

RADIOIMMUNOASSAY MEASUREMENT OF THE
SECRETION AND EXCRETION RATES OF
ALDOSTERONE* IN THE GUINEA PIG

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ABSTRACT

Following the subcutaneous injection of [³H]-aldosterone into guinea pigs, the pattern of tritiated metabolites of the hormone appearing in the urine has been studied. About 60% of the tritiated dose appeared in the urine, within 3 days of administration, indicating the urinary to be the major route of excretion of metabolites of the hormone. Fifty-one per cent of the tritiated dose appeared in the 0-24 urine, and 4.6% of the administered tritium could be extracted from this urine at neutral pH, 6% after pH 1.0 hydrolysis for 24 hr at R.T., 2.8% after glucuronidase hydrolysis and 26% with butanol. About 1.9% (corrected for recovery) of the administered dose was shown to be present in the neutral extract, specifically as aldosterone. No aldosterone could be found in the 24-48 and 48-72 hr urines.

Aldosterone secretion rates were determined using a radioisotope dilution procedure. 0-24 hr urine collections were made following [³H]-aldosterone administration to the animals, and the urine was extracted at neutral pH with methylene chloride. The aldosterone in the neutral extract was isolated and purified by celite partition column chromatography, and its mass was measured by the radioimmunoassay method of Underwood and Williams (J. Lab. Clin. Med. 79:848, 1972), and the tritium assayed by liquid scintillation counting. The secretion rate was calculated from the specific activity of the excreted aldosterone.

Excretion rates were determined from the amount of aldosterone measured in the neutral extract of a 0-24 hr urine. The value of the mass assayed was corrected for losses involved in the process of isolation and purification.

*Aldosterone = 11 β ,21-dihydroxy-3,20-dioxo-4-pregnen-18-al-11,18-hemiacetal.

tion by adding [^3H]-aldosterone to the urine immediately following collection to act as an internal recovery indicator. For nine animals, the mean value for the secretion rate was 2.9 ± 0.9 (S.D.), range 2.1-4.5 $\mu\text{g}/\text{day}$. The mean value for the excretion rate was 67 ± 34 (S.D.), range 31-136 ng/day . These results show that the metabolism of aldosterone in the guinea pig is very different from that in man, and on a body weight basis, the guinea pig secretes three times more aldosterone than the human.

INTRODUCTION

A previous study by Finkelstein (1) on the metabolism of [^3H]-aldosterone in the guinea pig revealed that, unlike in man (2,3), the major urinary metabolites of the hormone were not present as β -glucuronidase or acid hydrolysable conjugates, and were very water soluble and not readily extracted with organic solvents. Thus, a study was undertaken with a view to isolating and measuring a metabolite of aldosterone so that the secretion rate of the hormone could be determined, and with a particular objective of extracting, isolating, and identifying the polar water soluble conjugates. This paper describes the measurement of free aldosterone excreted in the urine and its use to determine the secretion rate of the hormone.

MATERIALS AND METHODS

1. Materials

[1,2- ^3H]-aldosterone, 52 Ci/mM, and [4- ^{14}C]-aldosterone, 46 mCi/mM (New England Nuclear Corporation, 575 Albany Street, Boston, Mass.). Purified by column partition chromatography using the Bush 5 solvent system. Aldosterone and aldosterone-18,21-diacetate* (Ikapharm, Ramat-Gan, Israel), were used without further purification. Methanol, cyclohexane, benzene (spectroquality-Matheson, Coleman & Bell, Curtin Scientific Co., Olympia Park, 3 Normac Rd., Woburn, Mass.). Skellysolve C (Skelly Oil Co., Kansas City, Missouri). Celite 545 - acid washed (Fisher Scientific Co., 461 Riverside Ave., Medford, Ma.).

*Aldosterone - 18,21 diacetate = 11β -hydroxy-18,21 diacetoxy-3,20 dioxo-4-pregnen-11,18-hemiacetal.

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Was further washed with acid, then with water until neutral. PCSTM Solubilizer (Amersham/Searle Corp., 2676 So. Clearbrook Drive, Arlington Heights, Illinois).

2. Paper chromatography

Chromatography was carried out at 31° constant temperature. Solvent systems used were: Bush 5 (benzene: methanol:water, 1:4:1); Bush 3 (skellysolve:benzene: methanol:water, 2:1:4:1) and P1 (cyclohexane:benzene: methanol:water, 1:1:7:3, Kliman, B. and Peterson, R.E., J. Biol. Chem. 235:1040, 1960).

3. Urine collection

Ken-Kal random bred male and females guinea pigs were housed in metabolic cages and maintained on an unrestricted diet of regular laboratory guinea pig lab chow (0.63% Na, 1.44% K) and water for seven days. Urine was collected over the third day, for 24 hours. At the beginning of the 4th day, 10 μ Ci [³H]-aldosterone in 0.4 ml physiological saline containing 20% ethanol was injected subcutaneously into every animal and urine collected in 0-24, 24-48, 48-72 hr batches.

4. Assay of radioactivity

Aliquots of urine were pipetted into Wheaton glass liquid scintillation vials and 10 ml PCS were added. In the case of urine extracts or paper chromatogram eluates, aliquots were first taken to dryness (vac., 55°) before adding the PCS. Every vial was counted for a total of 4000 counts minimum in a Nuclear Chicago Unilux II liquid scintillation counter. When both tritium and [¹⁴C] were present, the contributions from [³H] and [¹⁴C] were calculated according to the method of Okita et al. (4). The counting error was 2%.

5. Determination of the aldosterone secretion rate (ASR) from the specific activity of [³H]-aldosterone extracted from urine at neutral pH.

(a) Extraction of urine

1/4 vol of the 0-24 hr total urine volume collected from each animal following the administration of [³H]-aldosterone was adjusted to pH 7.0, made up to a total volume of 20 ml with water and extracted with 1 x 1 vol, 2 x 1/2 vol methylene chloride. The combined methylene chloride extract was washed with 5 ml water, then taken to dryness (vac., 55°).

(b) Column partition chromatography

The column and solvent system used was that described by Flood et al. (2). Aldosterone appears in fractions 10-12 from this column. The dried extract was dissolved in 5 ml mobile phase and added to the top of the column. The column was developed with mobile phase and the eluate collected in 5 ml fractions. An aliquot from every fraction (1-25) was taken to dryness and assayed for tritium. For every one of the nine extracts chromatographed, a tritium peak was found between fractions 9-13 and the three peak fractions for a particular extract were combined and taken to dryness.

(c) Determination of the radiochemical purity of the urinary [^3H]-aldosterone

Aliquots of the [^3H]-aldosterone from the column for every sample were combined (185,000 dpm [^3H] total) and 182,000 dpm [^{14}C]-aldosterone and 50 μg standard aldosterone to act as carrier were added and the sample submitted to successive paper chromatographies in the systems Bush 5, Bush 3, and PI. After chromatography in Bush 5 and before chromatography in Bush 3, the eluate was taken to dryness in a 25 ml r.b. flask and acetylated with 0.3 ml pyridine and 0.15 ml acetic anhydride for 16 hr at R.T. Excess pyridine and acetic anhydride were removed (vac., 55°), 1 ml ethanol was added and the mixture again evaporated. The carrier aldosterone and aldosterone diacetate were detected on the paper chromatograms by absorption of uv light in the short wavelength (240 m μ) region. An aliquot of the eluate from every chromatography was assayed for [^3H] and [^{14}C].

(d) Measurement of the specific activity (s.a.) of the purified urinary aldosterone.

The [^3H]-aldosterone from the column was chromatographed on paper in the Bush 5 system and an aliquot of the eluate from the paper chromatograms was assayed for radioactivity and duplicate aliquots were taken for radioimmunoassay. The complete procedure for chromatography and assay is described in the method of Underwood and Williams (3). The specific activity in dpm/ μg was calculated.

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(e) Calculation of the secretion rate

The ASR in $\mu\text{g/day}$ was calculated from the

formula:

$$\text{ASR} = \frac{[\text{3H}]\text{-aldosterone injected (dpm)}}{\text{s.a. urinary aldosterone (dpm } \mu\text{g)}}$$

6. Determination of aldosterone excretion rate (AER)

4475 dpm $[\text{3H}]\text{-aldosterone}$ to act as internal indicator were added to 1/10 vol of the 24-hr urine collected over the third day. The urine was extracted with methylene chloride and the extract processed and submitted to column chromatography by the procedure described above for measuring the secretion rate. The specific activity (dpm/ng) of the purified aldosterone was calculated and the AER (ng/day) determined from the formula:

$$\text{AER} = 10 \times \frac{[\text{3H}]\text{-aldosterone internal indicator added (dpm)}}{\text{s.a. urinary aldosterone (dpm/ng)}}$$

7. Recovery of the injected tritiated dose in urine.

A 0.3 ml aliquot from every 0-24, 24-48, 48-72 hr urine was assayed for radioactivity.

8. $[\text{3H}]\text{-aldosterone}$ metabolites extracted from the 0-24 hr urine.

(i) At pH 7.0 and then after acid hydrolysis (pH 1.0, 24 hr, 25°). 1/10 vol of the urine was adjusted to pH 6.5-7.0 and extracted with 1 x 1 vol, 2 x 1/2 vol methylene chloride. The combined extract was washed with 2 ml water and an aliquot assayed for tritium.

(ii) After $\beta\text{-glucuronidase}$ hydrolysis. 1/10 vol of the urine was extracted with 1 x 1 vol, 2 x 1/2 vol methylene chloride and the extract discarded. The urine was then adjusted to pH 5.0, 0.5 ml (2500 F.U.) "ketodase" (Warner-Chilcott, N.J.) added, and incubated at 38° for 48 hr. After the incubation, the urine was extracted with 1 x 1 vol, 2 x 1/2 vol methylene chloride, the extract washed with 2 ml water and then an aliquot assayed for tritium.

RESULTS AND DISCUSSION

The results on the radiochemical purity determination of the isolated [^3H]-aldosterone are shown in Table I. The percentage of the administered tritiated dose of aldosterone recovered in the crude urines and as metabolites extracted from the urine is shown in Tables II and III, respectively. Table IV shows the values obtained for the secretion rate in micrograms and the excretion rate in nanograms per day of aldosterone in the guinea pig.

The urinary excretion of 60% of the injected tritiated dose of aldosterone indicates the urine to be the major pathway for excretion of metabolites of the hormone in this animal. The percentage recovery of the injected tritiated dose from the urine by extraction with methylene chloride first at pH 7.0, then after acid hydrolysis, and also after β glucuronidase hydrolysis, 4.6, 6, and 2.8%, respectively, compared favorably with those from the study of Finkelstein (1), i.e., 2.5, 2.5, and 4%, respectively. More than half of the metabolites appearing in the 0-24 urine and representing 26% of the tritiated dose were extracted into butanol after first extracting the urine with methylene chloride. No aldosterone was reported as being found in any of the urine extracts in the previous study (1). However, we found 1.9% of the administered tritiated dose appearing in the urine as free aldosterone which was extracted from the urine at neutral pH. This figure is fully corrected for losses involved in the process of isolating and purifying the tritiated aldosterone prior to specific measurement. In the test for radiochemical purity of the isolated [^3H]-aldosterone, the constant [$^3\text{H}/^{14}\text{C}$] rates over three chromatographies confirmed radiochemical specificity at the stage of assaying both the mass and tritium of the [^3H]-aldosterone, (i.e., after chromatography in the Bush 5 system).

The mean value for the secretion rate was 2.9 ± 0.9 (S.D.) $\mu\text{g}/\text{day}$, range 2.1-4.5 and for the excretion rate 67 ± 34 (S.D.) ng/day , range 31-136.

The metabolism of aldosterone in the guinea pig is very different from that in man. In terms of percentage recovery of administered tritiated dose, 4.6% was extracted at neutral pH into methylene chloride and fractionation of this extract revealed aldosterone present to the extent of

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1.9%. When the urine was first extracted at neutral pH, then hydrolyzed at pH 1.0, 24 hr at 25° about 6% was then extracted into methylene chloride. However, when this extract was fractionated and specifically assayed for aldosterone, none of the hormone was found to be present. In contrast, in man, less than 0.2% is extracted from the urine at pH 7.0, and none of this tritium has been shown to be associated specifically with aldosterone. 14% is extracted after pH 1.0 hydrolysis and nearly all (> 95%) of the tritium in this extract is specifically associated with aldosterone.

When the daily secretion rate is calculated on a per kilogram body weight basis, then the guinea pig (500 gm, ASR 2.9 µg/day) compared with the human (80 kg, ASR 150 µg/day) secretes three times the amount of aldosterone.

ACKNOWLEDGEMENTS

This work was supported in part by the John A. Hartford Foundation, Grant 9893.

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TABLE I

Radiochemical purity of urinary free [^3H]-aldosterone. Ratios of [$^3\text{H}/^{14}\text{C}$] after successive paper chromatographies in Bush 5, Bush 3, P1 systems.

<u>Solvent</u>	<u>$^3\text{H}/^{14}\text{C}$</u>
Bush 5	0.29
Bush 3	0.29*
P1	0.27*

*As aldosterone diacetate

TABLE II

Per cent recovery of injected [^3H]-dose in urine.

<u>Animal</u>	<u>Urine collection, hr</u>			
	0-24	24-48	48-72	0-72
1	64.4	6.2	1.1	71.7
2	47.9	6.7	1.9	56.5
3	51.6	7.1	1.2	59.9
4	41.4	8.2	3.0	52.6
5	25.1	4.5	1.4	31.0
6	52.3	5.6	1.7	59.6
7	65.2	5.4	1.3	71.9
8	59.4	5.3	1.6	66.3
9	54.9	7.0	1.7	63.6
Mean	51.1	6.2	1.6	59.0

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TABLE III

Per cent recovery of injected [^3H]-dose in extracts from 0-24 hr urine: (1) at pH 7.0; (2) after acid hydrolysis (pH 1.0, 25 $^\circ$, 24 hr); (3) after β -glucuronidase hydrolysis; and (4) with butanol.

<u>Animal</u>	<u>Extract</u>			
	1	2	3	4
1	4.3	5.7	2.0	Combined urines 1-9
2	3.7	4.5	2.0	
3	3.6	4.2	2.4	
4	4.1	3.8	1.5	
5	4.0	8.7	4.9	
6	5.8	6.4	3.1	
7	5.8	7.9	3.2	
8	5.3	6.9	3.5	
9	4.9	8.0	2.4	
Mean	4.5	6.2	2.8	25.8

TABLE IV

Aldosterone secretion and excretion rates

<u>Animal</u>	<u>Excretion (ng/day)</u>	<u>Secretion $\mu\text{g/day}$</u>
1	64	2.8
2	63	2.1
3	85	2.2
4	86	2.3
5	42	2.4
6	83	2.9
7	136	3.1
8	31	4.2
9	47	4.5
Range	31-136	2.1-4.5
Mean	67	2.9
S.D.	± 34	± 0.9

