Hydrolysis of Pregnanediol Glucuronide by Sodium Periodate

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(Received 17 February 1967)

Pregnanediol (5 β -pregnane-3 α ,20 α -diol) is the major metabolite of progesterone found in human urine, and is present as a glucuronide. Hydrolysis of the glucuronide is an essential preliminary to the satisfactory determination of pregnanediol. This is usually achieved by enzymic or hot acid hydrolysis. Both methods have drawbacks. The enzyme can be expensive, or the raw material (e.g. limpets) difficult to obtain, and hot-acid hydrolysis leads to the formation of compounds that interfere with the estimation of pregnanediol if not eliminated by additional purification steps (Klopper, Michie & Brown, 1955). Norymberski & Sermin (1953) showed that treatment of a steroid glucuronide with sodium bismuthate gave a mixture of the parent alcohol and its formate, and Morris (1959) took advantage of this fact in his method for estimating urinary pregnanetriol (5 β -pregnane-3 α ,17 α ,20 α -triol). Similar considerations have been applied to the determination of urinary pregnanediol, substituting sodium periodate for sodium bismuthate. The advantages of sodium periodate over sodium bismuthate as an oxidizing agent have been given by Few (1961).

Method. The oxidation procedure is based on that of Metcalfe (1963). An aqueous solution of sodium metaperiodate (10%, w/v) (4.0 ml.) was added to urine (10 ml.) and the pH adjusted to 3.5 with N-HCl. The mixture was incubated at 50–60° for 40 min., aq. 5N-NaOH (1.0 ml.) added and the incubation continued for a further 15 min. After cooling, the mixture was extracted with benzene (3 $\times$ 20 ml.). The benzene solution was washed with 3N-NaOH (10 ml.) and water (3 $\times$ 10 ml.), filtered and evaporated to approx. 5 ml. Purification of the pregnanediol by column chromatography, the preparation of a derivative and colorimetric estimation were carried out as described by Klopper *et al.* (1955).

Results and discussion. The optimum pH for the oxidation of a steroid side chain by periodate is 6.5–7.0 (Few, 1961). At this pH, with pregnancy urine, the recovery of pregnanediol was only half that obtained after enzyme hydrolysis. Lowering the pH increased the recovery, the maximum yield, comparable with that after enzyme hydrolysis, being obtained at pH 3.5. Below this, recovery again fell. Under these conditions of oxidation pregnanediol was shown to be completely stable.

The time of oxidation was not critical above 30 min. After 10 min. 80% oxidation had occurred, and after 20 min. 91%. Between 30 and 60 min. complete oxidation took place. Recovery experiments were carried out by adding a known amount of pregnanediol to urine or water: recovery of pregnanediol from urine was satisfactory (mean 85% S.D. $\pm$ 15, range 65–109%). The precision of the method was determined from duplicate determinations, the standard deviation between duplicates being calculated by the method of Snedecor (1952). This was 0.19 mg./24 hr. Estimates ranged from 0.5 mg. to 64 mg./24 hr. Estimation after enzyme hydrolysis gave a standard deviation of 0.27 mg./24 hr. between duplicates. The specificity of the method was demonstrated by chromatography of the pregnanediol residue from the first alumina column on a thin layer of silica in the system chloroform–ethanol (19:1, v/v), and spraying with conc. H₂SO₄. A single spot corresponding to standard pregnanediol was found (*R_F* 0.52), together with spots near the solvent front. These latter however were also found in a blank after oxidation and column chromatography. The residue from the second alumina column was likewise chromatographed on a thin layer of silica, in the system ethyl acetate–benzene (1:9, v/v) with standard pregnanediol diacetate. Spraying with conc. H₂SO₄ showed both unknown and standard had the same *R_F* (0.48). As further proof of specificity, the absorption spectrum of the colour given by the diacetate after reaction with H₂SO₄ was closely similar to that given by pure pregnanediol diacetate.

There appeared to be no advantage in treating the urine with borohydride before oxidation, as in the estimation of 17-hydroxy corticosteroids. The 17-oxo steroids normally present and those formed after periodate oxidation do not interfere with the subsequent estimation of pregnanediol, being removed during the first chromatography. The interference of compounds that before oxidation have similar chromatographic properties to pregnanediol (e.g. 5 α -pregnane-3 β ,17 α ,20 β -triol and Δ^5 -pregnene-3 β ,17 α ,20 β -triol; Leon & Bulbrook, 1960), but that are oxidized to 17-oxo steroids, is also eliminated.

Table 1 gives the results of a comparison of periodate hydrolysis with enzyme hydrolysis. For enzyme hydrolysis, urine (10 ml.) was adjusted to

Table 1. *Urinary pregnanediol determined after periodate hydrolysis and enzyme hydrolysis*

Urine source	Urinary pregnanediol (mg./24hr.)	
	Periodate hydrolysis	Enzyme hydrolysis
Normal female	3.17	2.02
	1.25	0.91
	1.10	1.07
	0.52	0.50
	2.91	2.61
	3.54	3.45
	0.70	0.67
	1.51	1.85
	1.33	0.72
	0.82	0.63
Gonadotrophin treatment for infertility	0.47	0.47
	0.66	0.66
	0.37	0.95
	0.50	0.71
	1.00	1.13
	0.90	1.20
	0.92	0.74
	0.75	1.12
Secondary amenorrhoea	0.55	0.55
Pregnancy	33.2	31.6
	46.9	43.7
	51.7	64.1
	11.8	9.2
	28.6	30.1

pH 4.0 with acetic acid, and 1.1 ml. of 5M-acetate buffer, containing 15mg. (15000 units) of limpet β -glucuronidase, was added. The mixture was incubated overnight at 37°, cooled and extracted with benzene (3 × 20 ml.). The combined benzene extracts were washed with 3N-NaOH (10 ml.) and water (3 × 10 ml.), filtered and evaporated to

approx. 5 ml. Purification and estimation were as described by Klopper *et al.* (1955). Both methods give similar results.

While this work was in progress, Vanzetti & Valente (1966) published details of a method for pregnanediol determination that also used periodate oxidation for removing glucuronide. They also used acidic conditions (pH 4.5). Subsequent purification, however, was less satisfactory, the authors relying on the precipitation of pregnanediol from aqueous solution, followed by an H₂SO₄ reaction. The purification procedure of Klopper *et al.* (1955) is clearly superior, and this method, or modifications of it, has been generally adopted.

Inhibitors of β -glucuronidase activity are present in urine (Levy, 1956), a fact often advanced against the adoption of enzyme hydrolysis. On small-scale estimations (10 ml. of urine) the amount of enzyme used may be increased, or the glucuronides may be first extracted from urine (Edwards, Kellie & Wade, 1953), minimizing the risk of interference by inhibitors.

Periodate hydrolysis makes either of these procedures unnecessary.

The technical assistance of Miss Sheena Wallace is gratefully acknowledged.

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