Journal of Steroid Biochemistry, 1976, Vol. 7, pp. 661–664. Pergamon Press. Printed in Great Britain

ISOLATION OF TETRAHYDROALDOSTERONE 3β-GLUCOSIDURONIC ACID FROM URINE

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(*Received* 16 *January* 1976)

SUMMARY

Crystalline tetrahydroaldosterone 3β-glucosiduronic acid has been isolated from human urine, its structure has been determined, and several derivatives of it have been prepared and characterized.

INTRODUCTION

Tetrahydroaldosterone 3β-glucosiduronic acid* is the major urinary metabolite of aldosterone. Our knowledge concerning the structure of this compound, however, is based mainly on indirect evidence [1,2]. The intact conjugate has been characterized chromatographically by Pasqualini *et al.* [3], by Mohring [4], and by Grose and associates [5], but it has not been studied in detail.

In this paper we describe the isolation and characterization of crystalline tetrahydroaldosterone 3β-glucosiduronic acid from urine following the oral administration of d-aldosterone to normal subjects.

EXPERIMENTAL

The sources of chemicals, purification of solvents, techniques for chromatography, etc., have been described previously [6]. The following chromatography systems (composition by vol.) were employed.

1. Butyl acetate–toluene–methanol–water–acetic acid (60:40:50:45:5 by vol.)
2. Cyclohexane–benzene–methanol–water (5:2:5:1 by vol.)
3. KCl (0.40 M) in water/Aliquat 20% w/w on Celite
4. Butyl acetate–toluene–butanol–methanol–water–acetic acid (50:40:10:50:45:5 by vol.)
5. Isooctane–*t*-butanol–methanol–water (60:40:10:35 by vol.)
6. Benzene–methanol–water (40:20:10 by vol.)
7. KCl (0.20 M) in water/Aliquat 0.050 M (in chloroform)
8. Ethyl acetate–acetic acid (98:2 v/v)—ethylene glycol
9. Ethyl acetate–methanol–acetic acid (150:50:10 by vol.)
10. Benzene–acetone (30:10 v/v)
11. Ethyl acetate–isooctane (20:10 v/v)

Column chromatography. For systems 1 and 2, Celite 545 was impregnated with the more polar phase of the solvent system (0.50 ml polar phase/g Celite) and the less polar layer was used as mobile phase. System 3 was run by reverse phase technique as described for system N [6] except that 0.40 M KCl was used.

Paper chromatography. Bush technique was used with systems 4, 5 and 6, reverse phase technique [6] with system 7, and Zaffaroni technique with system 8.

Thin-layer chromatography (t.l.c.). Systems 9, 10 and 11 were used with unactivated silica gel G.

Isolation of 11β,18-epoxy-18,21-dihydroxy-20-oxo-5β-pregnan-3α-yl β-D-glucopyranosiduronic acid (**1**). After approval by the Human Studies Committee, Mayo Clinic, each of 4 normal subjects received 150 mg of D-aldosterone orally; one received, in addition, 65 μCi of [1,2,6,7-^{3}H]aldosterone. All urine passed during the first 12 h following ingestion of the aldosterone was pooled and its radioactivity was determined: 80% of the administered radioactivity was recovered. The urine was desalted on a column of Amberlite XAD-2, the effluent was extracted with chloroform and the aq. fraction was chromatographed on DEAE Sephadex A-25 and subsequently desalted on Amberlite XAD-2 to obtain a fraction consisting principally of steroidal glucosiduronic acids (85% of the urinary radioactivity). This fraction was chromatographed on a column as described in detail elsewhere [6]; the less polar peak (4.4 V_0) contained aldosterone 18β-glucosiduronic acid [6]; the more polar peak (8.8 V_0) contained tetrahydroaldosterone 3β-glucosiduronic acid **1** (which is described in this paper). The pooled material of the more polar peak (8.8 V_0) was concentrated and the residue was chromatographed on a column (274 g of Celite and 54 g of Aliquat) in system 3. The first peak (6.8 V_0) contained tetrahydroaldosterone 3β-glucosiduronic

*Trivial names are used in the discussion; systematic names in the experimental section.

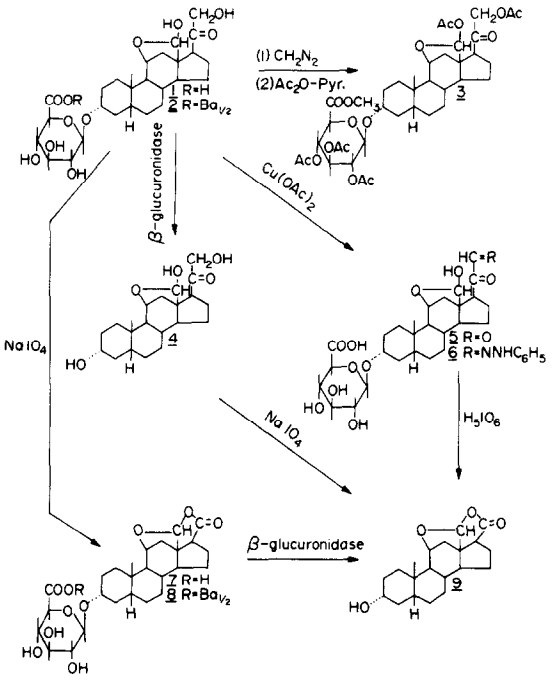

Fig. 1.

acid (22 μCi, 34% of the radioactivity administered to the subjects; 85% of the radioactivity placed on the column, 4.9×10^7 d.p.m.) and the second peak (10.3 V_0) contained additional tritium-labeled material (15% of the radioactivity) which was not investigated.

The aq. solution (6.8 V_0) was extracted with dichloromethane to remove a trace of ion exchanger (Aliquat) and desalted on 100 g of Amberlite XAD-2. An aliquot of the residue from the ethanolic eluate gave crystals of **1** (21 mg, 1.90×10^5 d.p.m./mg) from acetone–ethyl acetate. The product was homogeneous in chromatography systems 4 and 9; it gave positive color reactions with naphthoresorcinol and tetrazolium blue and a negative response with the Porter–Silber reagent; m.p. dec. at 183°; I.R. (KBr) 3430 (OH) and 1730 $cm.^{-1}$ (C=O). *Anal.* Calcd for $C_{27}H_{40}O_{11}$: C, 59.98; H, 7.46. Found: C, 59.88; H, 7.29.

Barium bis[(11β,18-epoxy-18,21-dihydroxy-20-oxo-5β-pregnan-3α-yl β-D-glucopyranosid)uronate] (**2**). To 19.5 mg of **1** in 50% ethanol (0.5 ml) was added a soln. of 0.20 M barium acetate in 50% ethanol (0.5 ml) and 10% acetic acid (1 drop); crystals of **2** (16.8 mg, 77% yield) were obtained; m.p. dec 283°; I.R. (KBr) 3420 (OH), 1720 (C=O) and 1600 $cm.^{-1}$ (COO^-). *Anal.* Calcd for $(C_{27}H_{39}O_{11})_2$ Ba · 6 H_2O: C, 48.96; H, 6.85. Found: C, 49.38; H, 6.95.

Methyl (18,21-diacetoxy-11β,18-epoxy-20-oxo-5β-pregnan-3α-yl 2,3,4-Tri-O-acetyl-β-D-glucopyranosid)-uronate (**3**). An excess of diazomethane in ether was added to glucosiduronic acid **1** (30 mg) in methanol. After 15 min, dil. acetic acid was added and the glucosiduronic ester was recovered and acetylated in acetic anhydride–pyridine (24 h). The product, chromatographed on a column in system 2, gave a band (5.2 V_0) which was homogeneous by t.l.c. in system 11 and which crystallized from carbon tetrachloride–cyclohexane (25.4 mg, 60% yield), m.p. 122–123°; I.R. (KBr) no OH band, 1760 (acetate and methyl ester), and 1230 $cm.^{-1}$ (acetate); n.m.r. ($CDCl_3$, 100 MHz), δ 0.97 (s, 19-CH_3), 1.92 (s, acetyl), 2.02 (s, 6H, acetyl), 2.04 (s, acetyl), 2.16 (s, acetyl), 2.60 (m, probably one of the two 16-H), 2.95 (t, C-17-H), 3.59 (m, C-3-H), 3.74 (s, OCH_3), 4.01 (m, C-5'-H), 4.49 (d, C-11-H, J = 6.5 Hz), 4.62 (d, C-1'-H, J = 7.5 Hz), 4.74 (s, 21-CH_2), 4.94 (m, C-2'-H), 5.22 (m, C-3'-H; C-4'-H) and 5.86 ppm (s, C-18-H). *Anal.* Calcd for $C_{38}H_{52}O_{16}$: C, 59.68; H, 6.85; OCH_3, 4.09. Found: C, 59.21; H, 7.02; OCH_3, 4.10.

11β,18-Epoxy-3α,18,21-trihydroxy-5β-pregnan-20-one (**4**). A soln. of **1** (10 mg) and β-glucuronidase (50,000 Fishman units, E.C. 3.2.1.31; Sigma Chemical Co., Type 1: bovine liver) in 0.10 M acetate buffer (pH 4.8, 10 ml) was incubated at 37° for 72 h. The product was partitioned between chloroform and water and the organic phase was concentrated. By paper chromatography in systems 5 and 6 the residue (98% of the radioactivity) was homogeneous and migrated at the same rate as **4**.

11β,18-Epoxy-18-hydroxy-20,21-dioxo-5β-pregnan-3α-yl β-D-glucopyranosiduronic acid (**5**). A soln. of 0.005 M cupric acetate in methanol (40 ml) was added to **1** (5 mg) in methanol (1 ml), a slow stream of air was passed through the soln. for 1 h and 0.026 M EDTA in water (8 ml) was added to quench the reaction. The soln. was concentrated, desalted on 3 g of Amberlite XAD-2, and the product was applied along a 26 cm. line and chromatographed (developed 9 h) on a sheet of paper in system 4. The principal product, located about 11 cm. from the origin, was eluted with methanol; crystals of **5** (2.5 mg) were obtained from acetone–ethyl acetate; m.p. dec. 214°. This substance, which was homogeneous by paper chromatography in system 7 and by t.l.c. in system 9, gave an immediate positive reaction with the Porter-Silber reagent, a positive reaction with naphthoresorcinol and a negative reaction with alkaline tetrazolium blue. Chromatographically, it was identical with a by-product which formed when a sample of **1** (possibly contaminated with a trace of Cu^{2+} that was derived [7] from the Celite 545 which was used in its chromatographic purification) stood in methanol for several days in the absence of EDTA.

11β,18-Epoxy-18-hydroxy-20,21-dioxo-5β-pregnan-3α-yl β-D-glucopyranosiduronic acid 21-(phenylhydrazone) (**6**). A soln. of 0.06 M phenylhydrazine hydrochloride in 50% acetic acid (0.4 ml) was added to **5** (1 mg) in 85% acetic acid (0.4 ml). A yellow color developed immediately; after 15 min the soln. was partitioned between water and ethyl acetate, the organic phase was concentrated and the residue was applied along a 12 cm. line on methanol-washed paper and chromatographed in system 8. The zone correspond-

ing to the principal product (R_F 0.65) was eluted with methanol and concentrated. The residue (**6**) had λ_{max}^{MeOH} 362 nm; in the Porter–Silber reagent λ_{max} = 408 nm. These spectral properties are characteristic [8] of steroidal 20-oxo-21-phenylhydrazones.

(18R)-17β-Carboxy-11β,18-epoxy-18-hydroxy-5β-androstan-3α-yl β-D-glucopyranosiduronic acid 17,18-lactone (**7**). An aq. soln. of 0.20 M $NaIO_4$ (0.6 ml) was added to **1** (20 mg) in methanol (0.60 ml) and the mixture was shaken. After 10 min the excess of oxidant was removed by addition of 0.20 M KI (4.2 ml) and 0.20 M HCl (4.8 ml) and the resulting iodine was reduced by addition of 0.20 M $Na_2S_2O_3$ (10 ml). The soln. was concentrated, desalted on Amberlite XAD-2 and the product was chromatographed on a column in system 1. The material (**7**) which was eluted at 6.2 V_0 (69% of the radioactivity) was homogeneous by t.l.c. in system 9 but could not be crystallized; it gave a positive reaction with naphthoresorcinol and negative reactions with tetrazolium blue and the Porter–Silber reagent: I.R. (pyridine) 3300(OH), 1778 cm.$^{-1}$ (C=O of γ-lactone).

Barium acetate (18R)-(17β-carboxy-11β,18-epoxy-18-hydroxy-5β-androstan-3α-yl β-D-glucopyranosid)-uronate 17,18-lactone monohydrate (**8**). Treatment of **7** (~12 mg) with barium acetate soln., as described above, gave crystals (12 mg) of **8** which separated as a hydrated double salt of steroidal glucosiduronate and acetate (homogeneous in system 9); m.p. dec. 280°; I.R. (KBr) 3320 (OH), 1780 (C=O of γ-lactone), and 1601 cm.$^{-1}$ (COO^-). *Anal.* Calcd for $(C_{26}H_{36}O_{10})$ $Ba_{0.5}\cdot(CH_3COO)$ $Ba_{0.5}\cdot H_2O$: C, 46.38; H, 5.98. Found: C, 46.85; H, 6.07.

(18R)-11β,18-epoxy-3α,18-dihydroxy-5β-androstane-17β-carboxylic acid γ-lactone (**9**). *A.* From **4**. An aq. soln. of 0.20 M $NaIO_4$ (0.25 ml) was added to **4** (~5.0 mg; prepared from **1** by treatment with β-glucuronidase) in 0.3 ml of methanol. Crystals started to form immediately. After 2 h the soln. was concentrated, the residue was partitioned between chloroform and water and the organic phase was resolved by preparative t.l.c. in system 10. The principal band (R_F 0.41) was eluted with acetone and crystallized from methanol; m.p. 255–256° (reported [1] m.p. 252–254°); I.R. (KBr) 3420 (OH), 1780 cm.$^{-1}$ (C=O of γ-lactone). This product was identical (chromatography, m.p., m.m.p. and I.R.) with a sample of **9** which had been prepared by periodate oxidation of authentic **4**.

B. From **5**. Oxidation of a sample of **5** (which formed spontaneously when a sample of **1** was stored at 4° as an oil) with H_5IO_6 by the procedure of Ulick *et al.* [1], followed by the purification and crystallization as described above, gave **9** (80% yield) which had properties identical (chromatography, m.p., and I.R.) with those of authentic **9**.

C. From **8**. Enzymatic hydrolysis of **8** (2.5 mg) followed by recovery and crystallization of the product gave material (80% yield) which was homogeneous and identical with authentic **9**.

DISCUSSION

In a study designed to isolate urinary glucosiduronates of aldosterone and its metabolites [6], 150 mg of aldosterone was administered orally to each of 4 normal individuals and one individual received, in addition, a tracer dose of [^{3}H]-aldosterone. The urine which was passed in the following 12 h was collected, pooled, desalted, and subjected to several chromatographic procedures which served to separate aldosterone 18β-D-glucosiduronic acid [6] and tetrahydroaldosterone 3β-D-glucosiduronic acid (**1**) from accompanying non-labeled impurities. The latter substance (**1**), which is considered to be the principal metabolite [1] of aldosterone, contained 34% of the administered radioactivity. This substance (**1**) is the first metabolite of aldosterone to be isolated from urine as a cryst. native steroidal glucosiduronic acid.

Tetrahydroaldosterone 3β-D-glucosiduronic acid (**1**) had properties typical of 20,21-ketols which are conjugated at C-3 with glucuronic acid [7]; it reduced alkaline tetrazolium blue, produced color with naphthoresorcinol and gave a negative test with the Porter–Silber reagent. It yielded a cryst. barium salt (**2**) when treated with barium acetate. The glucosiduronic acid (**1**) was hydrolyzed by β-glucuronidase to produce previously-prepared [2] tetrahydroaldosterone (**4**) which, upon treatment with periodate, gave the corresponding etiolactone (**9**) [2].

Upon treatment with diazomethane followed by acetylation, the acid (**1**) gave a crystalline pentaacetate (**3**). This substance (**3**) is considered to be a β-glucosiduronate because [9–11] the coupling constant ($J_{1'2'}$ = 7.5 Hz) for the protons at C-1′ and C-2′ is typical of that found for β-glycosides ($J_{1'2'}$ ~ 7.0 – 8.0 Hz) and distinctly different from that of α-glycosides ($J_{1'2'}$ ~ 3.0 – 4.0 Hz). It follows that compounds **1**, **2**, **5**, **6**, **7** and **8** also are β-glucosiduronates.

Brief treatment of glucosiduronic acid **1** with periodate leaves the glucuronate moiety intact and oxidizes the side chain of the steroid to give an androstanyl 17β-carboxylic acid which lactonizes to produce **7**; this product was obtained also as the barium salt (**8**). Removal of the glucuronate group from the lactone (**8**) with β-glucuronidase gives the known [1] etiolactone (**9**) and establishes C-3 as the locus of attachment of glucuronic acid to the steroid.

Additional evidence confirms that the glucosiduronate group in compound **1** is not attached to the steroid at either C-18 or C-21. Compound **1** does not undergo the facile hydrolysis at pH 1.0 that is characteristic of C-18 α- and β-glucosiduronic acids [6]. Treatment of glucosiduronic acid **1** with copper acetate [12] in methanol gives the C-21 aldehyde (**5**) which still retains the glucosiduronate moiety and has properties typical of a glyoxal; the product (**5**) does not reduce tetrazolium blue but it reacts with phenylhydrazine to give a phenylhydrazone (**6**) which absorbs maximally at 362 nm in methanol and at

408 nm in the Porter–Silber reagent [8]. Upon treatment with periodate, the glyoxal function in **5** is cleaved and C-20 is converted into a carboxyl group which lactonizes with the C-18 hydroxyl group; in addition, the glucuronyl ring is broken and its fragments are removed hydrolytically [1] so that the overall process gives etiolactone **9**.

Acknowledgements—We are indebted to Dr. Byron Arison, Merck & Co., Rahway, N.J., for the determination and interpretation of the n.m.r. spectra. Dr. J. C. Melby, Boston University, Boston, Massachusetts, kindly supplied us with a sample of tetrahydroaldosterone. This work was supported by grants from the USPHS, NIH, AM-05452 and AM-5628, and by a grant from the Northwest Area Foundation, St. Paul, Minnesota.

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