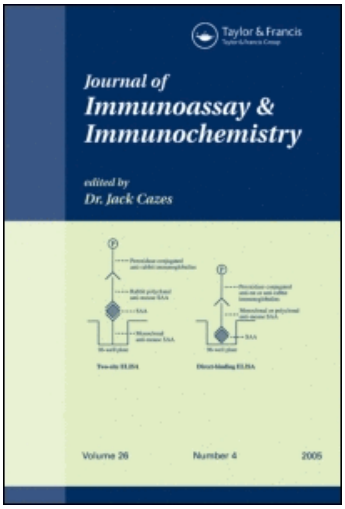

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Solid Phase Radioimmunoassay for the Determination of Aldosterone Excretion

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To cite this Article Török, Angela(1993) 'Solid Phase Radioimmunoassay for the Determination of Aldosterone Excretion', Journal of Immunoassay and Immunochemistry, 14: 4, 297 — 306
To link to this Article: DOI: 10.1080/15321819308019856
URL: http://dx.doi.org/10.1080/15321819308019856

JOURNAL OF IMMUNOASSAY, 14(4), 297-306 (1993)

SOLID PHASE RADIOIMMUNOASSAY FOR THE DETERMINATION OF ALDOSTERONE EXCRETION

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ABSTRACT

A solid phase radioimmunoassay (SP-RIA) was developed for the determination of urinary conjugated aldosterone. Acid-hydrolyzed urine was extracted with dichloromethane and the extract was analyzed by SP-RIA. Microtiter plates were coated with two different anti-aldosterone antisera (S-3: against aldosterone-21-hemisuccinate-BSA and 58: aldosterone-3-(-O-carboxymethyl)-oxime-BSA separately and preincubated with ^{3}H-aldosterone. Reference values were obtained using a liquid radioimmunoassay method including paper chromatography. Regression analysis revealed for S-3: $Y = 0.8\ X + 3.2$, $r = 0.84$; $p<0.0001$; $n = 36$ and for 58: $Y = 1.16\ X + 1.2$, $r = 0.89$; $p<0.0001$; $n = 36$.
The SP-RIA assay is useful for routine measurement of conjugated aldosterone excretion. The method is time-saving and does not require paper chromatography, and is adequate for many clinical purposes.

KEY WORDS: Aldosterone, solid phase radioimmunoassay

INTRODUCTION

The mineralocorticoid hormone aldosterone is a major factor in blood pressure homeostasis. Several disorders in blood pressure homeostasis exist related to alterations in aldosterone metabolism, including essential hypertension, hyperaldosteronism and adrenal hydroxylase deficiencies. Diagnostic measurement of aldosterone can be performed in a variety of body fluids: blood, urine, saliva. Concentrations in saliva are very low, requiring highly sensitive methods. Plasma concentrations are subject to a diurnal rhythm and to diet, posture, and the presence of binding proteins. Measurement of aldosterone in urine is a useful alternative (1-5).

In urine, aldosterone exists mainly in conjugated forms, especially as aldosterone glucuronide and tetrahydroaldosterone glucuronide, and free aldosterone comprises only a small proportion. Usually, excretion of aldosterone is determined by measuring its glucuronide in urine after hydrolysis at pH 1.0, extraction, with (6-8) or without chromatographic purification (9-13) and sub-sequent quantitation by radioimmunoassay (RIA).

In this study a simple method for the determination of urinary conjugated aldosterone was developed comprising extraction of hydrolyzed urine, followed by solid phase RIA (SP-RIA). Microtiter plates were coated with anti-aldosterone antiserum and pre-incubated with ^{3}H-labeled aldosterone. Two different antisera, against aldosterone-21-hemisuccinate and aldosterone-3-(-O-carboxymethyl)-oxime, were tested and the results were compared with a reference method comprising extraction, paper chromatography and liquid RIA (14).

MATERIALS AND METHODS

All solvents were of reagent or analytical grade. [1,2,6,7-^{3}H]-aldosterone (specific activity 2.90 TBq/mmol) was purchased from NEN GmbH (Dreierchenhain, Germany) or from Amersham-Buchler (Braunschweig, Germany). Before use the tracers were purified with paper chromatography in a (toluene/methanol/water, 2/1/1, v/v) descending solvent system.

Radioactivity was measured by liquid scintillation counting in a Packard Tricarb liquid scintillation spectrometer, types 2425 and 3255, with efficiencies of 40%.

Scintillation cocktail:

2,5 diphenyl-oxazole (PPO)		4.00 g
1,4-bis-[2-(5-phenyl-oxazolyl)]-benzene) (POPOP)		0.05 g
toluene	to	1.0 L

This solution was mixed with Triton X-100 (2:1,v/v).

Buffer solutions

Buffer A:

morpholino propane sulfonic acid, (MPSA)	20.39 g
sodium chloride	17.52 g

distilled water add to 1 L, pH= 7.2.

Buffer B: buffer A was adjusted to pH 2.0 with HCl.

Coating buffer:

solution (i) 0.2 M sodium carbonate	21.2 g/L

solution (ii) 0.2 M sodium bicarbonate 16.8 g/L
solution (iii) mix 170 mL of solution (i) and 80 mL of solution (ii) and make up to 1 L with distilled water, pH 10.6.

Wash solution:
Triton X-100 500 mL/L of phosphate buffered saline, (PBS) pH 7.2, 50 mmol/L.

Blocking solution: bovine serum albumin, (BSA) 10 g/L of PBS.

Urine was sampled as 24 h collections without preservative from normal subjects and from patients with suspected abnormal aldosterone secretion. Total volume was recorded and from each sample a 50 mL aliquot was taken and stored at -20°C until analysis.

Purification of antisera

Two different antisera were used for the experiments. S-3 was raised in sheep. The immunogen injected was the BSA conjugate of aldosterone-21-hemisuccinate (14). 58-antibody was raised in rabbits by injecting the BSA conjugate of aldosterone-3-(-O-carboxymethyl)-oxime as immunogen (15).

Protein A Sepharose CL-4B affinity chromatography was employed to isolate the IgG fractions of the antisera.

The procedure was as follows: 1 mL of antiserum was suspended in 3 mL of Protein A Sepharose CL-4B gel in buffer A in a small column and incubated for one hour at room temperature by end-over-end rotation. Subsequently, the gel was washed with buffer A and the IgG eluted with buffer B. MPSA was used to remove endogenous sheep aldosterone from antibodies. The IgG fractions (2-3 mL) were neutralized with NaOH and the protein content was quantitated with a protein-dye binding method (Commassie Blue). The IgG fractions were collected and concentrated with ultrafiltration and stored at -20°C in 1 mL portions.

Coating of solid phase

The antibodies were diluted with coating buffer (iii) to give a 50% binding capacity, quantitated by liquid RIA. U-shaped- bottom 96-well microtiter plates,(Maxisorp, Nunc Immuno Plate, Nunc AS, Kamstrup, Denmark) were coated with 100 μL of diluted antibody per well, covered with foil, and incubated overnight at room temperature.

Then the wells were emptied and the unbound antibody removed by washing with wash solution using an automated washer (Nunc AS, Kampstrup, Denmark).
The tracer binding sites were blocked with BSA by incubation with blocking solution, 200 μL/well, for 2 h at room temperature. Again the plates were washed with wash solution.

Ligand binding

After coating of the plates with antibody, 200 μL of [1,2,6,7-^{3}H]-aldosterone in PBS 20-25,000 dpm/200 μL was pipetted into each well. The plates were then incubated overnight at 4°C. The excess of tritiated aldosterone was removed and the plates were washed with PBS twice. The wells were emptied and dried by tapping the plates against filter paper.
Plates pre-incubated with tritium labeled aldosterone, covered with foil, could be stored at 4°C for up to 1 month without deterioration.

Extraction of urinary aldosterone (acid-labile conjugate)

Aliquots of 5 mL of urine were extracted with 15 mL of dichloromethane by shaking for 30 minutes to remove free corticoids. This procedure was according to the protocol of Steroid lab, University of Heidelberg. To 1 mL of the aqueous phase 5 mL of glycine/HCl buffer, pH= 1 was added. Hydrolysis was allowed to continue for 24 h at room temperature in the dark. Subsequently, 100 μL of PBS containing 3000 dpm of ^{3}H-aldosterone as internal recovery indicator was admixed and after 15 minutes incubation the mixture was extracted with 15 mL of dichloromethane by shaking for one hour. The aqueous layer was removed by aspiration and the organic layer transferred into another glass tube. After evaporation the residue was dissolved in 1 mL of PBS. Recovery calculation was made by counting 400 μL of extract.

SP-RIA procedure

In duplicate, 100 μL of aldosterone standards (0-100 pg) in PBS were pipetted into the wells, each microtiter plate containing one series of standards. Sample extracts were diluted 50 fold with PBS and 100 μL aliquots in duplicate were pipetted into the wells. The plates were covered with foil and incubated overnight at 4°C. The pre-incubated microtiter plates were put on crushed ice. Subsequently, 50 μL of the well contents was transferred into

counting vials, 10 mL of scintillation liquid was added and the vials were shaken and counted.

Reference method

The reference method was RIA after paper chromatographic purification of conjugated aldosterone. After extraction as described above the residues were dissolved in 250 µL of dichloromethane and spotted on Whatman 3 MM paper strips (2,5 x 46 cm). Chromatography was performed in a Bush B5 descending solvent system (benzene/methanol/water-2:1:1, v/v) for 16 h. The radioactive areas were located in a radiochromatogram spark chamber with the aid of two reference standards of ^{3}H-aldosterone. The appropriate spots were isolated, eluted in PBS and assayed by RIA (14).

Direct SP-RIA

For comparison, urine samples without hydrolysis were diluted (1:10 and 1:20) with distilled water (17). Aliquots of 100 µL were pipetted directly into the wells of the pre-incubated microtiter plates and assayed as described above.

RESULTS

The specificity of the antibodies was determined by measuring cross-reactivities for a series of steroids and derivatives. The cross-reactivities of the aldosterone antibodies were assessed by the standard procedure (14) using ^{3}H aldosterone as radioactive ligand. The displacement of ^{3}H-aldosterone was expressed as a percentage related to aldosterone being 100%.

From Table 1 it appeared that both antibodies showed negligible cross-reactivities for most steroids or derivatives, except that antibody 58 cross-reacted considerably with aldosterone-18-ß-glucuronide.

The precision of the SP-assay was determined by replicate measurement of samples of two urine pools in the same run (intra-assay) and in different runs (inter-assay).

	coefficient of variation %	
	Intra-assay	Inter-assay
Aldosterone	n=5	n=5
9.36 ± 1.8 SEM µg/L urine pool	4.8	10.5
3.6 ± 0.57 SEM µg/L urine pool	5.7	12.0

Blank values of SP-RIA were checked by using distilled water

TABLE 1
Cross-reactivities of anti-aldosterone antibodies (%) with various steroids and derivatives measured by radioimmunoassay.

Compounds	S-3-Ab	58-Ab
aldosterone	100	100
cortisol	0.08	<0.01
corticosterone	0.28	<0.1
deoxycorticosterone	--	<0.08
18-hydroxycorticosterone	0.004	<0.01
18-hydroxydeoxycorticosterone	0.12	<0.33
progesterone	0.1	<0.01
18-hydroxy-progesterone	0.2	<0.01
17α-hydroxyprogesterone	0.023	<0.01
testosterone	--	<0.33
3α,5ß-tetrahydroaldosterone	0.02	0.4
spironolactone	<0.008	<0.1
aldosterone-18ß-glucuronide	1.84	34.2
tetrahydro-aldosterone-3-glucuronide	<0.06	0.2
tetrahydro-aldosterone-18α-glucuronide	0.06	0.07

instead of the urine sample. The assay blank values were not significantly different from zero.

The recovery of aldosterone after the extraction procedure was calculated on the basis of counting the added internal recovery indicator. Mean recovery was 88 %, SEM= 0.6 %, n= 19.

The sensitivity was determined by calculating the minimum amount of aldosterone that could be significantly distinguish from zero standard ($\bar{B}_0$ ± 2SD). The detection limit of the SP-RIA corresponded to 1 μg/L undiluted urine.

Comparison of SP-RIA after extraction with reference method.

Thirty six urine samples of normal subjects and patients were extracted and the concentrations measured with the reference method and with SP-RIA employing microtiter plates coated with both antibodies. Regression analysis showed that for the S-3-Ab correlation with the reference method was:

$Y = 0.8\ X + 3.2$; $r = 0.84$; $n = 36$; intercept n.s. different from zero; slope (b) differs from 1.0, confidence interval $0.61 < b < 0.98$. For the 58 antibody this correlation was:

$Y = 1.16\ X + 1.2$; $r = 0.89$; $n = 36$; intercept n.s. different from zero; slope differs from 1.0, confidence interval $0.98 < b < 1.37$.

Comparison with direct SP-RIA

Both types of coated microtiter plates were also employed for

measuring urinary aldosterone directly after simple dilution of the samples. The results were again compared with those obtained with the reference method. Regression analysis revealed:
For the S-3-Ab:
$Y = 0.65\ X + 1.8$; $n = 22$; $r = 0.6$; intercept no different from zero; slope significantly differs from zero, $p<0.001$.
For the 58-Ab:
$Y = 0.34\ X + 7.46$; $n = 22$; $r = 0.28$; intercept no different from zero; slope significantly differs from zero, $p<0.001$.

Results of calculations for conjugated aldosterone excretion

		Mean ± SD μg/24 h	
Normal	Ab 3	10.7 ± 4.2	n= 28
	Ab 58	12.4 ± 4.4	n= 28
	Ref.	9.8 ± 2.6	n= 28
High	Ab 3	25.1 ± 5.4	n= 8
	Ab 58	32.3 ±10.0	n= 8
	Ref.	26.3 ± 6.5	n= 8

Normal range of conjugated urinary aldosterone varied between 3.5-17.5 with a mean of 8.0 ± 3.6 μg/24 h, n= 59 (15).

Diagnostic sensitivity and specificity of the SP-RIA

S-3 SP-RIA	Reference N	Reference H	
N	21	2	diagnostic sensitivity: 82%
H	4	9	diagnostic specificity: 84 %

58 SP-RIA	Reference N	Reference H	
N	22	2	diagnostic sensitivity: 80 %
H	4	8	diagnostic specificity: 85 %

N=normal and H=high values are from Figure 1, $\bar{X}$= 13.45 μg/24 h, $\bar{Y}$= 13.9 μg/24h for S-3 Ab and 16.8 μg/24h for 58 Ab.

To define the usefulness of this SP-RIA, using the reference method as a good standard, the diagnostic sensitivity and specificity for S-3 Ab were 82 and 84 % respectively; as for 58 Ab 80 and 85 % respectively.

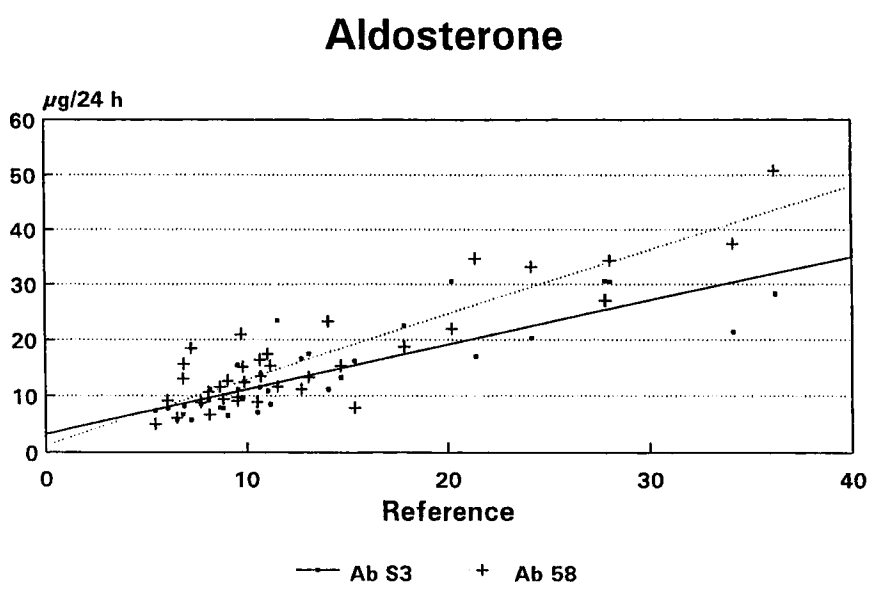

FIGURE 1
Urinary aldosterone excretion rate obtained with two antibodies, compared with reference method.

DISCUSSION

To avoid complex procedures, a solid-phase RIA (SP-RIA) was developed for the routine measurement of conjugated aldosterone in urine.

Most radioimmunoassays for urinary aldosterone have included either solvent extraction alone after hydrolysis (11,13) or extraction and purification steps necessitated by the lack of specificity of the aldosterone antisera used (6,7,8).

For the SP-RIA microtiter plates were coated with selected antibodies and preincubated with tritiated aldosterone. The stability of such coated plates offered a readily available system for the measurement of aldosterone in urine samples. Specific anti-aldosterone antisera have used with no appreciable cross-reactivities with various steroids.

To assess the applicability of the SP-RIA method, extracted urine samples were measured with this method employing both antibodies and also with a reference method including paper chromatography

for purification. Both antibodies in the SP-RIA gave results that correlated well with the reference values, although those produced with the 58 antibody were significantly higher than with the S-3 antibody.

The necessity of the extraction step was demonstrated by comparing direct SP-RIA of unextracted urine with the reference method. No correlation was found.

Measurement of urinary aldosterone is an important tool in the diagnosis of clinical disorders due to altered adrenal mineralocorticoid physiology. This simplified method of measuring urinary conjugated aldosterone represents, a significant advance in our capabilities for evaluating patients with hypertension and related disorders of mineralocorticoid physiology.

ACKNOWLEDGEMENTS

I am indebted to Prof. P. Vecsei, Department of Pharmacology, University of Heidelberg, for his advice.

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