* The urine sample (1-2 l.) is hydrolyzed by refluxing for 10 min. after addition of 100 ml. concentrated hydrochloric acid/l. urine. The urine is extracted for 24 hr. in a continuous benzene extractor, and the benzene extract concentrated by distillation from an oil bath into 10 ml. anisole. After removal of all the benzene, the volume of residue is made up to 15 ml. with anisole and the anisole solution transferred to a 35 ml. cylindrical shaking flask (fitted with a ground glass stopper held in position by lugs and small springs) and agitated 1-5 min. with 15 ml. of a 10% solution of aqueous sodium carbonate. After removal of the lower sodium carbonate layer, which is discarded, the process is repeated until less than 0.5 mg. of acid material is extracted by the carbonate from the anisole layer; usually 5-7 extractions are required. If desired these extractions may be carried out by hand. It is usually necessary to warm the anisole layer in warm water to effect rapid separation of the two layers.

The weight of acid material in the carbonate layer is determined as follows: After two hand extractions with 25 ml. portions of ether (to remove any material taken over mechanically from the anisole layer) the carbonate layer is made acid to congo-red paper by adding concentrated

hydrochloric acid, then extracted four times with 25 ml. portions of ether and the ether dried over anhydrous sodium sulphate. Evaporation of the ether into a small weighed flask followed by vacuum desiccation gives the weight of acid material extracted by the carbonate. The weight may be checked by titration against 0.01 N-alkali. However, the weighing and vacuum desiccation may be dispensed with by following the course of the extraction by titration alone. Since the oil thus extracted is not rapidly soluble in dilute alkali it is convenient to dissolve it in a few ml. of acetone before titration, then to titrate the acetone solution. Thymol blue has been found to be a suitable indicator for use in the presence of the brown-coloured solutions obtained. With experience, the determination of the acid material in the carbonate layer can be dispensed with, the colour of the carbonate extract being a sufficient guide.

(2) *Reaction with maleic anhydride.* The anisole solution thus exhaustively extracted with sodium carbonate is now ready for reaction with maleic anhydride. Solid maleic anhydride (1.0 g.) is added, and the solution agitated at room temperature overnight. Excess maleic anhydride is then removed by extracting with water in a similar manner to the initial carbonate extractions—15 ml. distilled water are added, the solution shaken for 10 min. and the water layer separated. Here again, warming is an aid to the rapid separation of the anisole and aqueous layers. The process is repeated with short hand washings until the extraction is complete as ascertained by titration of the separated water layer against 0.01 N-alkali; usually 5-6 water washings are sufficient.

(3) *Final sodium bicarbonate extraction.* The anisole solution thus exhaustively extracted with water is now extracted with 15 ml. saturated aqueous bicarbonate overnight, followed by two 15 ml. bicarbonate portions for 2 hr. each. The combined bicarbonate layers are made acid to congo red, with dilute hydrochloric acid, steam distilled for 30 min., the steam distillate being discarded, and then extracted with ether, and dried over sodium sulphate. The steam distillation removes the sodium bicarbonate soluble substances in the blank which interfere with the colour reaction described in the next section. After removal of the ether by distillation from a small weighed flask, the residue is vacuum-desiccated and weighed. If x is the weight of adduct recovered, then the original weight of dienoestrol = $266x/384$.

Experimental errors. Some losses of dienoestrol obviously occur during the preliminary extractions with sodium carbonate and water, but since the recoveries reached are approximately 60-70% of added dienoestrol where 3-20 mg./l. urine were present, they can be allowed for.

A more serious error becomes apparent when the dienoestrol concentration in the urine falls below about 3 mg./l. since there is a final blank yield of 0.8-1.2 mg./l. of acid-hydrolyzed urine. It is possible to reduce this blank when acidified, but unhydrolyzed, urines are used, but the method is obviously unsuitable where conjugated dienoestrol is present. The difficulty has been overcome by making use of the fact that the dienoestrol-maleic anhydride adduct couples with diazotized sulphanilic acid to give a red dye with absorption in the 480-510 m μ band. The blank gives little colour on coupling with diazotized sulphanilic acid provided the steam distillation previously referred to is carried out, so that by use of the Spekker absorptiometer,

the blank is eliminated and at the same time the method is made sensitive to less than 500 $\mu\text{g.}$ total dienioestrol present.

(b) *For concentrations of dienioestrol below 2 mg./l. urine: application of Spekker absorptiometer*

The urine is extracted and worked up exactly as under (a) but owing to the blank, the final weighing will give a high result. The final material is therefore made up to 5 ml. with 2N-NaOH and 1 ml. of it coupled with 2 ml. of diazotized sulphanilic acid; after 5 min. at room temperature the solution is diluted with 10 ml. N-NaOH; after a further 5 min. the Spekker reading is taken. Ilford spectrum green filters (510–620 $m\mu$) are used. From a graph constructed from readings with known additions of adduct, the amounts present in unknown samples may be determined.

Table 1. *Recovery of dienioestrol added to normal male urine*

Sample	Weight of dienioestrol added (mg./l.)	Weight of dienioestrol found (mg./l.)	
		Gravimetrically	Colorimetrically
1	77.8	62.2	—
2	60.0	42.2	—
3	8.33	9.5	7.5
4	6.5	5.4	8.0
5	5.5	4.1	5.9
6	5.0	5.8	5.5
7	5.0	4.8	5.0
8	4.0	5.3	3.1
9	4.0	4.1	4.0
10	4.0	2.1	3.4
11	3.33	3.2	2.3
12	3.33	2.1	2.3
13	3.0	4.8	3.8
14	2.0	1.1	1.0
15	1.49	1.8	0.8
16	1.0	1.6	0.6
17	1.0	1.6	0.6
18	0.4	1.8	0.4
19	0.2	1.7	0.2
20	0.2	0.9	0.2
21	0.1	1.1	0.1
22	0.1	1.1	0.2
23	0.0	0.7	0.0
24	0.0	0.6	0.0
25	0.0	1.5	0.0

Preparation of diazobenzenesulphonic acid solution. 1.0 g. sulphanilic acid is dissolved in 8.0 ml. N-NaOH and 50 ml. distilled water added. The solution is then acidified with 7.0 ml. 36% (w/w) hydrochloric acid and cooled to 0° in ice. 8.0 ml. 10% sodium nitrite solution at 0° are added, the solution made up to 100 ml. with distilled water. 2.0 ml. of this solution are used for each determination.

Preparation and properties of dienioestrol-maleic anhydride adduct (4:5-di-(p-hydroxyphenyl)-3:6-dimethyl- Δ^4 -tetrahydrophthalic anhydride)

2.66 g. dienioestrol (0.01 mol.) and 1.96 g. maleic anhydride (0.02 mol.) are refluxed together for 5 min. in 150 ml. benzene with just sufficient acetone added to effect solution, and then concentrated to one-third volume and set aside to crystallize. The crystalline product contains one molecule of benzene of crystallization which is readily lost on exposure to the atmosphere. After four crystallizations from benzene and acetone m.p. 197–200° (yield: 95%). (Found: C, 72.4; H, 5.6. $\text{C}_{22}\text{H}_{20}\text{O}_5$ requires C, 72.6; H, 5.5%.)

RESULTS

Table 1 shows the results of several determinations of dienioestrol added to normal male urine and Fig. 1 shows the variation of Spekker reading with concentration of dienioestrol-maleic anhydride adduct.

Fig. 1. Colour development of dienioestrol-maleic anhydride adduct with diazotized sulphanilic acid.

SUMMARY

A method for the determination of dienioestrol in urine has been evolved depending on the formation of its adduct with maleic anhydride. The adduct may be recovered and weighed, or determined colorimetrically, according to the amount present. High recoveries of added dienioestrol over a range of 0.2–80 mg./l. are obtained.

The preparation and properties of the adduct are briefly described.

We are indebted to the Council of the Middlesex Hospital Medical School and to Prof. Dodds for the provision of laboratory facilities, and to the Medical Research Council for a grant for our assistant Mr B. D. Shepherd.

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