The Metabolism of Aldosterone and 3a, 5β-Tetrahydroaldosterone in the Rabbit

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Abstract

Analysis of urinary metabolites of [1, 2-3H]-aldosterone and [1, 2-3H]-3α, 5β-tetrahydroaldosterone was performed in male rabbits. The preliminary separation of urinary metabolites was carried out by submitting these metabolites to countercurrent distribution. Further separation of each fraction thus obtained was achieved by means of DEAE-Sephadex A-25 column chromatography. The separated peak was then hydrolyzed with the enzyme and the free steroid released was identified on the basis of the mobilities of the steroid and its derivatives on paper chromatography.

After the injection of [1, 2-3H]-aldosterone, a major urinary metabolite was characterized as monosulfate of 3α, 5β-tetrahydroaldosterone. In addition, a small amount of the monoglucosiduronate fraction was found in the urine. 3α, 5β-tetrahydroaldosterone and 3β, 5α-tetrahydroaldosterone were detected as aglycones in this fraction. After the injection of [1, 2-3H]-3α, 5β-tetrahydroaldosterone, a similar pattern of urinary radiometabolites was observed.

The close similarity between the profile of urinary metabolites of [1, 2-3H]-aldosterone and that of [1, 2-3H]-3α, 5β-tetrahydroaldosterone suggests that the conversion of aldosterone to 3α, 5β-tetrahydroaldosterone is needed before the conjugation processes take place.

The metabolic fate and conjugation of aldosterone in man had been studied in detail and it was shown that a major urinary metabolite of aldosterone was monoglucosiduronate of 3α, 5β-tetrahydroaldosterone (3α, 5β-THAL) [For a comprehensive description of aldosterone metabolism in man, refer to a book by Ross (Ross, 1975)].

However, there has been, so far, no report on the nature of urinary metabolites of aldosterone or 3α, 5β-THAL in the rabbit. We have therefore attempted to characterize urinary metabolites of aldosterone as well as 3α, 5β-THAL in this species as described below.

Materials and Methods

Compounds

[1, 2-3H]-aldosterone (41–57 Ci/mmol) was purchased from The Radiochemical Centre, Amersham, England. [1, 2-3H]-3α, 5β-tetrahydroaldosterone ([1, 2-3H]-3α, 5β-THAL, 52.3–58.5 Ci/mmol) was obtained from New England Nuclear Corporation, Boston, MA, U.S.A. The radiochemical purity of these radioactive steroids was
checked by paper chromatography (PC). 3α,5β-THAL 3β,5α-tetrahydroaldosterone (3β, 5α-THAL) and 3β,5β-tetrahydroaldosterone (3β, 5β-THAL) were purchased from Sigma Chemical Co., St. Louis, Mo, U.S.A. Saccharo-1,4-lactone was a gift from Chugai Pharmaceutical Co, Tokyo, Japan.

Studies in vivo

Male, rabbits (albino strain) weighing 2–3 kg were used in the experiment. [1, 2-3H]-aldosterone or [1, 2-3H]-3α,5β-THAL was dissolved in a small amount of ethanol. The ethanolic solution was diluted with 0.9% (w/v) saline so that the final concentration was less than 5% with respect to ethanol. The solution thus prepared was injected as a bolus into the marginal ear vein and the urine was collected for 72 h using metabolic cages.

Preparations of urinary samples for analysis

The XAD-2 (Rohm and Haas Co., Philadelphia, PA, U.S.A.) columns were used to recover the radiometabolites from the rabbit urine by the technique described by Bradlow (Bradlow, 1968).

Separation of the urinary metabolites

The preliminary separation of the urinary metabolites was performed by means of counter-current distribution (CCD). Solvent systems used for CCD are shown in Table 1. Each fraction thus obtained was further separated into subfractions on DEAE-Sephadex A–25 column chromatography (Pharmacia Fine Chemicals, Uppsala, Sweden, K 9/60 Sephadex column was used) as described by Hobkirk et al. (Hobkirk et al., 1969).

Hydrolytic procedures

1. Beef liver β-glucuronidase (Tokyo Zoki Chemical Co., Tokyo, Japan)

The sample was dissolved in 0.1 M acetate buffer at pH 4.6. The enzyme concentration was 300 μMolar unit/ml. After the incubation was performed at 37°C for 48 h, the freed steroids were extracted with 3 vol of ethyl acetate.

2. Sulphatase from Helix pomatia (Type H-2, Sigma Chemical Co., St., Louis, MO., U.S.A.).

The sample was dissolved in 0.1 M acetate buffer at pH 4.6. The enzyme concentration was 69 Molar unit/ml. After the incubation was performed at 37°C for 48 h, the liberated steroids were extracted with 3 vol of ethyl acetate.

3. Hydrolysis at pH 1.

The sample was dissolved in 0.1 N HCl solution (pH was 1.0–1.1). After the solution was allowed to stand at room temperature for 48 h, the freed steroids were extracted with 3 vol of ethyl acetate. The ethyl acetate layer was washed with a small amount of saturated NaHCO3 solution.

Solvolytic procedure

This procedure was carried out as described previously (Miyazaki et al., 1978).

Separation of the free steroids and derivatives formation

The separation and purification of the free steroids and derivatives were performed by PC (see Table 1).

Oxidation of acetate by CrO3 was carried out as described by Kliman et al. (Kliman and Peterson, 1960).

Oxidation of the free steroids by NaIO4 was performed as mentioned by Kelly et al. (Kelly et al., 1962).

Results

[A] Analysis of urinary metabolites of [1, 2-3H]-aldosterone

After the injection of [1, 2-3H]-aldosterone, the largest amount of radioactivity appeared

| Table 1. The composition of solvent systems used for analysis (by volume). |
|----------------------------------------|-----------------------------|
| countercurrent distribution          |                            |
| System A:  n-butanol : ethyl acetate : 0.2%NH₂OH | (1 : 1 : 2) |
| System B:  n-butanol : 0.2%NH₂OH     | (1 : 1)                   |
| Paper chromatography                |                            |
| System I:  benzene : methanol : water | (2 : 1 : 1)                |
| System II: isoctane : methanol : water| (5 : 3 : 2)               |
in the urine during the first 24h of collection. Then, the excretion dropped off abruptly. After 72h of collection, 54–77% of the administered dose was excreted in the urine. Only the urinary samples voided during the first 24h were submitted to analysis and identification of metabolites.

As an example, the identification procedure for metabolites from rabbit-I will be described in detail.

An aliquot of the urinary sample voided during the first 24h was examined for the possible presence of the free steroids, whereupon no radioactivity could be extracted with ethyl acetate. Another aliquot of the urinary sample was applied to a XAD-2 column and radiometabolites were extracted with methanol.

When the extract was distributed in system A for 24 transfers, two radioactive fractions were present (a less polar fraction was designated as fraction C).

The polar fraction was redistributed in system A for 49 transfers and two radioactive fractions (fraction A and fraction B) were separated (Fig. 1).

**Analysis of fraction A**

This fraction was distributed in system B for 24 transfers and a single peak of radioactivity (K = 0.17) was observed. This material was then chromatographed on a DEAE-Sephadex A-25 column in a linear gradient of 0–0.2 M NaCl followed by 0.2–0.6 M NaCl, and at least five subfractions were found (Fig. 2).

Of these subfractions, subfraction A–2, which was eluted at an NaCl concentration of 0.06–0.10 M and comprised 60% of fraction A, was analyzed further. Other subfractions contained too little radioactivity to attempt further analysis (Table 2).

![Fig. 1. CCD pattern of polar urinary metabolites obtained from rabbit-1 injected with [1, 2-3H]-aldosterone in solvest system A (n=49).](image)

Table 2. Composition of urinary metabolites obtained from rabbits injected intravenously with [1, 2-3H]-aldosterone. [Expressed as % dose excreted]

<table>
<thead>
<tr>
<th>Fraction A (%)</th>
<th>Rabbit-II</th>
<th>Rabbit-III</th>
<th>Rabbit-I</th>
</tr>
</thead>
<tbody>
<tr>
<td>A–1</td>
<td>0.5</td>
<td>0.6</td>
<td>1.6</td>
</tr>
<tr>
<td>A–2</td>
<td>9.2</td>
<td>8.0</td>
<td>3.6</td>
</tr>
<tr>
<td>A–3</td>
<td>2.1</td>
<td>0.7</td>
<td>2.7</td>
</tr>
<tr>
<td>A–4</td>
<td>1.4</td>
<td>0.8</td>
<td>1.3</td>
</tr>
<tr>
<td>A–5</td>
<td>2.2</td>
<td>2.2</td>
<td>5.3</td>
</tr>
<tr>
<td>Fraction B</td>
<td>76.3</td>
<td>86.1</td>
<td>82.4</td>
</tr>
<tr>
<td>Fraction C</td>
<td>8.3</td>
<td>1.6</td>
<td>3.1</td>
</tr>
</tbody>
</table>

Table 3. Composition of urinary metabolites obtained from rabbits injected intravenously with [1, 2-3H]-3α, 5β-tetrahydroaldosterone. [Expressed as % dose excreted]

<table>
<thead>
<tr>
<th>Fraction A (%)</th>
<th>Rabbit-IV</th>
<th>Rabbit-V</th>
<th>Rabbit-VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>A–1</td>
<td>0.7</td>
<td>0.7</td>
<td>0.6</td>
</tr>
<tr>
<td>A–2</td>
<td>11.5</td>
<td>8.9</td>
<td>9.6</td>
</tr>
<tr>
<td>A–3</td>
<td>2.4</td>
<td>2.4</td>
<td>2.4</td>
</tr>
<tr>
<td>A–4</td>
<td>0.7</td>
<td>0.4</td>
<td>0.3</td>
</tr>
<tr>
<td>A–5</td>
<td>1.8</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>Fraction B</td>
<td>78.3</td>
<td>82.4</td>
<td>79.9</td>
</tr>
<tr>
<td>Fraction C</td>
<td>4.7</td>
<td>3.6</td>
<td>5.6</td>
</tr>
</tbody>
</table>
Fig. 2. Chromatographic profile of fraction $A$ in urinary metabolites obtained from rabbit-I injected with $[1, 2^{-3}H]$-aldosterone on a DEAE-Sephadex A-25 column.

Fig. 3. Chromatographic profile of fraction $B$ in urinary metabolites obtained from rabbit-I injected with $[1, 2^{-3}H]$-aldosterone on a DEAE-Sephadex A-25 column.
An aliquot of subfraction A-2 was incubated with beef liver \( \beta \)-glucuronidase; 72\% of radioactivity became ethyl acetate extractable. This hydrolysis was inhibited (\( >90\% \)) in the presence of saccharo-1, 4-lactone (2.5×10^{-2}M). When the aglycone thus obtained was chromatographed on paper in system I, a broad peak of radioactivity was observed and the mobility of this aglycone was close to that of standard [1, 2-\(^3\)H]-3\( \alpha \), 5\( \beta \)-THAL.

When the aglycone was mixed with 3\( \alpha \), 5\( \beta \)-THAL or 3\( \beta \), 5\( \alpha \)-THAL and chromatographed on paper in system I overnight*, the portion of radioactivity moved at the same rate as 3\( \alpha \), 5\( \beta \)-THAL and the remainder as 3\( \beta \), 5\( \alpha \)-THAL. Thus, subfraction A-2 was tentatively identified as a mixture of 3\( \alpha \), 5\( \beta \)-THAL monoglucosiduronate and 3\( \beta \), 5\( \alpha \)-THAL monoglucosiduronate.

**Analysis of fraction B**

When this material was chromatographed on a DEAE-Sephadex A-25 column using a linear gradient of 0–0.2M NaCl followed by 0.2–0.6M NaCl, a single peak of radioactivity was found, which was eluted at an NaCl concentration of 0.11–0.15M (Fig. 3). Aliquots of this material were submitted to various kinds of hydrolytic procedures or solvolysis. Incubation of this material at pH 1 or with beef liver \( \beta \)-glucuronidase was not effective in hydrolyzing this material. On the other hand, the solvolytic procedure was able to release the free steroid from this material (86\% release). Moreover, when this conjugate was incubated with sulphatase from Helix pomatia, about 50\% of the radioactivity became ethyl acetate extractable.

When the aglycone thus obtained was chromatographed on paper in system I, a radioactive peak with an Rf of 0.19 was found. This aglycone was mixed with 3\( \alpha \), 5\( \beta \)-THAL or 3\( \beta \), 5\( \alpha \)-THAL and chromatographed on paper in system I overnight; this material moved at the same rate as 3\( \alpha \), 5\( \beta \)-THAL but was slightly more mobile than 3\( \beta \), 5\( \alpha \)-THAL.

An aliquot of this aglycone was acetylated and the acetylated material migrated at the same speed as [1, 2-\(^3\)H]-3\( \alpha \), 5\( \beta \)-THAL-triacetate in system II (Rf=0.60). The acetylated compound was then oxidized with CrO\(_3\) and the product thus obtained was found to have the same Rf as the oxidized product of [1, 2-\(^3\)H]-3\( \alpha \), 5\( \beta \)-THAL-triacetate. When the aglycone thus obtained was chromatographed on paper in system I overnight, this material moved at the same rate as 3\( \alpha \), 5\( \beta \)-THAL but was slightly more mobile than 3\( \beta \), 5\( \alpha \)-THAL.

The urinary samples voided during the first 24hr of collection in rabbit-II and rabbit-III were analyzed similarly to that from rabbit-I. A similar pattern of urinary metabolites was observed (Table 2).


Discussion

The present studies have shown that a major urinary metabolite of [1, 2-³H]-aldosterone is monosulphate. The aglycone released from this conjugate was identified as 3α, 5β-THAL, although the possibility that this aglycone represented 3α, 5α-tetrahydroaldosterone could not be completely excluded. Thus, a major urinary metabolite of [1, 2-³H]-aldosterone in this species was characterized as 3α, 5β-THAL-monosulphate. In addition, a small amount of monoglucoconat of 3α, 5β-THAL as well as 3β, 5α-THAL was found in the urine. However, the position of hydroxyl in THAL nucleus, to which sulphuric acid or glucuronic acid was bound, could not be determined.

When analysis of urinary metabolites of [1, 2-³H]-3α, 5β-THAL was carried out and the metabolite profile was compared with that of [1, 2-³H]-aldosterone, there was observed a close similarity between these two profiles. This finding indicates that the conversion of aldosterone to 3α, 5β-THAL is needed before the conjugation processes take place.

The nature of urinary metabolites of aldosterone in the rabbit should be compared with that in man. In man, a major urinary metabolite of tritiated aldosterone was found to be the conjugate which was hydrolyzable with mammalian liver or bacterial β-glucuronidase (Ulick et al., 1958; Flood et al., 1961; Coppage et al., 1962; Luetzcher et al., 1963; Pasqualini et al., 1965) and this conjugate was characterized as 3-monoglucosiduronate (Pasqualini et al., 1965); a major component of aglycones was identified as 3α, 5β-THAL (Kelly and Lieberman, 1964). Thus, 3α, 5β-THAL-3-glucosiduronate was found to be a major urinary aldosterone metabolite in man. In addition, a small amount of acid-labile conjugate was found in human urine as a metabolite of tritiated aldosterone (Flood et al., 1961; Coppage et al., 1962; Luetzcher et al., 1963) and this conjugate was characterized as aldosterone-18-glucosiduronate (Underwood and Tait, 1964; Pasqualini et al., 1965; Carpenter and Mattox, 1976). The possibility exists that a portion of subfraction A-2, A-3 or A-4 from rabbit urine represents aldosterone-18-glucosiduronate. However, even if this is the case, this substance would comprise, at most, 3% of urinary tritium. Moreover, aldosterone-21-sulphate was identified as a minor aldosterone metabolite in the human urine (Grose et al., 1973). Although analysis of fraction C from the rabbit urine has not been performed in the present studies, it might be that a part of this material represents aldosterone-21-sulphate. In man, some 50% of urinary metabolites of aldosterone has not been identified. Therefore, it is possible that a portion of these unidentified metabolites represents 3α, 5β-THAL-monosulphate.

Furthermore, the finding that a major urinary metabolite of aldosterone as well as 3α, 5β-THAL in the rabbit is monosulphate is in striking contrast to those obtained for estrone and estriol, in which cases major urinary metabolites are diconjugates (glucosiduronate-N-acetylglucosaminide (Layne et al., 1964; Miyazaki, 1980).

References


