Journal of Steroid Biochemistry, Vol. 10, pp. 365 to 369
Pergamon Press Ltd 1979. Printed in Great Britain

COMPARISON OF THE URINARY EXCRETION OF FREE ALDOSTERONE AND ITS ACID-LABILE CONJUGATE IN RATS DURING EXPERIMENTAL HYPERALDOSTERONISM

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(*Received* 7 *September* 1978)

SUMMARY

A commercial antiserum was used in the development of a sensitive radioimmunoassay for the determination of free aldosterone as well as its acid-labile conjugate in rat urine. To determine which of the two products might best reflect variations of aldosterone secretion, they were both measured in the urine of rats with hyperaldosteronism induced by Na^+ depletion, K^+ loading, ACTH administration or spironolactone administration. Under each of these experimental conditions the excretion rate of both the free aldosterone and its acid labile conjugate increased. Since in the urine, free aldosterone has a higher concentration than the conjugate, a more rapid response to stimuli and is less time consuming to measure, it can be considered the compound of choice for the study of variations of aldosterone secretion in the rat.

INTRODUCTION

The lack of available methods of sufficient sensitivity has until recently, prevented the determination of aldosterone in the peripheral plasma and urine of the rat. Radioimmunoassay techniques have made it possible to measure aldosterone in both these biological fluids during *in vivo* studies [1, 2]. In the rat, blood sampling often requires anesthesia which induces stress and stimulates renin release. The urinary excretion of aldosterone permits the daily measurement of this steroid in the same unstressed, conscious rat.

As demonstrated by others [3, 4], the main route of aldosterone excretion in the rat is via the bile: 24 h after i.v. injection of [^{3}H]-aldosterone, about 40% of the radioactivity is found in the feces and 20–30% in the urine. Part of the urinary radioactivity comes from free aldosterone and the acid-labile conjugate of aldosterone.

In order to determine whether the excretion rate of these two urinary products could be used as a reliable index of the secretion of this steroid, we have measured these two compounds in situations of known hyperaldosteronism. Aldosterone secretion was stimulated by ACTH, K^+ loading, Na^+ depletion, and Na^+ depletion combined with spironolactone administration.

The method used essentially follows the method of Underwood *et al.*[5] which has been adapted to urine determination. Briefly it consists of extraction of free aldosterone followed by hydrolysis of the urine at pH 1, extraction of the acid-labile conjugate, purification of the two extracts by paper chromatography and quantitative determination by radioimmunoassay.

MATERIALS AND METHODS

Animals. Male Wistar rats (300–400 g) were housed under controlled temperature (23 ± 1°C) with an alternative 12 h light (07.00–19.00) and 12 h dark period (19.00–07.00). The animals were maintained on a *standard diet* (Na^+ 100 mmol/kg, K^+ 150 mmol/kg; VAR rat food, France) and tap water *ad libitum* or on a *low* Na^+ *diet* (Na^+ less than 22 mmol/kg, K^+ 59 mmol/kg rat chow, Altromin, Lage, Germany) and demineralized water. K^+ *loading* was achieved by supplementing the standard diet with a 5% glucose solution containing K^+ 300 mmol/l and Na^+ 15 mmol/l as drinking fluid. *ACTH* (Synacthen Depot, Ciba) was injected i.m. at 09.00 h at a dose of 0.05 mg/day per rat for 4 consecutive days to rats maintained on standard diet. *Spironolactone* (Boehringer, Mannheim) suspended in olive oil was injected subcutaneously for 6 days starting on the third day of Na^+ depletion, at a dose of 6 mg/day per rat. During the experiments, the rats were placed in individual metabolic cages. 24 h urines were collected, acidified to pH 4.5–5 and stored at −18°C until aldosterone determination.

Material. Analytical grade dichloromethane, toluene, benzene, methanol and hydrochloric acid were purchased from Merck (Germany). Liquid scintillation mixture was prepared from Permafluor I (Packard Instrument Company), NP 55/52 detergent (Lotti, Geneva) and toluene (4:30:66, by vol.).

The following compounds were used: [1,2,6,7-^{3}H]-aldosterone (specific activity: 120 Ci/mmol, Radiochemical Centre, Amersham); aldosterone (Steraloids, Inc., Wilton); rabbit antiserum against 3-oxime-aldo-

sterone (Endocrine Sciences, Tarzana, California); borate buffer (0.13 M, pH 8) containing 0.5% bovine serum albumine (Armour Pharmaceutical Company); Dextran-coated charcoal (2.5 g Dextran 70 (Pharmacia, Uppsala) and 2.5 g Norit (Fisher Scientific Company, New Jersey) in 1000 ml borate buffer brought to pH 7.8 with HCl.

Extraction and purification. About 2000 d.p.m. [^{3}H]-aldosterone (approx. 3 pg) were added to 3 ml of urine. After 10 min equilibration, the urine samples were extracted successively with 20 and 10 ml dichloromethane and the organic phases were pooled. Two ml of the extracted urine was hydrolyzed for 20 h at pH 1–1.2 at room temperature, after another addition of about 2000 d.p.m. [^{3}H]-aldosterone. Aldosterone derived from the acid-labile conjugate was extracted once with 10 volumes of dichloromethane. The aldosterone extracts were washed with 2 ml 0.1 M NaOH, 2 ml 0.1 M acetic acid, 2 ml distilled water and taken to dryness. They were purified by paper chromatography on Whatman 2 in a benzene–methanol–water (4:2:2, by vol.) system. About 20,000 d.p.m. [^{3}H]-aldosterone was run in parallel to localize the aldosterone zones. They were then eluted with methanol, taken to dryness, dissolved in 6 ml dichloromethane and washed with 1 ml water. After evaporation of the solvent, the extracts were redissolved in 2–5 ml ethanol. Recovery of radioactivity was determined by counting a portion of the extract.

Radioimmunoassay. When necessary, the urine extracts were diluted before assay. Duplicate, dried, aliquots of extracts and 0–150 pg aldosterone were mixed with 0.5 ml of binding solution containing 0.5% (w/vol.) bovine serum albumin, 9000 d.p.m. [^{3}H]-aldosterone and a final antiserum dilution of 1/20.000. After overnight incubation at 4°C, 1 ml of the Dextran-coated charcoal suspension was added; the mixture was kept for 10 min at 0°C, and centrifuged for 10 min at 3000 rev./min at 4°C. The supernatant was poured into 12.5 ml scintillation fluid and the radioactivity counted.

The percentage of bound radioactivity was calculated for the standards and the biological samples, taking into account the radioactivity coming from the recovery tracer. Excretion of aldosterone and its conjugate was expressed as ng per 24 h.

Statistics. All results are expressed as mean ± S.E.M. The paired *t*-test, except when noted, was used for the statistical analysis of the results.

RESULTS

Recovery of the tracer

The overall recovery was 79.0 ± 0.5% (n = 117, range 65.5–89.8%) for free aldosterone and 56.9 ± 0.8% (n = 96, range 42–68.1%) for the acid-labile conjugate.

Blank

Blank values for free aldosterone and for the acid-labile conjugate were measured in the urine of adrenalectomized rats. Both values were lower or equal to the detection limit of the standard curve.

Sensitivity

The detection limit of the standard curve was 2.5 pg (n = 8) when expressed as the dispersion of the 0 ± 2 S.D. pg value and 4 pg (n = 6) when expressed as the least detectable level. Therefore the minimum amount measurable is 0.2–0.3 ng/24 h if one assumes a 70% recovery and a 24 h excretion volume of 15 ml.

Accuracy

Increased amounts of unlabelled aldosterone (0–1200 pg) were added to 2 ml urine of adrenalectomized rats. The recovery of cold aldosterone was 95.7 ± 1.7% for free aldosterone and 101.1 ± 2.4% for the conjugate. Their respective regression line were $y = 0.94\ X + 0.4$ (n = 12, r = 0.99; $P < 0.001$) and $y = 0.97\ X + 18.4$ (n = 6; r = 0.98; $P < 0.001$).

Intraassay variation was tested in pooled urine samples with low concentration of aldosterone. It was 9.9% (n = 10) for free aldosterone and 14.4% (n = 12) for the acid-labile conjugate.

Interassay variation was calculated from samples containing various amounts of aldosterone which were analyzed twice in different assays. It was 11.8 ± 2.6% (n = 9) for free aldosterone and 14.1 ± 4.7 (n = 6) for the acid-labile conjugate.

Specificity

Steroid cross-reaction of the antibody was provided by Endocrine Sciences. Corticosterone and cortisol had the highest percentage of cross-reaction with the antibody [1–2%]. The first product, the only one present in the rat sample, was eliminated by paper chromatography.

Increasing amounts of free and acid-labile fractions were measured. The log-logit plot of the results was a linear curve parallel to the standard curve.

The possibility of an interference of free aldosterone with the measurement of the acid-labile conjugate was investigated. Less than 0.5% of free aldosterone remained in the urine before the hydrolysis, but considering the higher level of free aldosterone, as compared with the conjugate, determination of the latter could be affected by a factor of 10%.

The specificity of the method was also controlled by including a 2nd paper chromatography step in the routine procedure for both compounds (isooctane–terbutanol–water, 10:5:9, by vol.). The free aldosterone values were not affected by this modification except after ACTH stimulation, when they were found to be lower by a factor of 16% which is slightly higher than the interassay variation. The acid-labile conjugate values were not altered significantly by this additional purification step, although they tended to be lower.

No satisfactory specificity could be obtained using the aldosterone antibody Nr 088 from NIAMD.

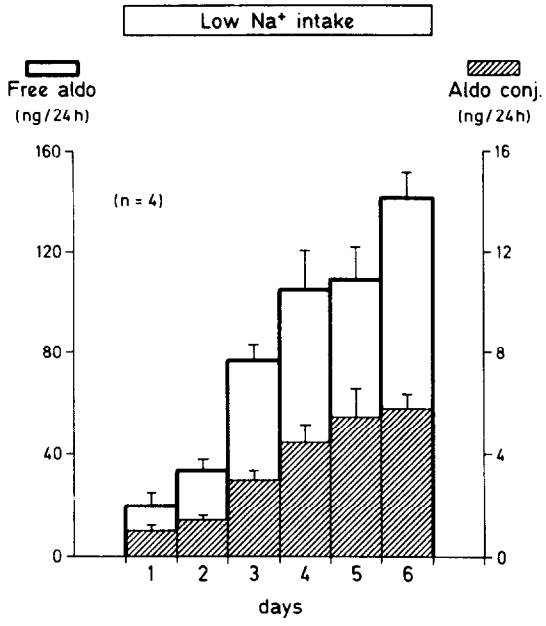

Fig. 1. Urinary excretion of free aldosterone and acid-labile conjugate during low Na^+ intake. Mean ± S.E.M.

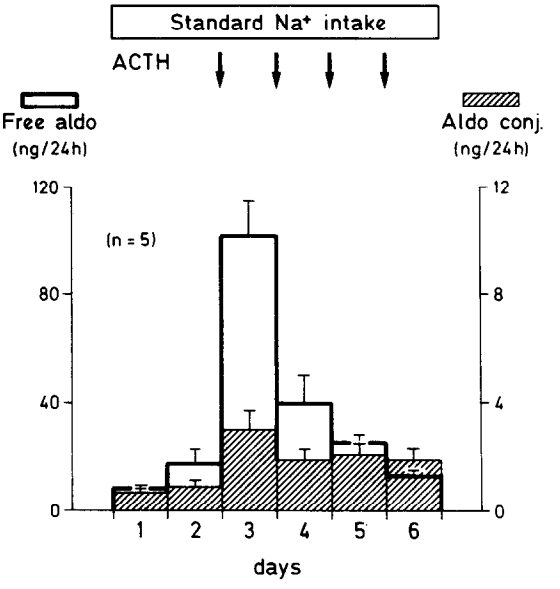

Fig. 3. Urinary excretion of free aldosterone and acid-labile conjugate during i.m. ACTH administration. Mean ± S.E.M.

Urinary results

Urinary levels of free aldosterone and of the acid-labile conjugate were 12.7 ± 1.7 ng/24 h and 0.9 ± 0.1 ng/24 h ($n = 20$), respectively during the control period with *standard diet*, 141.7 ± 9.4 ng/24 h and 5.8 ± 0.6 ng/24 h ($n = 4$) after 6 days of *low-Na^+ diet* and 73.5 ± 12.2 ng/24 h and 2.8 ± 0.4 ng/24 h ($n = 5$) after 3 days of *K^+ loading*. During *Na^+ depletion* and *K^+ loading* there was a regular and parallel increase of free aldosterone and the acid-labile conjugate (Figs 1 and 2). This increase was significant for both products on the second day of Na^+ depletion ($P < 0.001$ and $P < 0.025$ respectively, unpaired t-test), and on the first day of K^+ loading ($P < 0.001$ and $P < 0.01$ respectively, paired t-test) when compared with the mean values of the standard diet. The ratio of free aldosterone to acid-labile conjugate was constant during the experimental period and similar in the two groups of rats (Na^+ depletion: 24.6 ± 1.8, $n = 20$; K^+ loading: 26.2 ± 1.7, $n = 15$). During *ACTH* treatment (Fig. 3) the increased aldosterone excretion rate was not maintained. Free aldosterone levels increased about 10-fold on the first day of ACTH treatment (102.0 ± 13.0 ng/24 h, $n = 4$, $P < 0.001$) and decreased, reaching control value on the 4th day ($P > 0.95$). The variations were less marked for the conjugate, there was a 3-fold rise on the first day (3.0 ± 0.7 ng/24 h, $n = 4$, $P < 0.005$) and the values were still twice the mean control value on the 4th day (1.8 ± 0.1 ng/24 h, $P < 0.005$). The ratio of the two urinary compounds varied considerably, following the pattern of the free aldosterone excretion (range 40.7 ± 9.0 to 7.2 ± 0.7). *Na^+ depletion combined with spironolactone*, a renal antagonist of aldosterone, produced a rapid increase of the excretion of both products in two rats (Fig. 4). On the 4th day of spironolactone administration (6th day of Na^+ depletion) the values were respectively 408 ng/24 h and 273 ng/24 h for the free steroid and 9.8 ng/24 h and 6.3 ng/24 h for the conjugate. There was no interference of spironolactone or its metabolites with the measurement of aldosterone (paper in preparation).

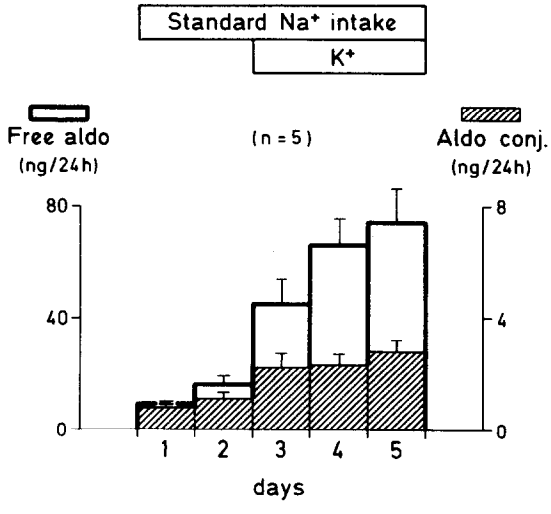

Fig. 2. Urinary excretion of free aldosterone and acid-labile conjugate during K^+ loading. Mean ± S.E.M.

DISCUSSION

Although both free aldosterone and its acid-labile conjugate are found in the urine of the rat, they nevertheless represent only a small fraction of the aldosterone excretion products. Both compounds are excreted rapidly, since more than 85% of the radioactivity eliminated through the kidney appears during the first

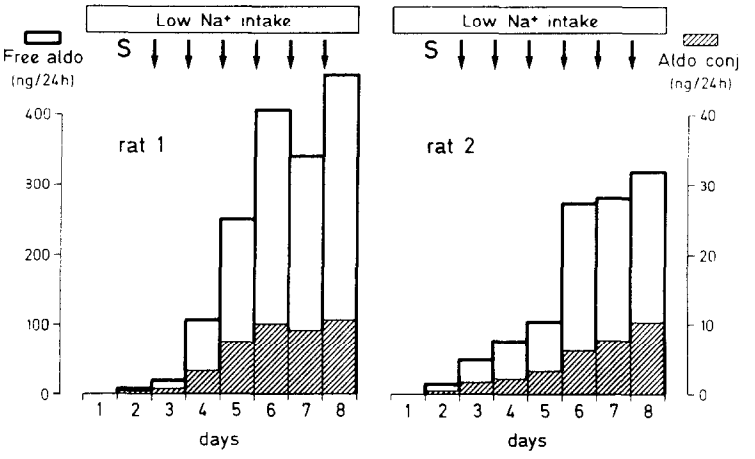

Fig. 4. Urinary excretion of free aldosterone and acid-labile conjugate during low Na^+ intake plus Spironolactone (S) in 2 rats.

24 h after the injection of labeled aldosterone [3, 6]. Measurement of daily urine samples should therefore provide a simple method of detecting changes in aldosterone secretion rate. Using a commercial antiserum the two compounds have been measured in rats in which aldosterone secretion had been stimulated by Na^+ depletion, K^+ loading, ACTH and spironolactone treatment. Their daily variations have been followed and compared.

During the control period with a standard diet, the mean values for both compounds were found to be consistent with the data of Hilfenhaus[1] who measured the free steroid, and Battarbee *et al.*[2] who measured the conjugate. Approximately 10 times more aldosterone was excreted in the free form than in the conjugate. This corresponds to the finding of Hilfenhaus who recovered a further 11% of aldosterone in a pool of rat urine, after pH 1 hydrolysis. However the blank value of the conjugate method is not negligible when the levels of the conjugate are low as is the case when the rat is on a standard diet. Therefore the ratio of the two urinary products can only be approximate in those conditions. During Na^+ depletion, the increases in the two products were comparable to those found on the 6th day of depletion by the same authors [2, 7]. K^+ loading increased both the free aldosterone, as has been shown by Corvol *et al.*[5], and the conjugate. During ACTH treatment, the increase in the excretion of the two compounds was not sustained. This phenomenon has already been observed in man with either the acid-labile conjugate [8] or tetrahydroaldosterone [9], but the reason is not well understood. ACTH may induce some changes at the adrenocortical cell level such as an enzymic blockade along the aldosterone synthetic pathway favoring the synthesis of glucocorticoids at the expense of mineralocorticoids [10]. Spironolactone administration combined with Na^+ depletion increased free aldosterone and the conjugate to levels which were more elevated than during Na^+ depletion alone.

Despite the fact that urinary free aldosterone and the acid-labile conjugate represent less than 0.5% of the secreted steroid [4], in the situations studied, the two compounds were increased in the urines. They can therefore be used to study changes in aldosterone secretion. However they may not always reflect the changes very accurately as suggested by the variations observed in the ratio of the two compounds during ACTH stimulation. These variations indicate alterations of metabolism which are the result of alteration of the numerous factors implicated in the excretion rate of either compound. These factors include binding to plasma proteins, liver and kidney enzymic systems and blood flow, enterohepatic circulation, diuresis. By analogy with the determination of free urinary cortisol in humans [11], one could predict that in some situations the measurement of free aldosterone may overestimate the changes in its secretion rate. With this reservation, free aldosterone seems to be the compound of choice since its levels are higher than those of the conjugate and thus less affected by the blank, its response to stimuli is rapid and its measurement is less time-consuming.

In conclusion both free aldosterone and the acid-labile conjugate can be measured in rat urine to study changes in the secretion of the steroid. The several advantages of the free steroid determination make it the preferred parameter for such a study.

Acknowledgements—We thank Miss V. Nicolet for her secretarial assistance and Mrs. J. Noebels for her editing help. This work was supported by the Swiss National Science Foundation (Grant No. 3.230-0.74 and No. 3.845-0.77). A financial support from Searle Company (Lausanne, Switzerland) is gratefully acknowledged.

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