Non-Chromatographic Radioimmunoassay Procedure for Urinary Aldosterone

Philip R. Walsh, Ming C. Wang, and Elizabeth A. Turner

We describe a sensitive, specific, and simple procedure for measuring aldosterone in human urine, which requires no chromatographic purification before quantification by radioimmunoassay but does include hydrolysis and extraction steps. Rabbit anti-aldosterone serum is used, generated against aldosterone-18,21-dihemisuccinate coupled to human serum albumin. The antibody cross reacted little with other structurally related steroids that are in human urine. Our procedure was validated by comparing values for urinary aldosterone in human urine, with and without preliminary purification by chromatography on either paper ($y = 0.92x + 2.9; r = 0.99; p < 0.01$) or (Sephadex LH-20) column ($y = 0.98x + 0.6; r = 0.99; p < 0.01$). Values by our procedure also correlated well ($y = 1.03x - 0.8; r = 0.99; p < 0.01$) with those obtained with use of a validated commercial “kit” for urinary aldosterone. All reagents for the proposed method are available commercially.

**Additional Keyphrases:** normal values, chromatographic results compared, steroids, “kit” methods

Aldosterone is present in human urine as both the unconjugated or “free” steroid and the acid-labile, aldosterone-18,21-dihemisuccinic acid conjugate (1). The unconjugated steroid comprises approximately 3% of the total aldosterone present in human urine (2). During the past 20 years, the quantification of urinary aldosterone has been an analytical challenge to clinical and research laboratories.

The advent of the radioimmunoassay (RIA) technique allowed sensitive and specific procedures to be developed for urinary aldosterone during the early 1970’s (2–7). Because highly specific antisera to aldosterone was lacking, most of these published procedures require a preliminary purification of the acid-hydrolyzed urine by paper chromatography (3–5) or differential solvent partition and derivatization (6–8) if separation of aldosterone from structurally related steroids present in urine is to be acceptable.

During the past five years, the availability of antisera to aldosterone of high specificity and avidity has facilitated rapid, sensitive, accurate, and precise RIA procedures (9–12) for measuring urinary aldosterone. Because of the high specificity of these anti-aldosterone sera, many of these RIA methods require no purification of the sample before RIA.

Here, we present data collected with use of commercially available anti-aldosterone serum prepared against aldosterone-18,21-dihemisuccinate-human serum albumin. The high specificity of this antisera is such that the chromatographic or solvent partition-derivatization steps frequently required as a pre-RIA purification procedure are obviated. The procedure requires both acid hydrolysis and organic solvent extraction before the RIA step. Furthermore, our results by the proposed method compare well with those by a validated “kit” assay for urinary aldosterone.

**Materials**

- **Phosphate buffer, 0.2 mol/L, pH 7.0,** containing, per liter, 1 g of gelatin (Difco Laboratories, Detroit, MI 48232), 8.5 g of sodium chloride, and 0.1 g of sodium azide.

- **Aldosterone standard.** We dissolved 10 mg of aldosterone (Research Plus Steroid Laboratories, Denville, NJ 07834) in 100 mL of methanol to give a stock solution of 100 mg/L, 1 mL of which was diluted to 100 mL with methanol to give a standard concentration of 1 mg/L. A working standard (5 µg/L) was prepared by evaporating 1.0 mL of the 1 mg/L standard and redissolving the residue in 200 mL of the assay buffer. This solution was further diluted with the phosphate buffer to obtain final standard concentrations of 4.0, 3.0, 2.0, 1.0, and 0.5 µg/L.

- **Tritiated aldosterone.** [1,2,6,7,3H(N)]aldosterone (80–105 kCi/mol) was obtained from New England Nuclear, Boston, MA 02118 and purified by chromatography on Sephadex LH-20 (Pharmacia Fine Chemicals, Piscataway, NJ 08854) before use. The steroid was eluted from the LH-20 by cyclohexane/benzene/methanol (60/40/10 by vol) as described under Methods. A working solution of the purified radioactive steroid was prepared for the RIA step by evaporating 0.1 mL of the cyclohexane/benzene/methanol solvent mixture eluted from the column and redissolving the residue in the phosphate buffer to a count of 8000/100 µL (about 40 pg of the tritiated aldosterone per 100 µL of phosphate buffer).

- **Rabbit, anti-aldosterone serum, lot no. 76-3-4.** The anti-aldosterone serum prepared against aldosterone-18,21-dihemisuccinate-human serum albumin was obtained from Radioassay Systems Laboratories, Inc., Carson, CA 90745. The 1 mL of antisem was shipped at a 100-fold dilution of the original antisem. We further diluted it to 10 mL with the phosphate buffer and froze it (−60 °C) in 1-mL aliquots for storage. A working dilution of 15000-fold was prepared by thawing one of these aliquots and adding 14 mL of the phosphate buffer. We used 0.1-mL aliquots of the 15000-fold dilution for the RIA procedure.

- **Dextran-coated charcoal.** We dissolved 62.5 mg of Dextran T70 (Pharmacia Fine Chemicals) in 100 mL of the assay buffer, on ice, with constant stirring. After the dextran had dissolved, we slowly added 625 mg of Neutral Norit A Charcoal (Schwartz/Mann, Orangeburg, NY 10962) and allowed the mixture to stir at 4 °C for 30 min before use.
Liquid scintillation fluid. “RIA-FLUOR” was used as obtained from New England Nuclear.

Solvents. Methylene chloride and benzene (distilled in glass) were used as obtained from Burdick and Jackson Laboratories, Muskegon, MI 49440. Cyclohexane and methanol (certified ACS grade) were used as obtained from Fisher Scientific Co., King of Prussia, PA 19406.

Nonradioactive steroids. Cortisol, cortisone, 11-deoxy-cortisol, corticosterone, progesterone, testosterone, and 17α-hydroxyprogesterone were obtained from The Upjohn Co., Kalamazoo, MI 49001.

Methods

Acid-catalyzed hydrolysis of the aldosterone conjugate. A 2.0 mL aliquot of each urine specimen and control was transferred to a 16 X 100 mm disposable test tube and acidified with 100 μL of 12 mol/L HCl. The individual samples and controls were mixed well, capped, and allowed to stand at room temperature in the dark for 18 h.

Organic solvent extraction of the aldosterone. After the 18 h acid hydrolysis, a 500-μL aliquot of each urine was transferred to a clean 16 X 100 mm disposable glass test tube, and 100 μL (40 pg) of chromatographically (LH-20) pure [3H]aldosterone was added to each hydrolyzed specimen, to estimate recovery. Similar aliquots, in duplicate, were placed into scintillation vials for total counts (control) and dried at 60 °C with air. After a 15-min incubation of the [3H]aldosterone with the hydrolyzed urine at room temperature, 5 mL of methylene chloride was added to each hydrolyzed urine and the tubes were rotated end over end on a “Roto-Rack,” Model 343 (Fisher Scientific) at a setting of 8. The specimens were then centrifuged at 1600 X g for 10 min at room temperature and the aqueous layers discarded. The individual solvent layers were quantitatively transferred to clean 16 X 100 mm disposable glass test tubes, evaporated at 60 °C in a stream of air, reconstituted with 3.0 mL of assay buffer, and placed in a 37 °C water bath for 10 min.

Radioimmunoassay procedure. Disposable polystyrene (12 X 75 mm) test tubes were marked for duplicate zero (aldosterone-free assay tubes), 50, 100, 200, 300, 400, and 500 pg/tube standards. Additional duplicate tubes were marked for each control and unknown to be assayed. Aliquots (100 μL) of the individual standards (prepared in the assay buffer), unknowns, and controls (prepared in the assay buffer as previously described) were added to the appropriately marked tubes, followed by the addition of 100 μL of tritiated aldosterone and 300 μL of the assay buffer. Duplicate total count and blank assay tubes were prepared by adding 100 μL of the tritiated aldosterone and 500 μL of the assay buffer to individual tubes. The contents of all of the tubes (except the total-count and blank tubes) were vortex-mixed gently and 100 μL of the anti-aldosterone serum was added. All of the tubes were then vortex-mixed gently for 3 to 5 s and incubated at 4 °C for 18 h. Then all of the tubes (except the total-count tubes) were placed in an ice bath. A 200-μL aliquot of ice-cold dextran-coated charcoal suspension was then added to each tube in rapid succession, and the tubes’ contents were gently vortex-mixed immediately and allowed to stand at 4 °C for 20 min. Then all of the tubes (except the total-count tubes) were centrifuged (1600 X g, 10 min, 4 °C) and the supernates were decanted into appropriately marked scintillation vials. Each scintillation vial then received 10 mL of RIA-FLUOR, was capped, inverted 10 times, and its radioactivity counted in an automatic liquid scintillation spectrometer (Model 3330; Packard Instruments Co., Downers Grove, IL 60515) for 2 min.

Calculations. The logit-log data-reduction method (13) was used to calculate the aldosterone concentrations of the controls and unknowns. The values computed were corrected for the percent recovery of the [3H]aldosterone from the unknowns and controls and for the total volume of urine excreted during the 24-h collection period. The results are reported as micrograms of aldosterone excreted per 24 h.

Sephadex LH-20 column chromatography. Eight hundred milligrams of dry Sephadex LH-20 (Pharmacia Fine Chemicals) was placed in a column (1 X 18.5 cm; Isolab, Inc., Akron, OH 44309) with a porous polyethylene filter disc positioned at its bottom. The column solvent, cyclohexane/benzene/methanol (6/4/1 by vol) was passed through the column, swelling the column so that its height was about 5 cm. To eliminate floating gel, a second polyethylene filter disc was positioned above the LH-20 bed. The average flow rate through the column was 0.33 mL/min. Acid-hydrolyzed specimens selected for chromatography were extracted with methylene chloride and evaporated in a stream of air, at 60 °C. The dried extracts were subsequently redissolved in 1 mL of the column solvent and applied to the column with a Pasteur pipette. The aldosterone fraction (5-15 mL of the eluate) was collected in a 16 X 150 mm disposable glass test tube, evaporated under nitrogen at 60 °C, and the residue was dissoluted in 2.0 mL of assay buffer and allowed to stand at 37 °C for 15 min before RIA. Analytical recovery of [3H]aldosterone through the methylene chloride extraction and LH-20 chromatography steps varied from 60 to 70% (mean, 67%).

Paper chromatography. Acid-hydrolyzed specimens selected for paper-chromatographic purification were subjected to the procedure of Bush (14) before RIA. (Aldosterone determinations by this technique were done by Endocrine Sciences, Tarzana, CA 91356.)

[125I]-labeled aldosterone method for urinary aldosterone. We used the “[125I]—Aldosterone Assay Kit” (Diagnostic Products Corp., Los Angeles, CA 90060) where indicated, for certain studies included in this report. It was done as described in the procedural insert that accompanied the reagents.

Results

Sensitivity. Figure 1 illustrates a typical standard curve for the assay. The detection limit of this method was approximately 25 pg per tube, which corresponds to a sensitivity of about 75 pg/mL. The percentage of antibody-bound [3H]-aldosterone at zero unlabeled aldosterone varied from 55 to 60% of the total radioactivity. The nonspecific binding (NSB) varied from 2 to 3% of the total counts. An excellent logit-log dose–response curve was routinely observed over the range from 20 to 500 pg/tube.

Specificity. For these studies we used the procedure of Abraham (15) to determine the specificity of the antiserum to aldosterone-18,21-dihemisuccinate–human serum albumin.

Fig. 1. Logit-log standard curve for the aldosterone assay

The percent antibody-bound [3H]aldosterone is plotted as a function of the quantity of aldosterone (pg/tube)
Fig. 2. Correlation of the urinary aldosterone concentrations (µg/24 h) as determined by the proposed RIA method with those determined by RIA after purification of the extracts on Sephadex LH-20.

The following percentage cross reactivities were calculated (aldosterone, 100%): 17α-hydroxyprogesterone, <0.01%; cortisol, 0.05%; cortisone, 0.2%; corticosterone, 0.2%; androstenedione, <0.1%; deoxycorticosterone, 0.8%; progesterone, 0.05%; and testosterone, <0.01%.

Assay blank. The assay blank was determined by substituting 0.15 mol/L NaCl for urine and performing the assay as described. The blank value was indistinguishable from the zero standard.

Sephadex LH-20 column chromatography. Figure 2 illustrates the correlation (y = 0.98x + 0.6; r = 0.99; p < 0.01) observed on analysis of 35 specimens by the proposed method, with and without a prior purification on LH-20 with cyclohexane/benzene/methanol (6/4/1 by vol) as the elution solvent. Mean values for the specimens analyzed with and without purification on LH-20 were 22.8 and 23.4 µg/24 h, respectively. Thus, values obtained without the prior purification on LH-20 agree very well with those obtained when LH-20 column chromatography is included before the RIA step.

Paper chromatography. Figure 3 illustrates the correlation (y = 0.92x + 2.9; r = 0.98; p < 0.01) observed when 19 specimens were assayed by the proposed method and by a method in which paper chromatography (System B5 in ref. 14) is used as a purification procedure before RIA. The mean values obtained on the specimens assayed with and without prior purification by paper chromatography were 31.8 and 32.2 µg/24 h, respectively. Thus the two methods demonstrated an acceptable correlation over the range from 6 to 180 µg/24 h.

Correlation of the proposed method with a commercial [125I]-labeled aldosterone procedure. Figure 4 illustrates the correlation (y = 1.03x = 0.8; r = 0.99; p < 0.01) obtained on analysis of 81 specimens by the proposed method and with the commercial assay kit for urinary aldosterone, which does not require chromatography. A [3H]aldosterone version of this commercial method has been shown (16) to be satisfactory for routine clinical testing. The mean values obtained by the proposed method and the commercial [125I]-aldosterone assay were 19.0 and 18.7 µg/24 h, respectively. The data indicate that the two methods correlate well over the range from 1 µg/24 h up to about 69 µg/24 h.

Incubation time. We assessed the duration of incubation for the antigen–antibody reaction. The standard curves demonstrated an approximately equivalent displacement of [3H]aldosterone with incubation periods of 2, 6, 18, and 24 h. The most consistent quality-control data were obtained with the 18-h incubation period.

Parallelism. The parallelism of the method was determined by analyzing various volumes of three different human urines. The excellent proportionality observed between the quantity of aldosterone quantified per tube and the volume (in microliters) of urine analyzed documented the immunochemical similarity between aldosterone measured in the acid-hydrolyzed urines and the crystalline aldosterone used as standard.

Intra- and inter-assay variations. Intra-assay variation of the assay was determined by assaying (n = 20) quality-control pools with low, normal, and above-normal concentrations on
the same day. The following data were obtained: low: $\bar{x} = 2.7 \times 0.3 \mu g/L$, CV = 11.1%; normal: $\bar{x} = 12.5 \pm 1.1 \mu g/L$, CV = 8.8%; and above-normal: $\bar{x} = 21.9 \pm 1.3 \mu g/L$, CV = 5.9%.

Inter-assay variation was determined by assaying the same control pools over a period of 33 days. The following data were obtained: low: $\bar{x} = 2.9 \pm 0.6 \mu g/L$, CV = 20.7%; normal: $\bar{x} = 12.8 \pm 2.1 \mu g/L$, CV = 16.4%; and above-normal: $\bar{x} = 22.3 \pm 3.0 \mu g/L$, CV = 13.5%.

Normal values. Urinary aldosterone was measured in 19 adult subjects (10 women and nine men) in apparently good health and receiving a standard sodium diet. The values ranged from 3 to 19 for men (mean, 11 $\mu g/24$ h) and 4 to 18 (mean, 10 $\mu g/24$ h) for women.

Analytical recovery. The percent recoveries of [3H]aldosterone from human urine were 93.0 $\pm$ 3.8% and 93.0 $\pm$ 3.9% for the within-assay (n = 20) and between-assay (n = 27) extractions, respectively.

Discussion

An evaluation of components available commercially for the RIA of urinary aldosterone indicated that the proposed method was sensitive, specific, and reproducible.

The assay demonstrated a detection limit of approximately 25 pg, corresponding to a sensitivity of about 75 pg/mL. The detection limit of the assay could be reduced to about 12 pg by appropriate dilutions of the anti-aldosterone serum and the [3H]aldosterone.

Our cross-reactivity data indicate that the specificity of the antiserum was such that the presence of those steroids in urine should not adversely affect the quality of the measurement as described. The data (Figures 2 and 3) comparing concentrations as determined by the proposed method with concentrations as determined by the preliminary LH-20 column chromatography and paper chromatography, respectively, support the reliability of the proposed method, which does not require a chromatographic or differential-solvent partition and derivatization before RIA. In addition, the excellent correlation observed (Figure 4) between results by the proposed method and with a prepackaged reagent kit for urinary aldosterone bolsters the acceptability of the proposed method. Demers et al. (16) published a very comprehensive evaluation of a [3H]aldosterone version of the kit from Diagnostics Products Corp., which validated the clinical utility of the reagents. The anti-aldosterone serum provided with the [3H] aldosterone kit from Diagnostic Products Corp. is the same as the antiserum supplied with the [125I]-aldosterone assay we used for these studies.

The purity of the [3H]aldosterone used as the radiolabeled ligand for the assay and the recovery check was a major factor regarding the low nonspecific binding (2–3% of the total radioactivity) and the negligible assay blank. Repurification of the tracer at monthly intervals as described in the text resulted in a well-controlled method.

The normal range (3-19 $\mu g/24$ h) of urinary aldosterone compares well with normal values reported by others: 2-16 $\mu g/24$ h (17), 5-19 $\mu g/24$ h (18), 5-20 $\mu g/24$ h (4), and 4-17 $\mu g/24$ h (7).

The assay we describe here is a sensitive and specific non-chromatographic radioimmunoassay that is practical and convenient for use in most clinical laboratories.

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References