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THE MEASUREMENT OF URINARY TETRAHYDROALDOSTERONE BY RADIOIMMUNOASSAY

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SUMMARY

A simple and specific radioimmunoassay is described for measuring urinary tetrahydroaldosterone (THAldo). The antiserum used is highly sensitive to aldosterone lactone, but is sufficiently sensitive to tetrahydroaldosterone lactone when the antibody dilution is reduced from 1:500,000 to 1:20,000. Specific activities of urinary THAldo determined by the radioimmunoassay were in close agreement with values obtained by a colorimetric method ($r = 0.966$). Subjects treated with ACTH showed a transitory increase in THAldo excretion when THAldo was quantitated by the radioimmunoassay. These findings differ from the sustained increase in THAldo excretion in response to ACTH reported on previously by this laboratory during a time when a colorimetric method was used to measure THAldo. The radioimmunoassay appears to be more specific than the colorimetric method when steroidogenesis is increased with ACTH. The measurement of urinary THAldo excretion by radioimmunoassay serves as a useful alternative to the more time consuming colorimetric procedures.

INTRODUCTION

Aldosterone secretion can be conveniently assessed by measurements of aldosterone metabolic products in urine. Excretion of the acid-labile, aldosterone 18-glucuronide has become representative of aldosterone secretion in most laboratories because of less complicated methodology involved in its quantitation. The conjugate of tetrahydroaldosterone (THAldo), whose discovery and characterization were carried out by Ulick and co-workers [1, 2, 3], is the most abundantly excreted aldosterone metabolite. Its formation in the liver is in contrast to the predominantly renal origin for the aldosterone 18-glucuronide [4]. A simple and specific tetrahydroaldosterone lactone (THAldo-lactone) radioimmunoassay developed as an alternative to the time consuming colorimetric methods [2, 5] is described here. The antiserum is highly sensitive to aldosterone lactone (aldolactone) but was found to have considerable sensitivity to THAldo-lactone when the antibody dilution was reduced to 1:20,000 from 1:500,000.

MATERIALS AND METHODS

Subjects. Following the intravenous injection of 5 μCi of [1,2-^{3}H]-aldosterone, 24 h urine samples were collected from 13 patients undergoing evaluation for hypertension, 1 patient with Cushing's disease, 2 patients with idiopathic edema, and 1 normal subject on a high NaCl intake. In addition, 5 normal adult men ingesting greater than 200 mEq of NaCl/d collected 24 h samples before and during a 3-day period when ACTH gel 60 units was administered at noon on day 1, at 8 am and 8 pm on day 2 and at 8 am on day 3.

Materials. [1,2-^{3}H]-aldosterone (60 Ci/mmol) and [1,2-^{3}H]-THAldo (54 Ci/mmol) were purchased from New England Nuclear. All solvents were reagent grade and used without further purification with the exception of dichloromethane and acetone which were distilled over anhydrous potassium carbonate. Thin layer chromatography plates were prepared by mixing silica gel GF-254 with 2% $Na_2B_4O_7 \cdot H_2O$ in water–0.1 *N* NaOH, 9:1 (v/v) with the slurry spread at a thickness of 375 microns. Periodic acid solution was prepared just prior to use by dissolving 0.57 g of periodic acid. (G. Frederick Smith Chemical Co.) in 25 ml of ethanol. In 100 ml solution of pH 7 phos-

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The following trivial names have been used: aldosterone = 11β,21-dihydroxy-18-oxo-4-pregnene-3,20-dione; aldosterone lactone (aldolactone) = 3-oxo-11β,18-epoxy-4-pregnene-17β,18-carbolactone; tetrahydroaldosterone (THAldo) = 3α,11β,21-trihydroxy-18-oxo-5β- pregnane-20-one; tetrahydroaldosterone lactone (THAldolactone) = 3α-hydroxy-11β,18-epoxy-5β-pregnane-17β,18-carbolactone; aldosterone 18-glucuronide = 11β, 21-dihydroxy-18-oxo-4-pregnane-3,20-dione-18-glucuronide; 18-hydroxy-11-dehydrotetrahydrocorticosterone (18-OH-THA) = 3α,18,21-trihydroxy-5β-pregnane-11,20-dione; 18-hydroxy-11-dehydrotetrahydrocorticosterone lactone (18-OH-THA lactone) = 3α-hydroxy-5β-pregnane-17β,18-carbolactone-11-one.

phate buffer containing 50 mg dextran, 500 mg of Norit A charcoal was suspended.

[1,2-^{3}H]-THAldolactone was purified by thin layer chromatography on silica gel plates in the solvent system benzene:ethanol, 90:10 (v/v).

THAldolactone standard was quantitated as follows: THAldo produced by the method of August[6] was combined with [1,2-^{3}H]-THAldo, and the S.A. determined after quantitation of THAldo by the modified Porter–Silber reaction [7]. Following oxidation with periodic acid and thin layer chromatography in the system benzene:ethanol, 90:10 (v/v), the radioactivity of the THAldolactone was counted. The quantity of THAldolactone was calculated from the previously established S.A. (300 c.p.m./μg).

The aldolactone antibody was prepared in sheep by injection of the 3-methoxine derivative of aldolactone conjugated to bovine serum albumin [8]. A solution containing 10,000 c.p.m./ml of [1,2-^{3}H]-THAldolactone and 0.5 mg/ml of bovine gamma globulin (Sigma Chemical Co.) was prepared in phosphate buffer in which the aldolactone antiserum was diluted 1:20,000. A "non-specific" solution was prepared in the same proportions except for deletion of the antibody.

18-Hydroxy-tetrahydro-11-dehydrocorticosterone (18-OH-THA) was obtained from human urine samples following extraction and purification procedures previously described [5]. Quantitation was performed by the Porter–Silber reaction [9].

Buffers. Acetate and phosphate buffers were prepared as follows: 50.4 gm sodium acetate and 36 ml of glacial acetic acid were diluted to 3 liters with water; 8.67 g Na_2HPO_4 and 4.97 g $NaH_2PO_4 \cdot H_2O$ were diluted to 1 liter with water.

Hydrolysis. Urine was pre-extracted with 5 vol. of dichloromethane. 1/400 of a 24 h sample was adjusted to pH 4.5 with acetate buffer containing freshly added β-glucuronidase (Glusulase®, Endo Lab Inc., 1,000 units/ml buffer with a ratio of urine to buffer 2:1) and incubated in a water bath at 37° for 18–24 h.

Extraction. When THAldo excretion rates were measured, approximately 5,000 c.p.m. of [1,2-^{3}H]-THAldo as tracer was added to the hydrolyzed urine. Samples were extracted with 10 vol. of dichloromethane, washed with 1/10 vol. 0.1 N NaOH, and then 1/10 vol. water. The dichloromethane was then dried down *in vacuo*. The addition of THAldo tracer was omitted when determining secretion rates.

Chromatography. Samples were spotted on borate thin layer plates and developed in the system: acetone:benzene:water, 90:10:8. Bromophenol blue dye was used as a marker for THAldo. The appropriate plate area was removed and suspended in 3.0 ml water, extracted with 30 ml dichloromethane and then washed twice with 1/10 vol. of water. The extracts were decanted into glass stoppered 50 ml tubes (oxidation tubes), and to each sample 0.5 ml of periodic acid solution was added. Samples were mixed and allowed to stand at room temperature for 30 min at which time oxidation was interrupted by shaking with 3.0 ml of 0.25 N $NaHCO_3$ solution. The aqueous phase was aspirated, and the samples were washed with 3.0 ml water, and finally dried down *in vacuo*.

Radioimmunoassay. Samples were dissolved in acetone and aliquoted as follows: radioactivity was counted in two-thirds for determining procedural losses, or for measuring specific activities; one-sixth was diluted 1:100, and 0.5, 1.0 and 2.0 ml aliquoted for quantitation. The standard curve was prepared over a range from 0 to 600 pg. All samples were dried under nitrogen, and 0.5 ml of antiserum added. To 2 tubes 0.5 ml of "non-specific" solution was added. Samples were incubated in a water bath at 37° for 1 h, and then at 4° for 16–18 h. The free from bound steroid was separated by addition of 0.5 ml of dextran coated charcoal. The samples were mixed, allowed to equilibrate in an ice bath for 20 min, and then centrifuged to separate the charcoal. Five-tenths ml of the supernate was pipetted into counting vials, 10 ml of counting solution added (a mixture of insta-gel, liquifluor and toluene) and the samples counted for a sufficient length of time to accumulate 20,000 counts. The "non-specific" radioactivity was subtracted from all samples, and the values for the 3 dilutions were averaged to calculate the amount of THAldo.

The S.A. of THAldo and the aldosterone secretion rate determined by radioimmunoassay were compared to values for the same specimens obtained by a colorimetric method [5]. These comparative data were subjected to linear regression analysis.

RESULTS

Recovery and standard curve. The recovery in 31 samples was 55.6 ± 7.8 (S.D.)% with a range of 40.4 to 71.4%. A typical standard curve plotted as free/bound vs concentration is depicted in Fig. 1 with a linear regression equation of $Y = 0.0091 \times + 0.588$. There was 64.8 ± 2.4 (S.D.)% binding to the antisera

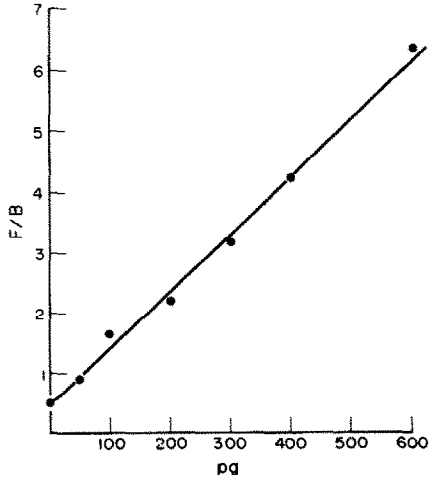

Fig. 1. Typical standard curve of tetrahydroaldolactone plotted as free/bound vs concentration.

of 1:20,000 dilution in the absence of unlabeled THAldo in 6 consecutive assays. "Non-specific" radioactivity was 45.7 ± 9.4 (S.D.) c.p.m. ($n = 6$).

Precision and sensitivity. The intra-assay variation, as determined by S.A. of THAldo after injection of labeled aldosterone, was 9.10% ($n = 7$) when the S.A. was 50,757 ± 4620 (S.D.) c.p.m./μg. The inter-assay variation was 10.29% ($n = 9$), when the S.A. was 30,590 ± 3149 (S.D.) c.p.m./μg. The useful range of the standard curve was 50 to 540 pg.

Specificity. The cross reaction of aldolactone antiserum with other steriods was negligible at a dilution of 1:500,000 [10]. Although in the present assay the dilution of the antiserum was considerably less, the likelihood of contamination seemed most unlikely with any of the known steroids after pre-extraction of urine, chromatography and oxidation to the lactone with the possible exception of 18-OH-THA which is excreted in quantities approximately twice that of THAldo [3]. The cross reaction of 1,000 pg of 18-OH-THA lactone was, however, only 5%. In addition, 18-OH-THA separated from THAldo in the thin layer chromatography step (R_F values 0.32 and 0.64 for 18-OH-THA and THAldo respectively). Additional proof of specificity of the method was shown by chromatographing the product after the oxidation step in the thin layer silica gel system benzene:ethanol, 80:20 (v/v). The S.A. of the THAldo lactone was 30,090 vs 30,850 c.p.m./μg, prior to and following the above procedure respectively.

Comparison of results by radioimmunoassay vs colorimetry. In Table 1, S.A. and secretion rate data obtained by radioimmunoassay and by colorimetry are presented for comparison. The correlation is close for essentially all samples throughout a wide range of values. A comparison of the two methods by linear regression analysis revealed close agreement with $r = 0.966$. The correlation, however, was not always good between the two procedures when measurements were performed on urine after ACTH stimulation (45.4 μg/24 h *vs* 83 μg/24 h by the RIA and colorimetric procedures, respectively). This suggests that the immunoassay may be more specific than the colorimetric procedure under conditions where large quantities of steroid metabolites, other than THAldo, are excreted.

Response to ACTH treatment (Fig. 2). Four of five subjects treated with ACTH showed an initial rise in the excretion rate of THAldo; however, by the third day of treatment, excretion of THAldo had decreased to basal levels. In one subject, THAldo excretion fell progressively with ACTH administration.

DISCUSSION

As an alternative to the time consuming colorimetric methods for measuring urinary THAldo [2, 5], a simple and specific radioimmunoassay is described. The aldosterone secretion rate data obtained by radioimmunoassay are essentially indistinguishable

Table 1. Comparison of radioimmunoassay vs. colorimetry in measuring THAldo S.A. and aldosterone secretion rate

	S.A., c.p.m./μg		ASR, μg/24 h	
	Immunoassay	Colorimetric	Immunoassay	Colorimetric
Normal renin HT* (128 mEq/d)†				
1	21,700	16,000	138	187
2	19,600	20,400	153	147
3	26,700	34,000	112	88.2
4	33,000	32,600	90.9	92.0
5	32,800	28,000	91.5	107
6	22,200	23,000	135	130
Normal renin HT (10 mEq/d)				
7	11,500	13,800	261	217
8	12,300	15,700	244	191
9	13,400	13,600	224	220
10	14,800	14,700	203	204
11	3,150	3,770	952	796
High renin HT (128 mEq/d)				
12	7,290	7,650	411	392
13	9,760	8,740	307	343
Cushing's disease				
14	22,110	23,300	136	129
Idiopathic edema				
15	31,700	26,000	94.6	115
16	39,500	39,000	75.9	76.9
Normal subject (> 200 mEq/d)				
17	218,140	266,000	13.7	11.3

* HT = hypertension. † Daily intake of NaCl.

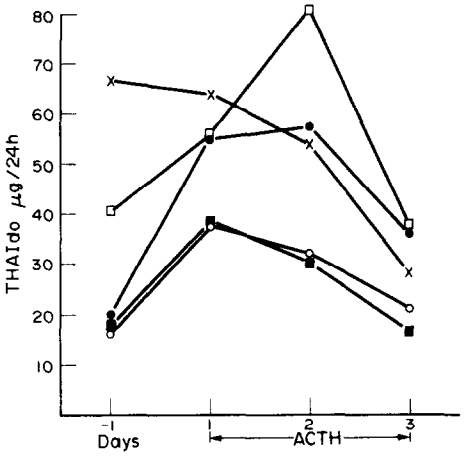

Fig. 2. Daily excretion of tetrahydroaldosterone in 5 normal men ingesting > 200 mEq of NaCl/d before and during treatment with repository ACTH.

from those found using a colorimetric method that has had extensive use in this laboratory over the past years [5]. Avoided is the extraction of large urine volumes (1/400 *vs* 1/10 of a 24 h urine), and a single chromatographic system replaces three with the older method.

Changes in metabolism of aldosterone arise primarily through alteration of events within the liver. Unlike the more commonly measured metabolite of aldosterone, the aldosterone 18-glucuronide formed primarily in the kidney, THAldo is hepatic in origin [4] and the most abundantly excreted aldosterone metabolite. Measurement of its excretion can point out disturbances in the normal processes of aldosterone metabolism. In patients with liver disease [11] and certain patients with hypertension [12], THAldo excretion is decreased relative to the 18-glucuronide. An unexplained shift toward a preponderance of the 18-glucuronide has been observed during pregnancy [13]. Similar studies can be more easily extended to other clinical situations with the addition of a simplified THAldo assay.

Earlier we showed that administered ACTH increases the metabolic clearance rate of aldosterone [14]. A sustained increase in THAldo excretion during prolonged ACTH treatment suggested that greater hepatic metabolism accounted for the increment in metabolic clearance. At that time in our laboratory, the colorimetric method was used for measuring THAldo [5]. In the present study, using the THAldo radioimmunoassay, individuals treated with ACTH showed a transient increase in THAldo excretion, consistent with the excretion pattern for the aldosterone 18-glucuronide in response to ACTH [15 19]. From this it appears that, in contrast to the colorimetric method [5], the radioimmunoassay is more specific when steroidogenesis is increased with ACTH. The immunoassay includes a chromatographic separation and lactone derivative formation, thus eliminating the major amount of interfering substances either by separation or removal by conversion to etio-acids by oxidation. The specificity of the antisera also assures that only THAldo is measured. The colorimetric method, while demonstrating validity under most circumstances, relies on three chromatographic steps for purification, oxidation to the C-21 aldehyde and measurement of the Porter–Silber chromogen produced. Slight contamination by one or more compounds capable of oxidation by cupric acetate, especially if steroidogenesis is increased with ACTH, would give invalid results. We must, therefore, retract statements made earlier in regard to what we considered a possible sustained aldosterone secretory response to ACTH and, in addition, our conclusions about the mechanism of how ACTH effects aldosterone metabolism. Although chronic ACTH treatment may increase liver blood flow and thus increase the rate of hepatic metabolism of aldosterone, this remains unproven. ACTH treatment has been found to decrease binding of aldosterone to plasma proteins [20–21], and to cause a redistribution of aldosterone from plasma to red cells [22]. Either effect could explain the increase in the plasma clearance of aldosterone observed with ACTH administration.

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