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SHORT COMMUNICATION

A NOVEL CHROMATOGRAPHIC RESOLUTION OF 21-HYDROXYSTEROID METABOLITES OF PROGESTERONE

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Summary—21-Hydroxysteroid metabolites of both progesterone and deoxycorticosterone are excreted in rabbit urine and are eluted from an alumina adsorption column after 21-deoxysteroids. The separation is independent of polarity and dependent on the interaction of the 21-hydroxyl function with the adsorbent. The group separation of these steroids allowed further analysis by high performance liquid chromatography and revealed different proportions of metabolites. This is the first report of the excretion of 21-hydroxysteroid metabolites of progesterone in rabbit urine.

INTRODUCTION

Progesterone 21-hydroxylation, which is an important reaction in the metabolism of this hormone by the rabbit, is catalysed primarily by a hepatic cytochrome *P*-450 (designated Form 1 [1], or P45011C5 [2]). Further side chain oxidation to the 21-oic acid has been shown to occur *in vitro* with hepatic microsomes [3, 4] and steroid acids are the major metabolites excreted in rabbit urine [5]. This suggests that the majority of the 21-hydroxylated metabolites are committed to the steroid acid pathway. Whether 21-hydroxylated metabolites are also excreted has not been determined to date, partly because of the complexity of the neutral polar metabolites in rabbit urine. Most studies on the neutral metabolites have been confined to the 21-deoxysteroid fraction which accounts for a relatively small proportion of the progesterone (P) metabolites in rabbit urine [6, 7]. In the present study we report a novel separation of the 21-deoxy and 21-hydroxysteroid metabolites of P and deoxycorticosterone (DOC) that allows further resolution of their constituents and may provide insight into the *in vitro* activity of the progesterone 21-hydroxylase.

EXPERIMENTAL

NZW rabbits, housed in metabolic cages, were injected via the marginal ear vein with a mixture of 1,2[^{3}H]DOC (50 μCi; 36 Ci/mmol) and 4[^{14}C]P (10 μCi; 57.2 mCi/mmol) dissolved in 50% ethanolic saline. Urines were collected for 24 h and the steroids either extracted on an Amberlite XAD-2 resin column [8] or small aliquots were processed on a C_{18} Sep–Pak (Waters, U.S.A.). The methanol eluates were evaporated and either processed directly or after hydrolysis for 24 h with Glusulase, a preparation from the snail, *Helix pomatia*, (Dupont, Canada) that contains β-glucuronidase (90,000 units/ml) and sulphatase (10,000 units/ml) activities. Units of activity were as defined by the manufacturer. Incubations were carried out at 37°C after addition of 900 units of β-glucuronidase and 100 units of sulphatase per ml of 0.2 M sodium acetate, pH 5.0 (total volume 10–20 ml). After hydrolysis for 48 h, ammonium sulfate (5 g per 10 ml) was added, the mixture was acidified to pH 2 with HCl and the steroids were extracted into ether–ethanol (3:1 v/v). The extracts were dried over sodium sulfate, and evaporated on a rotory evaporator. All traces of water were removed with a vacuum pump before the residue was redissolved in ether–ethanol (3:1 v/v, 50 ml). The extract was run on an alumina adsorption column as previously described [9]. In a typical run, neutral alumina (20 g, Fisher Scientific, U.S.A.) was deactivated with water (5% v/w) and packed as a slurry in benzene in a glass column (2 cm diameter). The dried steroid extract was applied in ether–ethanol (3:1 v/v; 50 ml) and the column eluted sequentially with the same solvent (50 ml) followed by ethanol (20 ml), 50% aqueous ethanol (v/v; 100 ml), and 0.1 M and 1.0 M sodium acetate buffers, pH 5.0 (100 ml of each). Fractions (10 ml) were collected in volumetric flasks and aliquots (0.2 ml) were added to Formula 963 scintillation fluid (10 ml; Dupont, Canada) and the radioactivity counted in a Beckman counter (model LS-3801). The elution profiles were highly reproducible when appropriate volumes of solvents were used on columns containing 5–50 g alumina.

Thin-layer chromatography (TLC) was carried out on precoated silica gel plates (250 μm; 5 × 20 cm; Anasil OF, U.S.A.) and developed with benzene–ethanol (8:2 v/v). Radiometabolites were located with a radiochromatogram scanner (Packard, U.S.A., model 7201). Non-labelled ring A-saturated steroids eluted from alumina columns or after HPLC were spotted on TLC plates and visualised with either ethanol–sulphuric acid (1:1 v/v) or a methanolic blue tetrazolium in aqueous sodium hydroxide spray [10].

High performance liquid chromatography of neutral steroids isolated from alumina columns was carried out on a Chromegabond diol straight phase column (4.6 mm × 30 cm, 10 μ). The steroids were dissolved in isopropyl alcohol, filtered through nylon filters (0.45 μm) and 20 μl aliquots injected with DOC (800 ng) as an internal standard. The

Nomenclature: P, 4-pregnene-3,20-dione; DOC, 21-hydroxy-4-pregnene-3,20-dione; THDOC, tetrahydroDOC,3α,21-dihydroxy-5β-pregnan-20-one; HHDOC, hexahydroDOC, 3α, 20β, 21-trihydroxy-5β-pregnane; 6-OHTHDOC; 3α,6α,21-trihydroxy-5β-pregnan-20-one.

Table 1. Group separation of reference 21-deoxy and 21-hydroxysteroids by alumina adsorption column chromatography

Alumina column fractions	
A (ether–ethanol 3:1 v/v)	B (50% aqueous ethanol)
3α-20α-Diol-5β-pregnane (pregnanediol)	21-Hydroxy-4-pregnen-3,20-dione (Deoxycorticosterone; DOC)
3α,6α-Diol-5β-pregnan-20-one	20β,21-Dihydroxy-4-pregnen-3-one
3α,6α,20-Triol-5β-pregnane (pregnanetriol)	3α,21-Dihydroxy-5β-pregnen-20-one (Tetrahydro DOC; THDOC)
21-Acetoxy-4-pregnen-3,20-dione (DOC acetate)	3α,20β,21-Trihydroxy-5β-pregnane (hexahydro DOC; HHDOC)
4-Pregnen-3,20-dione (progesterone)	

column was developed with a linear 5–20% isopropyl alcohol/hexane gradient for 60 min after 5 min with the 5% solvent. The flow rate was 2 ml/min and 1 min fractions were collected with a Fract-100 fraction collector for analysis of radioactivity or colorimetric visualisation of standards. Other HPLC conditions, equipment and suppliers were as previously reported [11].

RESULTS AND DISCUSSION

The metabolites of [^{3}H]DOC and [^{14}C]P excreted in rabbit urine were hydrolysed with Glusulase and fractionated on an alumina adsorption column. Figure 1 shows a typical elution pattern of radiometabolites and the close similarity in distribution of the DOC and P metabolites. Elution of the alumina column, which had been modified for the group fractionation of P metabolites [12, 9] was known to sequentially elute free neutral steroids (A in ether–ethanol), steroid acids (C in 0.1 M buffer) and steroid glucuronides (D in 1 M buffer). The identity of the B fraction steroids had not been determined but it was known not to contain steroid sulphates which were eluted in fraction numbers 15–17. Fraction B was eluted as a sharp peak in fraction number 13 which corresponded to the break through of the 50% ethanol eluant (hold back volume 14.5 ml).

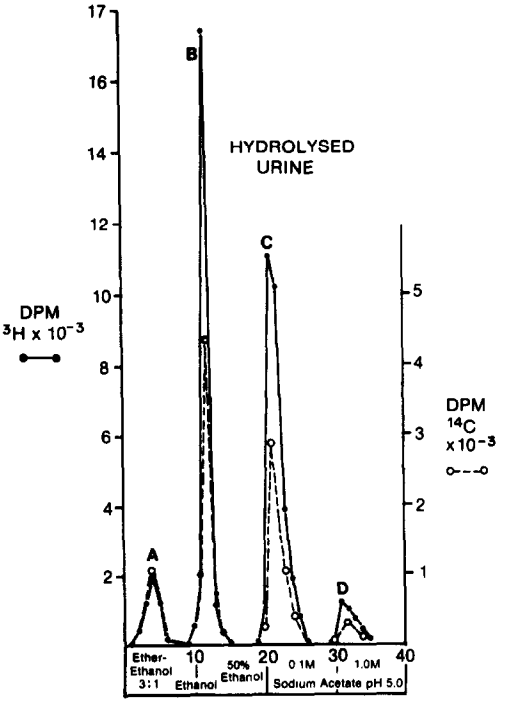

Fig. 1. Alumina adsorption column chromatogram of [^{3}H]DOC and [^{14}C]P metabolites isolated from rabbit urine after Glusulase hydrolysis. Fractions A–D correspond to the elution of 21-deoxy,21-hydroxy,21-oic acid and steroid glucuronides respectively. The abscissa units refer to fraction numbers (10 ml each).

The radiometabolites in alumina fractions A and B had the properties of neutral steroids when analysed by TLC. An unexpected finding was the intermediate mobility of a major fraction B metabolite (R_f 0.51) between two fraction A zones (R_f 0.45; 0.64). This indicated that resolution of the alumina A/B fractions was not dependent on differences in polarity. Furthermore, the [^{3}H]/[^{14}C] ratio of the fraction B TLC zone (24.4:1) was higher than the two fraction A TLC zones (4.2 and 4.8:1 respectively) which suggested that 21-hydroxylated metabolites derived from [^{3}H]DOC predominated in the alumina fraction B. Representative 21-deoxy and 21-hydroxysteroids were, therefore, chromatographed on alumina columns. Table 1 shows that steroids with a 21-hydroxyl function are retained by the alumina and eluted in fraction B whereas polar 21-deoxysteroids are eluted in fraction A. The retention of the 21-hydroxysteroids appears to be related to the 21-hydroxyl function since DOC 21-acetate is eluted in fraction A. An important methodological consideration is the removal of all traces of water from the original extract applied to an alumina column. Elution with 50% ethanol–water gives a sharp peak, however, elution is also effected if the proportions are reduced to 90:10 respectively.

HPLC of the 21-deoxy and 21-hydroxysteroids was carried out on a straight phase silica column developed with similar linear solvent gradients. Figures 2 and 3 show that without the group separation by alumina chromatography there would be considerable overlap with 21-deoxysteroids ranging in polarity from pregnanediol to prenanetriol and 21-hydroxysteroids from DOC to 6-hydroxyhexahydro DOC. Figure 3 also indicates the presence of 21-hydroxylated metabolites of [^{14}C]P with similar mobilities to 6-hydroxylated tetra and hexa hydro DOC standards. By contrast, the major [^{3}H]DOC metabolites had similar mobilities to the lesser polar tetra and hexa hydro DOC standards. [^{3}H]DOC metabolities were also present in the 21-deoxysteroid fraction, though of minor quantitative significance. These may have arisen by 21-dehydroxylation, a reaction which has been reported previously in the rabbit [13], though the metabolites were not identified in the present study.

The majority of the 21-hydroxysteroid metabolites of [^{14}C]P were excreted in rabbit urine as steroid glucuronides and eluted in the alumina fraction D. Unconjugated 21-hydroxysteroids accounted for 2.7 and 4.1% of the radiometabolites before hydrolysis and 14.8 and 21.6% respectively after hydrolysis of the urine from two rabbits. Rabbits exhibited considerable individual variations with the total 21-hydroxysteroids ranging from 7.1 to 32.9%

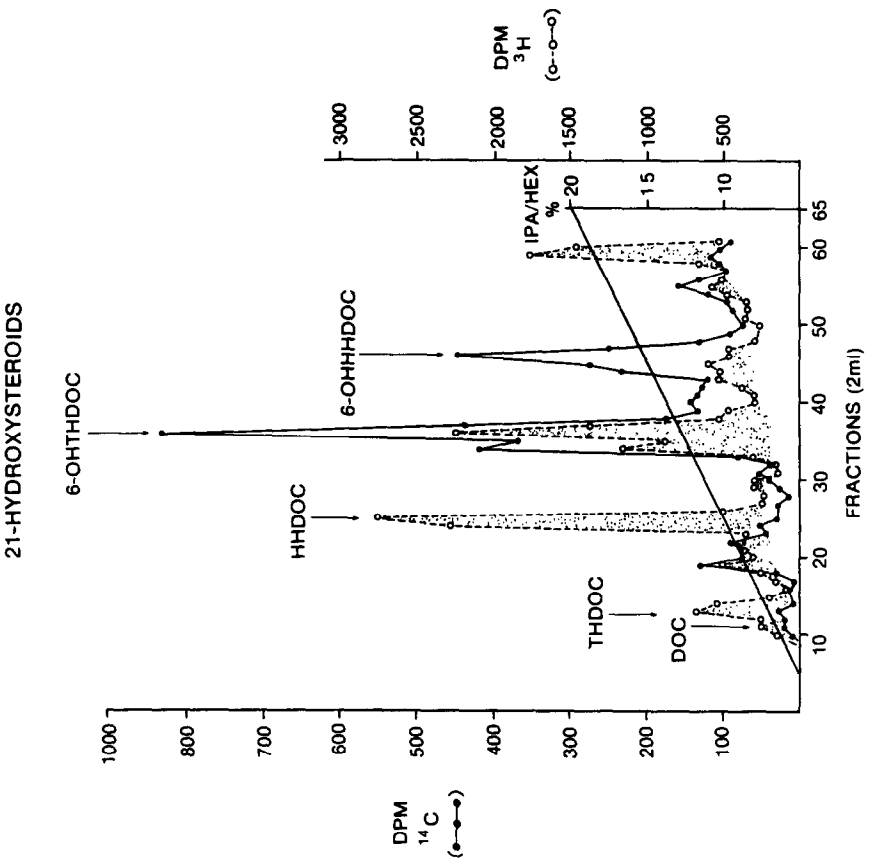

Fig. 3. HPLC of 21-hydroxysteroid metabolites of [^{3}H]DOC and [^{14}C]P isolated from alumina fraction B and chromatographed under the same conditions as in Fig. 3.

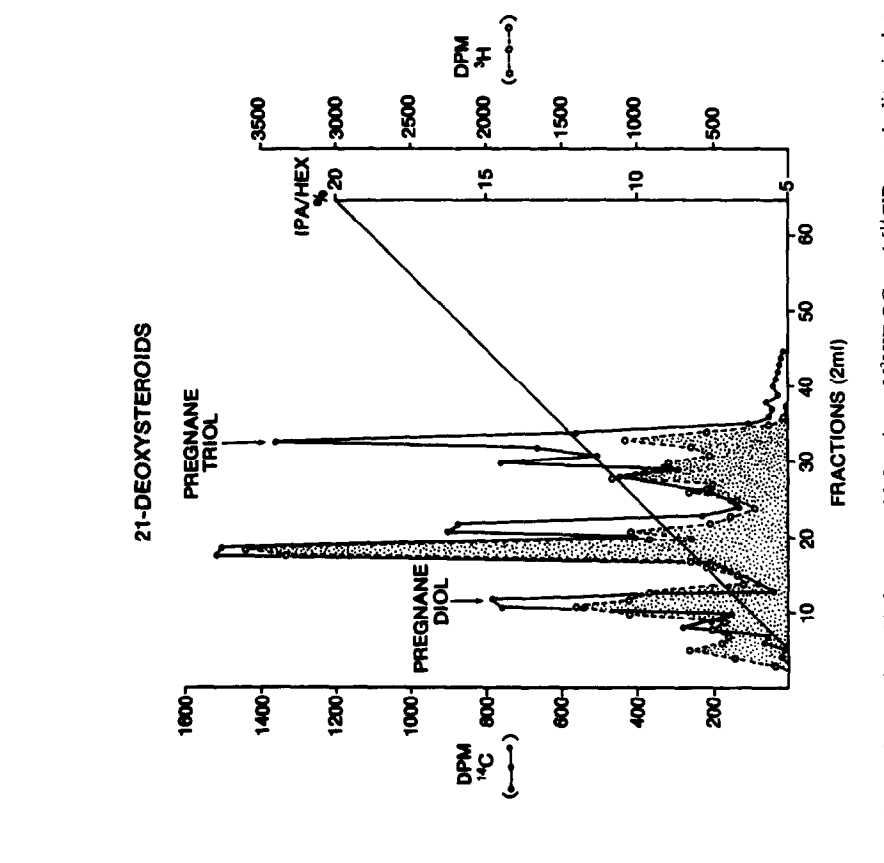

Fig. 2. HPLC of the 21-deoxysteroid fraction of [^{3}H]DOC and [^{14}C]P metabolites isolated from alumina fraction A. A Chromegabond diol column was developed with a linear isopropanol/hexane gradient at a flow rate of 2 ml/min. Aliquots of fractions (2 ml) were counted for radioactivity. Arrows indicate the elution of reference steroids that were detected colorimetrically.

(16.2% mean; $N = 8$) and 21-deoxysteroids 3,3–19.6% (11.7% mean). Whether these differences reflect differences in progesterone 21-hydroxylase activity or fluxes through different metabolic pathways remains to be determined.

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