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EVALUATION OF RADIOIMMUNOASSAY FOR ALDOSTERONE IN URINE AND PLASMA OF RATS*

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SUMMARY

A method is described for the measurement of aldosterone in urine and plasma of rats and the sensitivity, specificity, accuracy and precision of the assay are assessed. The method includes one extraction step by methylene chloride, purification of the extract by one thin layer chromatography and determination of the purified aldosterone by radioimmunoassay. [^{3}H]-Aldosterone is used for the radioimmunoassay as well as for the determination of the recovery of the individual sample. A modified calculation of the radioimmunoassay results is described to correct for the varying amounts of [^{3}H]-aldosterone of the recovery preexisting in the unknowns but not in the standards of the calibration curve. Normal values of plasma aldosterone concentration in adult male Sprague–Dawley rats (23–288 pg/ml depending on the time of day) as measured with our assay are in good agreement with the values reported in the literature. Normal values of urinary aldosterone excretion rate in these rats (3.9–5.8 ng/24 h) are much lower than those reported in the literature possibly due to the higher specificity of the present assay.

INTRODUCTION

In the rat, *in vivo* studies on aldosterone have been restricted for a long time to the determination of aldosterone secretion in surgically stressed animals (for review see [1]). Due to the development of highly sensitive radioimmunoassays (RIA) the determination of physiological concentrations of aldosterone in the blood of intact, i.e., conscious and unstressed rats has become possible [1–6]. We recently also reported on aldosterone excretion rates in the urine of rats [7, 8].

Since data on urinary aldosterone in the rat as measured by RIA have not been published yet, we herein describe a RIA for the measurement of aldosterone in urine and plasma of rats using extraction and thin layer chromatography (t.l.c.) for separation of aldosterone. This assay has been used routinely in our laboratory during the last 4 years and has proven to be comparatively rapid and simple.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats (200–250 g) were kept in a room with controlled temperature (23–24°) and humidity (>50%) under a lighting regime of 12 h light (06.00–18.00) and 12 h dark (18.00–06.00). The animals were maintained on a standard diet (Na^+ 96 mmol/kg, K^+ 143 mmol/kg; Altromin. Lage, Germany) and tap water *ad libitum* or on a low-sodium diet (Na^+ 4.4 mmol/kg, K^+ 60 mmol/kg; Altromin) and demineralized water *ad libitum*. For determination of plasma aldosterone rats were decapitated without anaesthesia and blood was collected from the trunk using EDTA (1 mg/ml) as anticoagulant. Plasma was separated by centrifugation at 0° and stored at −20° until assayed. For measurement of urinary aldosterone excretion rats were placed in individual metabolic cages made of plexiglas with free access to food and water, the urine—separated from feces and spilled food—was collected in 25 ml-Erlenmeyer flasks. After that urine samples were stored at −20° until assayed. Plasma corticosterone concentrations were identical in rats kept in usual cages or kept in metabolic cages indicating that sitting in a metabolic cage was no stress for the animals.

Materials. Methylene chloride, benzene, acetone (all technical grade) were obtained from Merck (Darmstadt) and distilled over a 120 cm packed column filled with wire-mesh rings. Toluene (technical grade, Merck) and Triton-X-100 (technical grade, Serva, Heidelberg) were used without further purification. Precoated t.l.c. plates containing a fluorescence indicator (DC-Alufolien, Kieselgel F 254, Merck) were used as purchased. Chromatography was performed in the solvent system benzene–acetone–H_2O (3:2:0.025 by vol) (system A) or in the system ethylacetate–acetone–H_2O (6:1:0.025 by vol) (system B). [1.2.6.7-^{3}H]-Aldosterone (S.A. 103 Ci/mmol, Radiochemical Centre, Amersham) was stored in benzene:ethanol (9:1 by vol) at 4°. Radiochemical purity was checked by t.l.c. at 3 months intervals. A measureable decomposition of 5% was observed after 6 months of storage and the tracer was purified by t.l.c.

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using solvent system A. D-aldosterone was kindly supplied by Ciba and was used without further purification. The antiserum against aldosterone-21-hemisuccinate was raised in a sheep [10]. Borate buffer (0.05 M, pH 8), γ-globulin buffer (borate buffer containing 1% of bovine immunoglobulin, Behring, Marburg) and Dextran-coated charcoal (6 g Norit A, Serva, and 0.2 g Dextran T 70, Pharmacia, Uppsala, suspended in 100 ml of distilled water) were stored at 4° and were stable for at least 1 month. The scintillation fluid consisted of 40 g PPO (2.5-diphenyloxazole, Merck), 1 g POPOP (1.4-bis-2-(5-phenyloxazolyl)-benzene, Merck), 2 l of Triton-X-100 and 8 l of toluene.

Extraction and purification of plasma and urine samples. 0.05-4 ml of plasma or urine were mixed with known amounts of [^{3}H]-aldosterone (about 5000 d.p.m. = 8 pg) as internal standard for determination of individual recovery. After an equilibration period of 12 h the samples were extracted with 20 ml of methylene chloride by magnetic stirring for 15 min. The aqueous layer was discarded and the filtered organic extract evaporated. The dry extracts were dissolved in acetone (3 × 0.2 ml) and transferred to the t.l.c. plates using a Desaga-autoliner. The plates were developed in the solvent system A. Aldosterone was located by running spots of unlabeled aldosterone (about 0.5 μg) with each plate and examining the plates under U.V. light at 254 nm. The areas corresponding to aldosterone were cut out and eluted with 5 ml of acetone. The eluates were evaporated and redissolved with 0.35 ml of borate buffer. For determination of recovery 0.1 ml of the eluates were transferred to counting vials and 0.4 ml of borate buffer plus 10 ml of the scintillation fluid were added to achieve counting conditions identical to those of the RIA samples. For determination of the aldosterone concentration preexisting in the antiserum 50 μl of a 1:100 antiserum dilution were treated as described above but extraction with methylene chloride was repeated three times resulting in a complete extraction as checked by radioactive recovery.

RIA procedure. Duplicate aliquots (0.1 ml) of the eluates and triplicate standards containing 0–1000 pg of aldosterone in 0.1 ml of borate buffer were mixed with [^{3}H]-aldosterone (10,000–15,000 d.p.m. = 16–24 pg in 0.5 ml of borate buffer) and antiserum diluted with γ-globulin buffer (0.05 ml, final antiserum dilution in the 0.65 ml sample 1: 500,000). The tubes were mixed and incubated at 0° for 20 h. Separation of free and bound aldosterone was achieved by 0.05 ml of the Dextran-coated charcoal suspension simultaneously mixed with all assay tubes by shaking the coal from polyethylene caps fitted to the assay tubes. All samples were centrifuged simultaneously for 5 min at 0° and 2500 *g*. 0.5 ml-Aliquots of the supernatant together with 10 ml of the scintillation fluid were transferred to counting vials and counted in a Mark II Liquid Scintillation Counter (Nuclear Chicago).

Construction of the standard curve. "Absolute % bound" were calculated according to the formula: $B - N/T - N \times 100$, where B = c.p.m. bound, T = total c.p.m., N = non specific c.p.m. (all counts based on the 0.5 ml-aliquot out of 0.7 ml of supernatant) and plotted vs. the total pg of the standards on a logit/log paper. Total pg of the standards were calculated as the sum of pg of standard aldosterone added plus pg of [^{3}H]-aldosterone (calculated from the S.A.) plus pg of aldosterone preexisting in the antiserum (5 μg/ml in the original antiserum corresponding to 6.5 pg/0.65 ml in the 1:500,000 antiserum dilution). By this procedure a linear logit/log calibration curve was obtained in the range of 30–530 pg of total aldosterone, i.e., 0–500 pg of standard aldosterone added (Fig. 1).

Calculation of the unknowns. Since the total counts

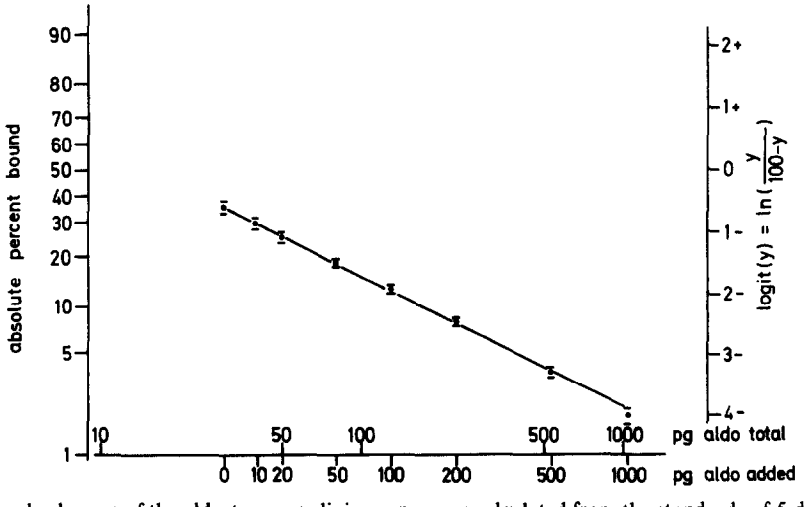

Fig. 1. Standard curve of the aldosterone radioimmunoassay calculated from the standards of 5 different assays performed during 1 week ($\bar{x} \pm 2$ S.D., $N = 15$). "Absolute percent bound" are plotted on a logit scale (ordinate) vs. the total amount of aldosterone (standard aldosterone added *plus* pg of [^{3}H]-aldosterone *plus* pg of endogenous aldosterone preexisting in the antiserum) on a log scale (abscissa). Linear regression line (least square method): $Y = -2.13\,x + 2.53$.

of the unknown samples were increased by the counts of the internal standard added for determination of individual recovery, it was necessary to compute "individual % bound": $(B - N) \times 100 : (T + 5/7\,R - N)$, where B = c.p.m. of an unknown, N = non specific c.p.m., T = total c.p.m. added to the RIA-sample, R = c.p.m. recovered in the 0.1 ml-aliquot of the respective unknown (R was reduced by 5/7, because all other c.p.m. were based on the 0.5 ml-aliquot out of 0.7 ml supernatant). Total pg of aldosterone were read of the standard curve, the pg of preexisting aldosterone (pg labeled aldosterone corresponding to the individual total counts plus pg endogenous aldosterone of the antiserum) were substracted and the netto pg obtained were corrected by the recovery of the respective 0.1 ml-aliquot of the unknown. A programable mini-computer (Hewlett–Packard, HP 65) was used for all calculations including logit transformation, linear regression and interpolation of the assay data. Plasma aldosterone concentrations were calculated as pg/ml and urinary aldosterone excretion rates were computed by multiplying urinary aldosterone concentration by the respective urine vol.

Determination of the specific activity of [^{3}H]-aldosterone by RIA. Increasing amounts of extra [^{3}H]-aldosterone were added to zero-standards and processed together with a usual standard curve (see Table 1). Individual total counts (samples with extra [^{3}H]-aldosterone, but without charcoal separation) and individual non specific counts (samples with extra [^{3}H]-aldosterone, but without antiserum) were also measured. Individual % bound of the samples containing extra [^{3}H]-aldosterone were computed and the netto pg of aldosterone corresponding to extra [^{3}H]-aldosterone were calculated (total pg read of the standard curve minus total pg of zero-standards). S.A. was calculated as pg of aldosterone per 1000 d.p.m. at a counting efficiency of 49%. Mean S.A. of [1.2.6.7-^{3}H]-aldosterone as measured by RIA was 1.60 pg/1000 d.p.m. (=98 Ci/mmol) as compared to 1.59 pg/1000 d.p.m. (=103 Ci/mmol) as specified by the manufacturer.

RESULTS

Radioactive recovery. Overall recovery (after extraction and t.l.c.) of the internal radioactive standard was 73.2 ± 9.1% ($\bar{x}$ ± S.D., N = 108; range 54–83%) from rat plasma samples and was 76.6 ± 4.0% ($\bar{x}$ ± S.D. N = 100; range 67–85%) from rat urine samples.

Blank and sensitivity. Water blanks assayed in 10 different experiments gave a mean value of 10 ± 7.5 pg/sample ($\bar{x}$ ± S.D.) with a reading on the standard curve of 0–4 pg/tube. The sensitivity was calculated from 5 standard curves (see Fig. 1) according to [8]; it was 5 pg at the 95% confidence level (2.145 S.D., N = 15). Therefore, the least-detectable dose was 25 pg/original sample.

Accuracy. Aqueous standards of unlabeled aldosterone (500 pg or 1000 pg) were processed through the entire procedure in 13 different experiments. Recovery of unlabeled aldosterone ($\bar{x}$ ± S.D.) was 104 ± 6.5% (N = 6) and 97 ± 6.9% (N = 7), respectively.

Within-assay variation. Ten 3 ml samples of a pool of rat plasma (mean aldosterone concentration 63 pg/ml) and then 1 ml samples of another pool of rat plasma (mean aldosterone concentration 197 pg/ml) were run in a single experiment. The coefficients of variation were 9.5% and 5.6%, respectively. In eight 1 ml samples of a pool of rat urine (mean aldosterone concentration 403 pg/ml) the coefficient of variation was 4.3%.

Between-assay variation. 1 ml samples of a pool of rat plasma (mean aldosterone concentration 228 pg/ml) were assayed in 10 different experiments during a period of 3 months. The coefficient of variation was 8.8%. When twenty-two 1 ml samples of urine of different rats (aldosterone concentrations ranging from 200–700 pg/ml) were determined in 2 different assays, the coefficient of variation was 6.9%.

Specificity. Specificity is obtained by the chromatographic purification involved in the assay. As can be seen from Table 2 there is a definite separation of aldosterone from corticosterone, progesterone and 18-OH-deoxycorticosterone, which are the steroids most probable to crossreact with the antibody used [10]. In plasma samples (4 ml) of rats adrenalectomized 48 h prior to sacrifice and in urine samples (6–8 ml) of rats adrenalectomized 7 days prior to sampling no aldosterone could be detected. In some experiments samples of plasma and urine obtained from rats with experimentally induced low and high aldosterone levels were assayed after an additional purification by a second t.l.c. using solvent system B. The values measured after the second t.l.c. were compared with those obtained after a single t.l.c. using solvent

Table 1. Determination of the specific activity of [1.2.6.7-^{3}H]-aldosterone by radioimmunoassay

Sample No.	Extra [^{3}H]-aldo (c.p.m.)	Individual* total counts (c.p.m.)	Individual non specific counts (c.p.m.)	Counts bound (c.p.m.)	Individual % bound	Mass of [^{3}H]-aldosterone total pg	Netto pg†	S.A. pg aldo/ 1000 d.p.m.‡
1	25,362	32,736	210	4919	14.5	114	84	1.62
2	38,715	46,089	339	5185	10.6	158	128	1.62
3	51,147	58,521	454	5494	8.7	200	170	1.63
4	66,360	73,734	557	5898	7.3	235	205	1.51

* Individual total counts = c.p.m. of extra [^{3}H]-aldosterone plus 7374 c.p.m. of [^{3}H]-aldosterone added to the RIA as tracer. †Netto pg corresponding to extra [^{3}H]-aldosterone = total pg read of the standard curve minus total pg of zero standard (30 pg). ‡Calculation based on a counting efficiency of 49%.

Table 2. Separation of several steroids in the chromatographic systems used in the aldosterone assay

Steroid	Solvent system A (benzene–acetone–H_2O, 3:2:0.025, v/v) R_F-value	Solvent system B (ethylacetate–acetone–H_2O, 6:1:0.025, v/v) R_F-value
Progesterone	0.96	0.96
11-Deoxycorticosterone	0.84	0.91
Corticosterone	0.58	0.79
Cortisone	0.56	0.84
18-OH-Deoxycorticosterone	0.52	—
Cortisol	0.48	0.80
Aldosterone	0.38	0.50
18-OH-Corticosterone	0.28	—

system A (Table 3). The differences were within the variability of the assay and no systematic deviation was observed ($t > 1$, paired t-test).

Acid hydrolysis of rat urine. Four 1 ml-aliquots of a pool of rat urine were acidified to pH 1 with concentrated HCl and allowed to stand for 20 h at room temperature. Aldosterone concentration was determined and compared with unacidified samples of the same urine pool. Acid hydrolysis increased the concentration of urinary free aldosterone by 11% only.

Stability of aldosterone in rat urine. Since urine of rats was sampled without any preservative at 24° over a period of 24 h, the stability of urinary aldosterone was checked. A loss of more than 10% was not observed until 4 days of exposure to these conditions.

Normal values. Mean plasma aldosterone concentration ($\bar{x} \pm$ S.D., $N = 6$) in rats fed the standard diet and sacrificed at 08.00 or at 18.00 was 40 ± 17 pg/ml (range 23–72) and 153 ± 76 pg/ml (range 59–288), respectively. 24 h-Urinary aldosterone excretion ($\bar{x} \pm$ S.D., $N = 6$) in these rats measured on two subsequent days was 5.0 ± 0.9 ng/24 h and 4.5 ± 0.7 ng/24 h (range 3.9–5.8), respectively.

In rats under dietary sodium restriction for 2 weeks plasma aldosterone concentration ($\bar{x} \pm$ S.D., $N = 3$) was 9730 ± 710 pg/ml at 08.00 and was $17{,}210 \pm 1440$ pg/ml at 18.00. 24 h-urinary aldosterone excretion ($\bar{x} \pm$ S.D., $N = 5$) was 223 ± 34 ng/24 h in these rats.

Table 3. Comparison of aldosterone values obtained by the use of either a single t.l.c. (solvent system A) or of 2 subsequent t.l.c. (solvent system A followed by solvent system B)*

Treatment	Plasma aldosterone concentration pg/ml 1. t.l.c.	2. t.l.c.
Control (08.00)	52	57
Control (08.00)	47	51
Control (18.00)	116	106
Control (18.00)	118	110
ACTH 250 μg/kg†	365	327
ACTH 500 μg/kg†	479	456
Furosemide 25 mg/kg‡	451	681
Furosemide 25 mg/kg‡	388	368
	Urinary aldosterone excretion ng/2h h	
	1. t.l.c.	2. t.l.c.
DOC-Depot 2 mg/kg§	0.86	0.58
DOC-Depot 2 mg/kg§	0.84	0.61
Control	5.49	4.49
Control	4.69	4.64
ACTH 2 × 25 IU/kg/day‖	15.11	16.46
ACTH 2 × 25 IU/kg/day‖	15.79	16.11
Furosemide 25 mg/kg/day¶	20.71	21.74
Furosemide 25 mg/kg/day¶	19.36	20.54

* Data from experiments published elsewhere in full detail [8]. † ACTH (Synacthen®, Ciba) was injected i.v. 1 h prior to blood sampling. ‡ Furosemide (Lasix®, Hoechst) 25 mg/kg was injected s.c. 90 min prior to blood sampling. § DOC-Depot (Cortiron–Depot®, Schering) 2 mg/kg was injected s.c. 7 days prior to urine sampling. ‖ ACTH (Acethropan®, Hoechst) 2 × 25 IU/kg/day was injected s.c. for 2 days prior to urine sampling. ¶ Furosemide (Lasix®, Hoechst) 1 × 25 mg/kg/day was injected s.c. for 7 days prior to urine sampling.

DISCUSSION

Since 1970 several RIA have been developed for the determination of aldosterone in biological fluids in man (for review see [11]), some of which have been also applied to rat plasma [1, 2, 3, 6], but not to rat urine. The RIA described in this study has been used for the measurement of physiological amounts of aldosterone in plasma as well as in urine of rats and has proven to be valid for both purposes.

Despite the rather high specificity of the antiserum used [10] the minute amount of aldosterone in biological fluids necessitates the separation of aldosterone from other corticosteroids before the binding assay. In agreement with others [12] we found t.l.c. to suit this purpose quite well, because it is rapid and simple. However, inclusion of a chromatographic step demands the correction for procedural losses by individual recovery. When using internal radioactive standards for determination of individual recovery the problem arises, how to correct for the radioactive aldosterone preexisting in the unknown samples submitted to RIA. This problem has been solved [13] by calculating the bound fraction of the unknown sample as percentage of the individual total counts of the same sample. In addition, this calculation method enables the determination of the specific activity of the tracer by RIA (Table 1).

Linearization of the standard curve was not obtained by logit transformation when total percent binding was plotted vs. the log of inert aldosterone added. Even when the log of total amount of antigen (inert plus labeled antigen) was used as the dose parameter [14], a slight flattening persisted at the upper part of the standard curve. Therefore, the aldosterone content of the antiserum itself was determined after

extraction by methylene chloride and was found to be remarkably high (5 μg/ml) as has also been shown in rabbit antisera against several corticosteroids [15]. Repeated treatment of the antiserum with Dextran-coated charcoal did not significantly reduce this high aldosterone concentration. Therefore, the amount of endogenous aldosterone of the antiserum was added to the total amount of antigen ("total pg"). This procedure resulted in a linearization of the calibration curve allowing the use of a mini-computer for calculation of all assay data.

Specificity of the aldosterone assay is difficult to establish. No aldosterone was detected in plasma or urine of rats after adrenalectomy. However, those substances most probable to cross-react with the antibody are expected to originate from the adrenal cortex and therefore to be eliminated by adrenalectomy as well. On the other hand, several corticosteroids known to cross-react with the antibody used [10] are clearly separated by the t.l.c. involved in our method (Table 2). Nevertheless, there may be steroids or metabolites, which are not identified thus far and hence can not be tested directly. Specificity of the assay is demonstrated more likely by the results obtained with plasma and urine samples purified by two subsequent t.l.c. using different solvent systems (Table 3). There was no systematic decrease in the aldosterone concentrations after the second t.l.c. and the differences between the values obtained after one or two t.l.c. were not significant.

In man, urinary aldosterone excretion rate is usually measured after acid hydrolysis of the aldosterone-18-oxo-glucuronide. In the rat, acid-hydrolysable aldosterone metabolites and, in particular, aldosterone-18-oxo-glucuronide are excreted in the urine in minimal amounts only [16, 17]. In agreement with these findings aldosterone concentration increased by only 11%, when rat urine was subjected to acid hydrolysis. Since the RIA used is sensitive to detect free aldosterone in small samples of urine, hydrolysis of rat urine was omitted and aldosterone excretion rates were based on the determination of urinary free aldosterone.

Normal values of plasma aldosterone concentration in male Sprague–Dawley rats showing a pronounced circadian rhythm [4, 5, 7] ranged from 23 to 288 pg/ml, when rats were kept on a standard diet, and from 7000 to 20,000 pg/ml, when rats were kept on a sodium-poor diet for 14 days. These values are in good agreement with those reported in the literature for male adult rats regardless whether an isotope derivative method [18] or a radioimmunological method [2, 3, 6] was used. On the other hand, our method shows that normal values of urinary aldosterone excretion are lower than levels of 100–380 ng/24 h [19] or 40–50 ng/24 h [20] reported previously for rats. The differences obtained appear to result from the improved isolation technique and the use of RIA instead of polarography [19] or double isotope derivative technique [20] for measurement of aldosterone.

The determination of urinary aldosterone excretion rate is a useful parameter when investigating the control of aldosterone secretion in intact, unstressed rats. The biological reliability of this parameter has been established in experiments in rats using DOC, ACTH, furosemide and changes in sodium balance to alter the activity of the renin–angiotensin–aldosterone system [8].

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