Determination of Oestrogen Monoglucuronides by the Brown Method

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In the Brown (Brown, 1955; Brown, Bulbrook & Greenwood, 1957) procedure for the determination of urinary oestrogens, losses may occur during the hydrolysis of the oestrogen conjugates and during the purification of the oestrogens thus formed. Losses during the latter stages were measured previously by adding free oestrogens to the hydrolysed urine. In the present study losses during acid and enzyme hydrolysis were measured by adding synthetic oestrogen monoglucuronides to urine.

During the last 10 years, the method of Brown (Brown, 1955; Brown, Bulbrook & Greenwood, 1957) for the measurement of urinary oestrone, oestradiol and oestriol has become widely accepted. Though the accuracy of the method has been examined in detail, the lack of pure oestrogen conjugates made it difficult to assess the overall recovery. An attempt was made to determine losses by measuring the recovery of free oestrogens added after hydrolysis and by assessing, indirectly, losses during the hydrolysis step. Thus Brown & Blair (1958) established optimum conditions for the hydrolysis of urinary conjugates by hot-acid hydrolysis and by enzymolysis. They concluded that under hot-acid conditions 20% of the oestrogen was destroyed; no loss was observed during enzyme hydrolysis.

More detailed information is now available about the forms of conjugation of oestrogens in pregnancy urine. Oestrone has been found as the 3-glucuronide (Beling, 1963) and as the 3-sulphate (McKenna, Menini & Norymberski, 1961). Oestradiol has been identified as the 17 β -glucuronide (Smith & Kellie, 1967), but, though there is circumstantial evidence of the excretion of an oestradiol sulphate (McKenna *et al.* 1961), the position of conjugation has not been established. Oestriol 16 α -glucuronide is the major oestrogen conjugate of late-pregnancy urine (Neeman & Hashimoto, 1962), but significant amounts of oestriol 3-glucuronide are also excreted (Beling, 1963; Goebelsmann, Sjöberg, Wiquist & Diczfalussy, 1965). Oestriol 3-sulphate has been reported as a component of pregnancy urine (Hähnel & Ghazali bin Abdul Rahman, 1967). Several workers have reported the excretion of the bis-conjugate of oestriol, oestriol 3-sulphate 16 α -glucuronide (Straw, Katzman & Doisy, 1955; Troen, Nilsson, Wiquist & Diczfalussy, 1961; Diczfalussy, Barr & Lind, 1964). The presence of an

oestriol bisglucuronide in commercial preparations of oestriol glucuronide has been reported (Felger & Katzman, 1961).

All the theoretically possible monoglucuronides of oestrone, oestradiol and oestriol have now been synthesized (Elce, Carpenter & Kellie, 1967) and these have now been used to test the validity of earlier conclusions.

METHODS

Oestrogen monoglucuronides. The following compounds were used as the sodium salts: oestrone 3-glucuronide, oestradiol 3-glucuronide, oestradiol 17 β -glucuronide, oestriol 3-glucuronide, oestriol 16 α -glucuronide, oestriol 17 β -glucuronide. Though the samples were all white crystalline solids, the analytical data (% C and H) were not all satisfactory (Elce, 1965); it is recognized that steroid glucuronides frequently crystallize with the incorporation of solvent molecules (Schapiro, 1939; Wotiz, Smakula, Lichtin & Leftin, 1959).

Acid hydrolysis. Hydrolysis was achieved by boiling the urine under reflux with conc. HCl (30 ml./200 ml. of urine) for 60 min. (Brown & Blair, 1958).

Enzymic hydrolysis. The conditions were those recommended previously, except that an extract from *Helix pomatia*, which contains both β -glucuronidase and a phenol sulphatase, replaced the enzyme preparation from *Patella vulgata* used by Brown & Blair (1958). The hydrolysis was carried out at pH 4.7 by incubating with 600 units of β -glucuronidase/ml. for 96 hr. at 37°.

Extraction and measurement of the hydrolysed oestrogens were as described by Brown (1955) or Brown *et al.* (1957).

RESULTS

Comparison of the Kober colour intensity of oestrogen monoglucuronides and free oestrogens

Standard solutions of the oestrogen monoglucuronides were prepared by weighing the solids and dissolving in 30% (v/v) ethanol, and portions

Table 1. *Comparison of the Kober colour intensity of oestrogen glucuronides and the molar equivalent of free oestrogen*

Oestrogen glucuronide	Wt. (μ g.)	Corrected <i>E</i> value	
		Glucuronide	Free oestrogen (molar equivalent)
Oestrone 3-glucuronide	9.7	0.369	0.360
	19.4	0.748	0.720
Oestradiol 3-glucuronide	8.4	0.252	0.280
	16.8	0.507	0.570
Oestradiol 17 β -glucuronide	7.76	0.238	0.261
	15.5	0.470	0.525
Oestriol 3-glucuronide	8.16	0.179	0.198
	16.32	0.360	0.384
Oestriol 16 α -glucuronide	8.4	0.178	0.190
	16.8	0.350	0.380
Oestriol 17 β -glucuronide	10.4	0.222	0.236
	20.8	0.440	0.474

Table 2. *Comparison of the percentage recoveries of oestrogen glucuronides added to urine before enzymic hydrolysis with those of free oestrogens added after hydrolysis*

Results are shown as means $\pm$ s.d. with the numbers of determinations in parentheses, and are corrected for the endogenous blank values.

Oestrogen glucuronide	Recovery (%)		
	Oestrone	Oestradiol	Oestriol
Oestrone 3-glucuronide	81 $\pm$ 4.2 (12)	—	—
Oestradiol 3-glucuronide	—	81 $\pm$ 3.6 (12)	—
Oestradiol 17 β -glucuronide	—	82 $\pm$ 3.2 (12)	—
Oestriol 3-glucuronide	—	—	77 $\pm$ 5.1 (11)
Oestriol 16 α -glucuronide	—	—	78 $\pm$ 4.3 (11)
Oestriol 17 β -glucuronide	—	—	75 $\pm$ 4.9 (11)
Free oestrogen			
Before enzyme hydrolysis	80 $\pm$ 5.5 (7)	81 $\pm$ 3.2 (7)	81 $\pm$ 2.9 (16)
After enzyme hydrolysis	82 $\pm$ 2.6 (4)	84 $\pm$ 3.4 (4)	79 $\pm$ 2.3 (7)

containing 8–20 μ g. were determined by the Kober colour reaction. The corrected extinction values given by the conjugates are shown in Table 1 and are compared with those produced by the equivalent amounts of pure oestrogens. The results are the means of triplicate determinations and show reasonable agreement between the extinction values produced by the glucuronides and the equivalent amounts of free oestrogen. It follows that the Kober determination can be applied directly to measure the oestrogen content of monoglucuronide solutions.

Recovery of oestrogen glucuronides added to urine

Oestrogen monoglucuronides (15–20 μ g.) were added to samples of normal female urine (200ml.) representing one-quarter of a 24hr. sample. The urine was then subjected to hydrolysis and the

oestrogen content was determined by the method of Brown (1955). The recoveries were corrected for endogenous oestrogen.

Acid hydrolysis. The percentage recoveries of oestrogens from the added monoglucuronides were as follows: oestrone 3-glucuronide, 75 $\pm$ 8.6% (11); oestradiol 3-glucuronide, 60 $\pm$ 4.6% (9); oestradiol 17 β -glucuronide, 80 $\pm$ 4.5% (12); oestriol 3-glucuronide, 61 $\pm$ 3.7% (12); oestriol 16 α -glucuronide, 69 $\pm$ 8.5% (12); oestriol 17 β -glucuronide, 58 $\pm$ 4.0% (10). These results are expressed as means $\pm$ s.d. with the numbers of determinations in parentheses. The loss of oestrogen represents the sum of losses occurring during hydrolysis and losses during the preparation of the material for Kober measurement.

Enzymic hydrolysis. The percentage recoveries of oestrogens from the added oestrogen monoglucuronides when enzyme hydrolysis was used are shown in Table 2. For comparison Table 2 also

includes data on the recoveries of free oestrogens added before and after enzymic hydrolysis taken from the paper by Brown & Blair (1958).

DISCUSSION

The main purpose of the present work was to test earlier conclusions on the efficiency of hydrolysis steps used in methods for measuring oestrogens in human urine. Though losses arise both from the hydrolysis step and from the subsequent work-up, no attempt was made to study or change the latter.

The overall recovery of oestrogen added as glucuronides before acid hydrolysis and processed by the method of Brown (1955) was 58–80%. As oestriol 17 β -glucuronide, if present in urine, is only a trace component, perhaps a more realistic range would be 60–80% (Smith & Kellie, 1967) (Table 1). Brown & Blair (1958) assessed the recovery as 60–75%, and though the components of this range were not investigated it is likely that hydrolysis was complete. These losses represent destruction during hydrolysis and work-up loss.

The recovery of oestrogens added as glucuronides before enzymic hydrolysis was 75–82% (77–82% excluding oestriol 17 β -glucuronide), a range that is very similar to that of free oestrogens added after hydrolysis, namely 79–84% (Table 2). This finding suggests that the enzymic hydrolysis of the glucuronides is complete and that no destruction of the oestrogen occurs during this step.

The colour intensity given by the oestrogen conjugates in the Kober determination agreed closely, on a molar basis, with that of corresponding free oestrogens. This confirms the purity of the

glucuronides used and indicates that the Kober reaction can be applied directly to oestrogen conjugates without preliminary hydrolysis.

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