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URINARY METABOLITES OF 18-HYDROXYLATED CORTICOSTEROIDS: MICROBIAL PREPARATION OF REFERENCE COMPOUNDS

C. H. L. SHACKLETON*, J. W. HONOUR†, J. WINTER‡ and V. D. BOKKENHEUSER‡
*Biomedical Mass Spectrometry Resource, Space Sciences Laboratory, University of California, Berkeley, CA 94720, U.S.A.
†Division of Clinical Chemistry, Clinical Research Centre, Harrow, Middlesex, England and
‡Department of Microbiology, St. Luke's Hospital Center, New York, NY 10025, U.S.A.

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SUMMARY

Clostridium paraputrificum quantitatively reduced 18-hydroxy-11-deoxycorticosterone, 18-hydroxy-11-dehydrocorticosterone and 18-hydroxycorticosterone to their 3α-hydroxy-5β metabolites. This microbial transformation is eminently suited for the preparation of rare or expensive reference steroids. Mass spectra of the methyloxime-trimethylsilyl ethers of the metabolites are illustrated.

INTRODUCTION

During the last decade, interest has developed in the secretion of the 18-hydroxylated steroids, 18-hydroxy DOC§ and 18-hydroxycorticosterone by the adrenal glands. In *low renin hypertension* 18-hydroxy DOC has been shown to be hypersecreted [1], an observation suggesting that the steroid may have mineralocorticoid properties. 18-Hydroxycorticosterone is the immediate stable precursor of aldosterone and measurement of this compound and its metabolites has become important in the investigation of defects in the biosynthesis of aldosterone [2]. The role of 18-hydroxycorticosterone as a hormone in its own right has been discussed, and present knowledge of its biochemistry is contained in a recent view [3].

In previous communications we have described the identification and measurement by gas chromatography/mass spectrometry of metabolites of 18-hydroxylated corticosteroids [4, 5]. Hampered by lack of authentic reference compounds, we identified urinary 18-hydroxytetrahydro DOC [5] by demonstrating that its mass spectrum was identical to that published for the structure by Bournot *et al.*[6]. Urinary 18-hydroxytetrahydroCompound A [4], in the absence of a reference compound, was tentatively identified by the specific fragmentation pattern of 18-hydroxylated corticosteroids. Determination of these compounds in clinical medicine makes the availability of appropriate reference compounds desirable. The tetrahydro derivatives are usually obtained by reduction of the 3-oxo-4-ene steroids with rat liver enzymes, an expensive procedure often with low yields [7]. In this work we show that they may equally well be obtained, inexpensively and with high recovery, by metabolism of the same compounds with *Clostridium paraputrificum* in a special medium [8].

§ *Steroid abbreviations*: *18-hydroxy DOC*, 18,21-dihydroxy-4-pregnene-3,20-dione (20,21-dihydroxy-18,20-epoxy-4-en-3-one); *18-hydroxyCompound A*, 18,21-dihydroxy-4-pregnene-3,11,20-trione (20,21-dihydroxy-18-20-epoxy-4-pregnene-3,11-dione); *18-hydroxycorticosterone*, 11β,18,21-trihydroxy-4-pregnene-3,20-dione (11β,20,21-trihydroxy-18,20-epoxy-4-en-3-one); "tetrahydro" refers to the 3α-hydroxy-5β products of the above mentioned steroids.

MATERIALS AND METHODS

Source of steroids

18-Hydroxy DOC and 18-hydroxycorticosterone were obtained from the Medical Research Council Steroid Reference Collection, Westfield College, Hampstead, London.

18-HydroxyCompound A was synthesised by the Makor Chemical Co., Ltd., Israel. It was obtained from Uniscience Ltd., Cambridge, England.

Media

Bacterial conversion of steroids was examined in prereduced broth (PR; 45 ml Brain Heart Infusion broth supplemented with 0.05% cysteine HCl in 60 ml vials) from Scott Laboratories Inc., Fiskeville, R.I. Cultures were propagated in supplemented peptone broth (SPB; 18 ml in a tube with rubber stopper) obtained from Baltimore Biological Laboratories, Division of Becton, Dickinson & Co., Cockeysville, MD.

Source of microorganism

Clostridium paraputrificum, recovered from human faecal flora [8], was maintained in lyophilised form. Prior to use, the culture was rehydrated and passaged 2–3 times in SPB at 37°C. A 24 h broth culture was employed for conversion experiments.

Conversion experiments. One mg of steroid was dissolved in 0.25 ml methanol and added to the PR to give a concentration of 20 μg steroid/ml medium and

0.5% methanol (v/v). The medium was then seeded with 0.25 ml *C. paraputrificum* and incubated at 37°C for 7 days. At the end of the incubation, pH and Eh were measured (Beckman Zeromatic II). A platinum electrode was used for the Eh measurements. The instrument was checked regularly with quinhydrone-saturated buffer at pH 4 and 7. Invariably the readings were within 10 mV of the theoretical values.

Thin layer chromatography was used to demonstrate completion of transformation. Five-ml portions of incubated culture were extracted with 5 ml methylene dichloride. The extracts were dried under nitrogen and applied in acetone to fluorescent silica gel plates. Good resolution of precursor steroids and products was obtained with the solvent system benzene–acetone (1:1, v/v). The steroids were located by ultraviolet light or by spraying with blue tetrazolium or sulphuric acid–ethanol (1:1, v/v).

Extraction. The steroids were extracted as previously described [9].

Preparation of derivatives. Portions of the crude extract were heated for 8 h at 60°C with methoxyamine hydrochloride in pyridine (2%). Silylation was carried out for 8 h following addition of 100 μl hexamethyldisilazane/trimethylchlorosilane (4:1, v/v). The products were dried under nitrogen, sonicated with 1 ml of cyclohexane which, following centrifugation, was transferred to a vial.

Gas chromatography/mass spectrometry. Preliminary gas chromatographic analysis was carried out on a 25 m OV-1 glass capillary column linearly programmed between 160°C and 260°C, and retention times (methylene units, MU) were determined. GC/MS analysis was undertaken on a Varian MAT 731 instrument with 7 ft packed OV-1 column. Scans (40–800 mass units) were obtained repetitively and stored on tape in mass converted form for subsequent processing.

RESULTS

Thin layer chromatography and gas chromatographic analysis of the unpurified extract from the incubation of *C. paraputrificum* with each of the 18-hydroxycorticosteroids demonstrated that a single product was formed.

The separation of the reduction product of 18-hydroxyCompound A is shown in Fig. 1. The two peaks (ratio 4:1) represent the *syn-* and *anti*-forms of the oxime derivatives of the metabolite. Such isomers are known to be formed in the preparation of the methyloxime derivatives of 17-deoxy-21-hydroxylated corticoids [10]. Methylene unit values for the two peaks were 30.64 and 31.44, respectively. The mass spectrum is shown in Fig. 2a. The molecular ion was at *m/z* 609 and prominent fragments were *m/z* 578 (M-31), 519 (M-90), 488 (M-90-31), 457 (M-152) and 103. The mass spectrum of a urinary metabolite essentially identical to that of 18-hydroxytetrahydroCompound A has been published [4].

The mass spectrum of the metabolite of 18-hydroxycorticosterone is shown in Fig. 2b. The two peaks gave retention times of 31.00 and 31.45 MU. The molecular ion is at *m/z* 683. Other principal ions: *m/z* 652 (M-31), 593 (M-90), 562 (M-90-31), 531 (M-152), 472 (M-90-90-31) and 103. An essentially identical spectrum of a urinary steroid, tentatively identified as 18-hydroxytetrahydrocorticosterone, has also been published [4].

The metabolite of 18-hydroxy DOC gave the mass spectrum shown in Fig. 2C. The retention times of the two peaks were 29.73 and 30.50 MU. The molecular ion was at *m/z* 595 and major ions at *m/z* 564 (M-31), 505 (M-90), 474 (M-90-31), 445 (M-150), 443 (M-152) and 103. The spectrum is typical for 18-hydroxytetrahydro DOC [6].

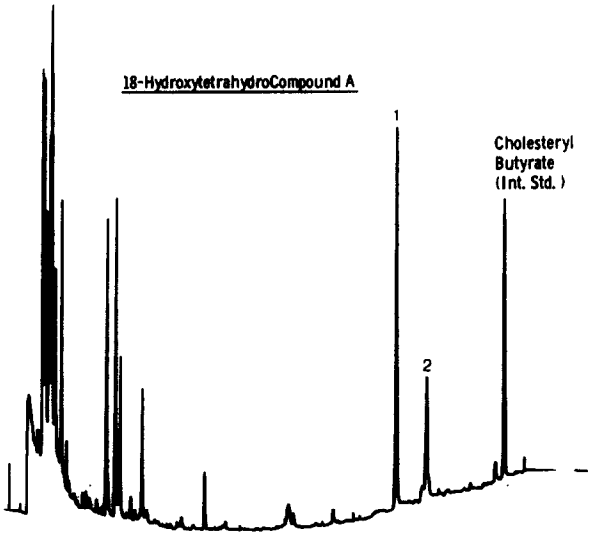

Fig. 1

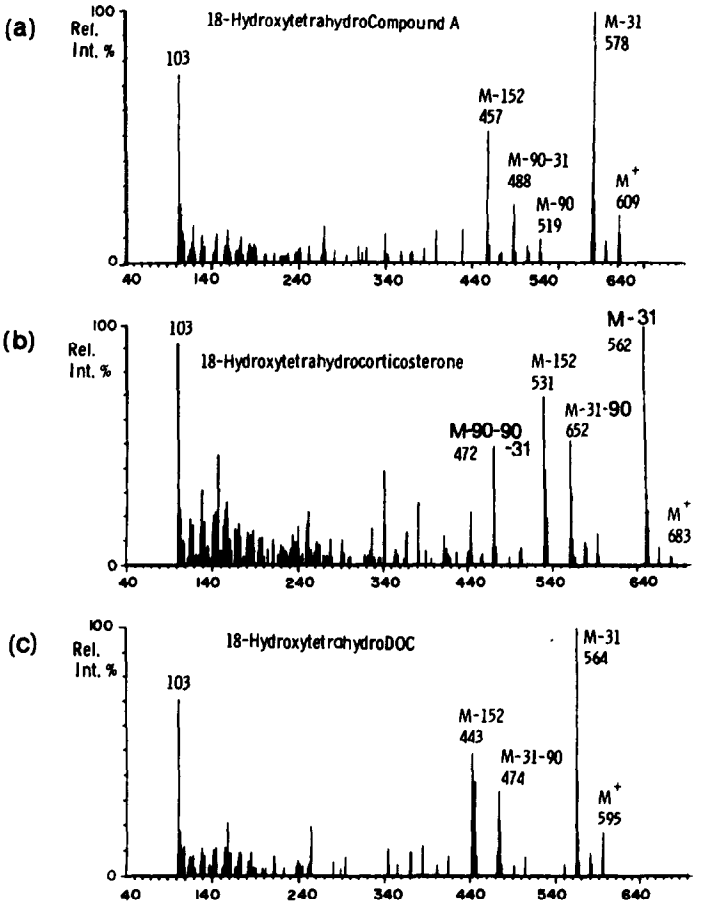

Fig. 2

DISCUSSION

In 1976 Bokkenheuser and co-workers [8] showed that at least ten bacterial species reduce DOC to the 3α-hydroxy-5β products in yields ranging from 1–10%, but *C. paraputrificum* was the only organism which converted DOC to tetrahydro DOC quantitatively. More recent experiments demonstrated that *C. paraputrificum* also transformed Compound A, corticosterone, cortisone, cortisol [11], aldosterone [12] and 6β-hydroxy DOC (unpublished) to the corresponding 3α-hydroxy-5β reduction products. No other products were formed during these incubations.

In the present experiments we have shown that *C. paraputrificum* reduces 18-hydroxycorticosteroids to their tetrahydro derivatives equally well, as demonstrated by reduction of t.l.c. R_f values, disappearance of U.V. absorbance at 240 nm and specific mass spectrometric fragmentation patterns. For reference purposes, the mass spectra of methyloxime-trimethylsilyl ethers of 18-hydroxytetrahydro DOC, 18-hydroxytetrahydrocorticosterone and 18-hydroxytetrahydro-Compound A are depicted in Fig. 2a–c.

The most significant features of the spectra of 18-hydroxylated corticosteroids compared with those of other 17-deoxy-21-hydroxylated corticoids was the absence of the characteristic side chain D-ring fragments *m/z* 175 and 188. On the other hand, the 18-hydroxy steroids exhibited an important ion at *m/z* (M-152). Thus the presence of a derivatised 18-hydroxy group clearly influences the fragmentation of the side chain and the D-ring. A possible mechanism of this phenomenon has been described previously [4]. Another common characteristic of the mass spectra of the three C-18 hydroxylated steroids is the presence of a major ion at *m/z* 103. This can be formed by the removal of the C-18 or C-21 primary trimethylsilyl groups.

The efficiency of the reduction of the 3-oxo-4-ene structure is independent of carbonyl or hydroxyl groups at C-11, C-17, C-21 and C-6. In contrast, hydroxyl groups at both C-20 and C-21 protect the C-3 carbonyl against the reductase of *C. paraputrificum* [8]. Under these circumstances only the 4-ene structure is reduced.

The present study also confirms that 18-hydroxytetrahydrocorticosterone is a urinary metabolite in man. The mass spectrum of the isolated steroid [4] tentatively identified in a previous communication is identical to the mass spectrum shown in Fig. 2b. In unreported studies we have shown that the urinary excretion of *21-deoxy metabolites* of 18-hydroxylated corticosteroids is unlikely, in contrast to the situation obtaining for other 17-deoxy-21-hydroxylated steroids

[11, 12]. Incubation of the tetrahydro-products produced during this investigation with *Eubacterium lentum*, an active 21-dehydroxylating organism, did not result in any transformation. Presumably the C-18-C-20 cyclic forms of 18-hydroxylated steroids prevent further microbial side chain reduction.

Preparative reduction of corticosteroids to 3α-hydroxy-5β steroids is usually carried out by a rat liver enzyme [7]. The method only permits small amounts of substrate to be metabolised. Moreover, several products are formed requiring extensive fractionation before pure materials are obtained. Thus it is a costly and wasteful procedure. In contrast, the microbial reduction under our conditions results in quantitative and specific conversion to 3α-hydroxy-5β structure; considerable amounts of substrate can be metabolised at each experiment and minimal fractionation is necessary.

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