The Excretion of Synthetic Oestrogens as Ethereal Sulphates and Monoglucuronides in the Rabbit and in Man

By S. A. SIMPSON AND A. E. WILDER SMITH (Countess of Lisburne Memorial Fellow)
Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W. 1

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Mazur & Schorr (1942) found that when stilboestrol (4:4'-dihydroxy- $\alpha\beta$ -diethylstilbene) was administered to the rabbit the main product excreted in the urine was stilboestrol monoglucuronide, in amounts corresponding to about 30% of the dose. This finding was confirmed by Wilder Smith & Williams (1948) and extended to hexoestrol and dienoestrol. Prof. R. T. Williams (private communication) found that, in the rabbit, probably up to about 10% of administered synthetic oestrogen was excreted as the ethereal sulphate. This figure was based on increases of total urinary ethereal sulphate on administering synthetic oestrogen, increases which were, however, not always unequivocal.

In man, the excretion of stilboestrol as the monoglucuronide in substantial amounts was reported by Dodgson & Williams (1948) and by Wilder Smith (1947), whilst detection of a small excretion as the ethereal sulphate was claimed by Wilder Smith (1947).

Further experiments on excretion of synthetic oestrogens by the rabbit and by man are reported here together with the methods used for separation and assay of the ethereal sulphate, monoglucuronide and free oestrogen in urines.

METHODS

In the case of man the oestrogen was given in the form of tablets by mouth and the urine collected for 48 hr. and preserved with toluene. In the rabbit the oestrogen was given in solution in ethyl lactate by stomach tube; the urine was collected for 4 days and preserved with toluene. In both cases the urines were stored at 3° until use. The period of storage never exceeded 1 week.

The method we have used for the ethereal sulphate and other determinations has the disadvantage that it is dependent on bioassay. It has been found that if slightly acidified urine or water containing hexoestrol, hexoestrol disulphate and hexoestrol monoglucuronide is extracted with ether the hexoestrol and hexoestrol monoglucuronide pass almost quantitatively into the ether layer. They may be separated from each other by extraction of the ether solution with aqueous NaHCO_3 , in which the glucuronide is soluble, followed by dilute aqueous NaOH in which the hexoestrol is soluble. The urine or water remaining contains the ethereal sulphate from which the free oestrogen may be liberated by hydrolysis by acid; the amount of the ethereal sulphate originally present was determined by bioassay.

In order to carry out the preliminary recovery experiments it was necessary to prepare the pure monoglucuronide and disulphate of hexoestrol. The monoglucuronide was obtained from the metabolism experiments reported previously (Simpson & Wilder Smith, 1948) while the disulphate (3:4-diphenylhexane-4':4''-disulphuric acid) was prepared by treating the free phenol at -10° in carbon disulphide and dimethylaniline with chlorosulphonic acid (cf. Burkhardt & Lapworth, 1926; Short & Oxley, 1946) followed by crystallization from 2N-NaOH.

Preliminary recovery experiments

(a) *Preparation of solutions.* The synthetic mixtures for test were made up by weighing out the hexoestrol monoglucuronide and the sodium salt of hexoestrol disulphate and adding them to a known volume of water or urine. The free oestrogen was weighed out and dissolved in a known volume of ethanol; the required portion was then pipetted from this solution directly into the centre of the prepared aqueous solution of sulphate and monoglucuronide. Care was taken to prevent precipitation losses due to the insolubility of the free oestrogen.

Hexoestrol and its derivatives were used in preference to those of stilboestrol or dienoestrol on account of their greater stability (Wilder Smith & Williams, 1947).

(b) *'Working up' procedure.* The sample was acidified with HCl until just acid to Congo-red paper and was then well shaken three times with equal volumes of ether. Foams were broken by adding traces of surface-active agents (e.g. Cal-solene). In general, the use of fairly large quantities of ether tended to reduce emulsion formation. Residual foams after this treatment were broken by filtration. Salt may not be used to break foams since the aqueous layer left after ether extraction is used directly for bioassay after suitable dilution.

After the three hand extractions the aqueous layer was extracted for 24 hr. in a continuous ether extractor and the combined extracts evaporated to dryness, finally under reduced pressure. The residue was treated with 40 ml. saturated aqueous NaHCO_3 solution and the resultant mixture extracted with 3×40 ml. ether. The bicarbonate layer, containing the monoglucuronide, was made acid to Congo-red paper with HCl, extracted with 5×40 ml. ether, the ether removed by evaporation and the residue made up to 100 ml. with acetone for bioassay.

The ethereal solution which had been extracted with bicarbonate solution was then washed three times with 2N-NaOH and the aqueous layer, containing the free oestrogen, acidified to Congo-red paper, extracted with ether three times and the ether extract made up in the same way for bioassay as the ether extract from the acidified bicarbonate layer.

The remaining original aqueous layer (containing the ethereal sulphate) from the continuous ether extraction, was refluxed for 1 hr. after the addition of 1% by volume of concentrated HCl, neutralized with dilute NaOH and assayed on rats directly after suitable dilution with water. Where the concentration of oestrogen present was too low for direct assay after this procedure, the solution was extracted with ether and the concentrated extract assayed.

(c) *Bioassay*. The extracts of free oestrogen and monoglucuronide in standard volumes in ethanol or acetone were diluted with water to give doses corresponding to recoveries of 10, 25, 50, 75 and 100%. Thus a rough range of recovery efficiency was obtained which was later narrowed by intermediate dosing.

In the case of ethereal sulphate excretion in actual metabolism experiments much lower dose levels were required than in the case of the recovery experiments. In these cases the two nearest dose levels giving 80–100% and 0% response on five rats were reported in the Tables since the total excretion was so low that it was obviously not worth while narrowing the range to give figures which would allow of statistical treatment. The procedure of subcutaneous injection and vaginal smearing was the standard one used in this Institute (Wilder Smith & Williams, 1948). It will be seen that by this method only very approximate figures can be given.

Table 1. *Recovery of hexoestrol, hexoestrol monoglucuronide and sodium hexoestrol disulphate from water and urine**

Medium used	Recovery of hexoestrol (1 mg.) (%)	Recovery of Na hex-oestrol disulphate (10 mg.) (%)	Recovery of hexoestrol monoglucuronide (10 mg.) (%)
Water (1 l.)	67 (49–91)	48 (33–71)†	80 (63–105)
Normal male urine (1 l.)	91 (77–117)	65 (51–84)	104 (89–124)
Normal male urine (1 l.)	88 (73–106)	74 (64–85)	—
Normal male urine (1 l.)	103 (79–135)	82 (57–114)	108 (90–129)

* The mean responses and fiducial limits (in parentheses) were determined by the methods given in Fisher & Yates (1943).

† The low figure was due to insufficient hydrolysis.

RESULTS

Table 1 shows the results of some recovery experiments of hexoestrol monoglucuronide, hexoestrol disulphate and hexoestrol added to water and to urine. Tables 2 and 3 give the results of metabolism

Table 2. *Ethereal sulphate excretion of synthetic oestrogens in the rabbit*

Rabbit no.	Dose (mg.)	Excretion as ethereal sulphate* (%)	Monoglucuro- nide excretion for comparison† (%)
Stilboestrol			
R 30	500	0.04-0.2	(30-40)
R 30	500	0.04-0.2	
R 30	240	0.04-0.4	
R 30	240	0.08-0.4	
R 20	275	0.1-0.4	
R 20	275	0.04-0.1	
Hexoestrol			
R 8	500	0.1-0.5	(30-46)
R 9	500	0.05-0.1	
Dienoestrol‡			
R 6	500	0.1-0.5	(24-48)
R 7	500	0.1-0.5	
R 8	400	0.2-0.5	
R 9	525	0.2-0.5	

* The lower percentage quoted gave 80–100% response in five rats, while the higher figure gave 0% response in five rats; these figures are not fiducial limits.

† Cf. Wilder Smith & Williams (1948).

‡ The bioassay calculation for ethereal sulphate was made on the basis that the dienioestrol was cyclized to the corresponding indene during acid hydrolysis (cf. Hausmann & Wilder Smith, 1948).

experiments carried out in man and the rabbit. Excretion of synthetic oestrogens as glucuronides in the rabbit has been reported previously (Mazur & Schorr, 1942; Wilder Smith & Williams, 1948).

Table 3. *Ethereal sulphate and monoglucuronide excretion of synthetic oestrogens in man*

Case	Patient no.	Oestrogen used	Dose (mg.)	Percentage* excreted		
				As ethereal sulphate	As mono-glucuronide	As free oestrogen
Prostatic cancer	1	Stilboestrol	25	1–5	50–60	1–2
"	1	"	20	3–9	10–25	1–5
Normal woman	4	Hexoestrol	5	1–5	75–100	10
"	4	"	15	<0.5	25–50	<1
"	5	"	5	<1.5	25–50	1–5
"	5	"	50	<0.2	10–20	<1
"	6	"	10	0.1–1	30–40	0.1–1
"	7	"	10	0.1–1	25–50	0.1–1

* The lower percentage quoted gave 80–100% response in five rats, while the higher figure gave 0% response in five rats; these figures are not fiducial limits.

DISCUSSION

In preliminary experiments it was found that, if the continuous ether extraction of the urine or water was continued for much more than 24 hr., low recoveries of sulphate with correspondingly higher recoveries of free oestrogen were obtained. This is due to the fact that under the acid conditions of the experiment, the ethereal sulphate is hydrolyzed to the free oestrogen which is then extracted by the ether. The somewhat high apparent recoveries of glucuronide are due to the slight solubility of free oestrogen in saturated aqueous bicarbonate. The high biological activity of the free oestrogen compared with that of the glucuronide makes noticeable the presence of even a trace of free oestrogen. During preliminary experiments also it was found that low sulphate figures resulted unless care was taken to ensure complete hydrolysis of the ethereal sulphate before bioassay.

From Table 2 it is seen that the ethereal sulphate excretion in the rabbit was very low. Although the method was worked out for hexoestrol and its derivatives the similarity in chemical and physical properties of stilboestrol and its derivatives make it likely that the method can be applied in their case also without much probability of serious error. In the case of dienoestrol it is necessary to make an additional correction to compensate for the conversion of dienoestrol to the corresponding indene (Hausmann & Wilder Smith, 1948) under the acid conditions used for the hydrolysis of the ethereal sulphate.

It can be seen from Table 3 that large variations in glucuronide and ethereal sulphate excretion occur in man as in the case of the rabbit. Owing to the small dosage of oestrogen given in the case of man the experimental errors are likely to be larger. Nevertheless, it is quite clear that the excretion as monoglucuronide in man is much larger than the excretion as free oestrogen or ethereal sulphate.

In both the human subject and in the rabbit fairly high recoveries of monoglucuronide are obtained, both by our method and that of Dodgson & Williams (1948), but we have consistently found that, if the urine of patients treated with stilboestrol or hexoestrol is hydrolyzed under conditions known to give high yields of the free oestrogen

from the glucuronide, only about 10–15% of the original oestrogen administered can be detected afterwards as the free oestrogen. Thus, urines showing the presence of about 40–50% of the original oestrogen as monoglucuronide by the method given above will, on hydrolysis and redetermination as free oestrogen in the crude urine, show only about 10–15% present. Although it is known that the monoglucuronide figure determined by our method tends to be high, yet this is scarcely sufficient to account for the discrepancy, for which we can offer at present no other explanation than to suggest that some other oestrogen, soluble in sodium bicarbonate, may be present which has a higher oestrogenic potency than that of the monoglucuronide.

The figures reported show that the general pattern of excretion of synthetic oestrogens is similar both for the rabbit and man in that the vastly increased doses used in animals for these metabolism experiments do not give a substantially changed picture from that obtained in therapeutic dosing in man.

SUMMARY

1. A method of separating the ethereal sulphates of certain synthetic oestrogens from their monoglucuronides and free forms is described.

2. A small excretion of synthetic oestrogen as ethereal sulphate has been found in both man and the rabbit.

3. The method used for working up gives reasonably quantitative results on known synthetic mixtures in urines, so that there is little doubt that the comparatively low overall recoveries obtained in metabolism experiments are not due entirely to inadequate technique, but do indicate a real metabolic breakdown.

4. Results obtained by determination of the metabolized oestrogen as the monoglucuronide are consistently higher than those obtained by hydrolysis of all conjugated forms of oestrogen present followed by determination as free oestrogen. A possible explanation for this is offered.

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