A Simplified Radioimmunoassay for Plasma Aldosterone

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ABSTRACT

The analytical evaluation is described of a \([^{125}I]\)-aldosterone radioimmunoassay method for measuring aldosterone in human plasma, which requires no chromatographic purification before quantification by radioimmunoassay but does require organic solvent extraction. Rabbit anti-aldosterone serum is used, generated against aldosterone-3-oxime coupled to thyroglobulin. The antibody demonstrates negligible cross reactions with structurally-related steroids. The proposed method uses \([^{125}I]\)-labeled aldosterone as the radioactive ligand. The recovery of D-aldosterone added to human plasma averaged 98 percent. The parallelism and precision of the method are excellent. Seventy specimens were assayed by the proposed method and a \([^3H]\)-aldosterone reference procedure, which includes LH-20 column chromatographic purification prior to the radioimmunoassay step \((y = 1.03X - 3.0; r = 0.99; p < 0.01)\). The \([^3H]\)-aldosterone reference procedure uses an anti-aldosterone serum prepared against aldosterone-3-oxime coupled to rabbit serum albumin.

Introduction

The accurate measurement of aldosterone in human plasma represents a considerable analytical challenge. This is due to both the extremely low concentration of this steroid in human plasma and to the presence of structurally-related steroids, usually present in much higher concentrations.

During the past ten years, numerous procedures for measuring plasma aldosterone by radioimmunoassay (RIA) have been reported. Owing to the lack of highly specific anti-aldosterone serum, the majority of these procedures utilized paper chromatography\(^3,11\) thin-layer chromatography,\(^19\) partition chromatography on LH-20 columns\(^5,9\) or a combination of solvent partition and derivative formation\(^8\) to improve the specificity of the measurement.

Data are presented which were obtained with a simplified radioimmunoassay (RIA) method for plasma aldosterone. Since the proposed method uses an anti-aldosterone serum which is highly...
specific for aldosterone, chromatography or derivative formation are not required. Furthermore, the proposed method uses $^{125}$I-aldosterone as the radioactive ligand, rather than $^3$H-aldosterone, a feature which greatly simplifies the procedure.

**Materials**

**Phosphate Buffer (0.2 M, pH 7.0, 0.1 Percent Gelatin)**

One g of gelatin* is dissolved in 900 ml of deionized water. The gelatin is heated to dissolve it (do not boil). It is cooled to room temperature and 8.7 g of Na$_2$HPO$_4$·H$_2$O, 5.4 g of Na$_2$HPO$_4$, 1 g of sodium azide, and 9 g of NaCl are added. The pH is adjusted to 7.0 and diluted to one liter.

**Aldosterone Standard-$^3$H Aldosterone Method**

Ten mg of aldosterone† are dissolved in 100 ml of methanol to give a stock solution of 100 mg per liter. One ml of the 100 mg per liter standard is then diluted to 100 ml with methanol to give a standard concentration of one mg per liter. A working standard (5 µg per liter) is prepared by evaporating 1.0 ml of the 1 mg per liter standard to dryness and redissolving the residue in 200 ml of the phosphate buffer. The working standard is further diluted with the phosphate buffer to obtain final concentrations of 4000, 2000, 1000, 500, 250, 125, and 62.5 ng per liter.

**Anti-alderosterone Serum, #HSC-23B-$^3$H ALDOSTERONE METHOD**

The anti-alderosterone serum prepared against aldosterone-3-oxime coupled to rabbit serum albumin was a gift.‡ The antiserum was received as a 1.0 ml aliquot. The 1.0 ml aliquot is diluted to 250 ml with phosphate buffer and frozen at -60°C in 0.5 ml aliquots. A working preparation of the anti-aldosterone serum is prepared by diluting the 0.5 ml aliquot to 15.0 ml with phosphate buffer.

**Dextran-coated Charcoal (DCC)-$^3$H Aldosterone Method**

Exactly 62.5 mg Dextran T-70§ is dissolved in 100 ml of phosphate buffer on ice with constant stirring. After the dextran is dissolved, 625 mg of Neutral Norit A Charcoal¶ is slowly added. The mixture is allowed to stir at 4°C for 30 minutes prior to use.

**Tritiated Aldosterone**

$^{1,2,6,7}$-H (N)-aldosterone¶ with a specific activity from 80-105 Ci per mM is purified by chromatography on Sephadex LH-20 prior to use. The steroid was eluted from LH-20 as described under Methods. A working solution of the purified $^3$H-aldosterone was prepared for the RIA step by evaporating 0.1 ml of the solvent mixture eluted from the column to dryness and redissolving the residue in phosphate buffer to 8000 cpm per 0.1 ml (approximately 40 pg of tritiated aldosterone per 0.1 ml of buffer).

**Nonradioactive Steroids**

Cortisol, cortisone, corticosterone, testosterone, progesterone and 17α-

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* Dišo Laboratories, Detroit, MI 48232.
† Research Plus Steroid Laboratories, Denville, NJ 07834.
‡ Dr. John K. McKenzie, University of Manitoba, Health Science Centre, Winnipeg, Manitoba, Canada.
§ Pharmacia Fine Chemicals, Piscataway, NJ 08854.
¶ Becton-Dickinson Diagnostics, Orangeburg, NY 10962.
¶ New England Nuclear Corp., Boston, MA 02118.
hydroxyprogesterone were obtained* and the aldactone was a gift.†

**Solvents**

Methylene chloride and benzene (distilled in glass) were used as obtained.‡ Cyclohexane and methanol (certified ACS grade) were used as obtained.§

**Methods**

*[^125I] Aldosterone Method for Plasma Aldosterone*

The *[^125I]*-Aldosterone Assay Kit† was used as the proposed method. The assay was performed as described in the procedural insert that accompanied the reagents. All of the standards, quality control pools, and patient specimens were assayed in duplicate.

**Organic Solvent Extraction of Plasma Aldosterone—[^3H] Aldosterone Method**

An aliquot (2.0 ml) of each plasma is transferred to a clean 16 × 150 mm disposable glass test tube and 0.1 ml (4 pg) of[^3H]-aldosterone is added to each specimen to estimate recovery. Similar aliquots of[^3H]-aldosterone, in duplicate, are placed into scintillation vials for total counts (controls). After a 15-minute incubation of the[^3H]-aldosterone with the plasma at 25°C, 15 ml of methylene chloride is added to each specimen and the tubes are rotated end-over-end on a "Roto-Rack"* at a setting of 8 for 15 minutes. The specimens are then centrifuged at 1600 × g for 10 minutes at 25°C and the aqueous layer discarded. The individual solvent layers are quantitatively transferred to clean 16 × 100 mm disposable glass test tubes, evaporated at 60°C in a stream of air, reconstituted with 1.0 ml of cyclohexane/benzene/methanol (6/4/1), and vortex-mixed for 3 to 5 seconds. Each extract is then purified by LH-20 column chromatography.

**Sephadex LH-20 Column Chromatography—[^3H] Aldosterone Method**

Eight-hundred mg of dry Sephadex LH-20† is placed in a column¶ with a porous polyethylene filter disc positioned at its bottom. The column solvent, cyclohexane/benzene/methanol (6/4/1, by volume) is passed through the column, swelling the column so that its height is about 5 cm. To eliminate floating gel, a second polyethylene filter disc is positioned above the LH-20 bed. The average flow rate through the column was 0.33 ml per minute. The individual 1.0 ml extracts, which have been prepared by reconstituting the original methylene chloride extracts with cyclohexane/benzene/methanol, are then applied to appropriately labelled LH-20 columns with a Pasteur pipet. The aldosterone fraction, which elutes between 5 to 15 ml of the column solvent, is collected in a 16 × 150 mm disposable glass test tube, evaporated under nitrogen at 60°C, the residue dissolved in 3.0 ml of phosphate buffer, and allowed to stand at 37°C for 15 minutes. Duplicate aliquots (0.3 ml) of each specimen are removed from the appropriate tubes at this step to estimate recovery.

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* From The Upjohn Co., Kalamazoo, MI 49001.
† From Searle Laboratories, Chicago, IL 60680.
‡ From Burdick and Jackson Laboratories, Muskegon, MI 49440.
§ From Fisher Scientific Co., King of Prussia, PA 19406.
¶ From Diagnostic Products Corp., Los Angeles, CA 90060.

† Pharmacia Fine Chemicals, Piscataway, NJ 08854.
¶ 1 x 18.5 cm; Isolab, Inc., Akron, OH 44309.
The recovery of $[^3$H]$\text{-}$aldosterone through the methylene chloride extraction and the LH-20 chromatography varied from 63 to 73 percent (mean, 68 percent).

**SIMPLIFIED RADIOIMMUNOASSAY FOR PLASMA ALDOSTERONE**

**RADIOIMMUNOASSAY PROCEDEUDR-**  
$[^3$H] ALDOSTERONE METHOD

Disposable polystyrene (12 × 75 mm) test tubes are marked for duplicate zero (aldosterone-free) assay tubes, 6.25, 12.5, 25, 50, 100, 200, and 400 pg per tube standards. Additional duplicate tubes are marked for each control and unknown to be assayed. Aliquots (0.1 ml) of the individual standards (except the total-count and blank tubes) are vortex-mixed gently and 0.1 ml of the anti-aldosterone serum is added. All of the tubes are then vortex-mixed gently for 3 to 4 seconds and incubated at 4°C for 18 hrs. Then, all of the tubes (except the total-count tubes) are placed in an ice bath. A 0.2 ml aliquot of ice-cold dextran-coated charcoal suspension is then added to each tube in rapid succession. Each tube is gently vortex-mixed immediately and allowed to stand at 4°C for 20 minutes. Then all of the tubes (except the total-count tubes) are centrifuged (1600 × g, 10 minutes, 4°C) and the supernates are decanted into approximately marked scintillation vials. Each scintillation vial then receives 10 ml of RIA-FLOUR, is capped, inverted 10 times and counted in an automatic liquid scintillation spectrometer§ for two minutes.

**Calculations**

The logit-log data-reduction method$^{15}$ was used to calculate the aldosterone concentrations of the unknowns and controls. The values computed were corrected for the percent recovery of the $[^3$H]$\text{-}$aldosterone through the methylene chloride extraction and LH-20 column.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Dilution</th>
<th>Aldosterone* (ng/liter)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>X 5</td>
<td>70</td>
</tr>
<tr>
<td></td>
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<td>2</td>
<td>X 5</td>
<td>250</td>
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<td></td>
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</table>

*Concentrations corrected for dilution RIA = Radioimmunoassay

**Results**

**SENSITIVITY AND SPECIFICITY**

In figure 1 is depicted a typical semi-logarithmic standard curve for the $[^1$25$I]$-aldosterone method and the cross-reactivity determinations which were performed to check the specificity of the anti-aldosterone serum (Lot #1003). The detection limit, defined as the smallest quantity of aldosterone which could be distinguished from zero aldosterone, was approximately 5 pg per tube. This detection limit corresponds to an aldosterone

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Aldosterone (Picograms)</th>
<th>Present (A)</th>
<th>Added (B)</th>
<th>Assayed (C)</th>
<th>Percent Recovery*</th>
</tr>
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<tbody>
<tr>
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<td>50</td>
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<td></td>
<td>210</td>
<td>200</td>
<td>390</td>
<td>90</td>
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</tbody>
</table>

*Percent recovery = $\frac{C - A}{B} \times 100$

Average recovery = 98 percent

§ Model 3330; Packard Instruments Co., Downers Grove, IL 60515.
concentration of approximately 10 ng per liter. The percentage of antibody-bound [125I]-aldosterone at zero unlabeled aldosterone varied from 50 to 60 percent of the total radioactivity. The nonspecific binding was routinely <3 percent of the total counts. An excellent log-log dose-response curve was routinely observed from 5 to 480 pg per tube. Aldosterone levels >400 pg per tube were diluted, re-extracted and re-analyzed. The cross-reactivity data (figure 1) indicated that quantities of cortisone, aldactone, testosterone, progesterone, and 17a-hydroxyprogesterone as high as 500 ng per tube did not affect the accuracy of the aldosterone measurement. Cortisol (not shown) and corticosterone cross reacted at approximately 0.006 percent, calculated according to the method of Abraham.1

Correlation of the [125I]-Aldosterone Method with a [3H]-Aldosterone Sephadex LH-20 Column Method

In figure 2 is illustrated the correlation ($y = 1.03x - 3.0; r = 0.99; p < 0.01$) obtained on an analysis of 70 specimens by the proposed method and a [3H]-aldosterone method which included LH-20 column chromatographic purification prior to the RIA step. The mean aldosterone concentrations obtained by the [125I]-aldosterone (LH-20) procedure were 237 ng per liter and 231 ng per liter, respectively. The data indicate that the two methods correlate well over the range from nondetectable to approximately 1400 ng per liter.

Assay Blank

The assay blank was determined by substituting 0.15 mol per liter of NaCl for plasma and performing the assay as described. The blank value was indistinguishable from the zero standard.

Parallelism

In table I are summarized studies performed to determine the parallelism of the [125I]-aldosterone method. The four plasma specimens assayed at $\times 5$ and $\times 10$ dilutions demonstrated acceptable parallelism.
ANALYTICAL RECOVERY

In table II are summarized the recovery data obtained following the additions of known quantities of D-aldosterone to three different plasma specimens. The recovery varied from 78 to 108 percent, with a mean recovery of 98 percent.

INTRA- AND INTER-ASSAY VARIATION

The inter-assay variation of the $[^{125}I]$-aldosterone method was determined by assaying quality control pools with normal, intermediate, marginally elevated and elevated aldosterone concentrations over a period of 40 days. The data accumulated from these studies are summarized in table III. The inter-assay variation was generally <12 percent at aldosterone concentrations from approximately 60 to 700 ng per liter. Aldosterone concentrations <60 ng per liter generally demonstrated an inter-assay variation of approximately 15 percent by the proposed method. Studies performed to evaluate the intra-assay variation of the method are also summarized in table III. Routinely, the intra-assay variation was approximately 60 percent of that observed with the inter-assay variation.

NORMAL VALUES

The expected values for this assay with a sodium intake of 100 to 200 mEq per day are as follows: 7 A.M. fasting: 30 to 90 ng per liter; 9 A.M. upright: 40 to 300 ng per liter. Aldosterone values for a sodium intake of approximately 10 mEq per day are as follows: 7 A.M. fasting: 120 to 360 ng per liter; 9 A.M. upright: 170 to 3170 ng per liter.

DISCUSSION

The proposed $[^{125}I]$-aldosterone radioimmunoassay technique described is a simple, precise, sensitive, and specific method for the quantification of plasma aldosterone. The elimination of column chromatography or other tedious purification procedures prior to the RIA step should make the proposed method attractive to clinical laboratories interested in accurate and, at the same time, convenient procedures for this very important measurement.
Sources of Error

Plasma is the preferred specimen for this procedure. It is important to separate the plasma from the cells within 20 minutes of collection. The steroid is stable in plasma for up to four hours at room temperature. If the assay will be performed after this time, it is advisable to freeze the specimen at −20°C. Aldosterone is stable at this temperature for at least six months.

Specimens with cholesterol concentrations greater than 600 mg per dl and/or triglyceride concentrations greater than 800 mg per dl should be extracted with methylene chloride as described and then subjected to LH-20 chromatography, to eliminate the interference which is frequently experienced with elevated lipids. The reader is advised to review two excellent articles which address this problem.8,14

Specimens expected to contain high concentrations of progesterone such as specimens collected from pregnant females should be pre-extracted with n-hexane to eliminate the interference from high concentrations of this steroid. The procedure for the pre-wash with n-hexane requires extraction of 1 ml of plasma with 5 ml of n-hexane. The plasma and solvent mixture is then placed on a Roto-Rack® at a setting of 8 for 15 minutes. The tube is then centrifuged at 1600 × g for 15 minutes at 25°C, the lower phase (plasma) is frozen in an acetone-dry ice bath and the n-hexane is discarded. The specimen is subsequently processed by the proposed method.

The individual solvents utilized for this method should be of the highest purity possible. Furthermore, the solvent mixture prepared for the LH-20 procedure should be prepared fresh for each experiment. It is recommended that latex surgical gloves be worn throughout the entire procedure. The use of gloves will decrease the likelihood of aldosterone contamination from excretions of the skin and reduce the risk of radiation exposure.2 In order to eliminate the contamination of the individual specimens with detergents, the routine use of disposable glassware and pipet tips is advised.

Many factors affect the concentration of plasma aldosterone. The following are known to increase the concentration of plasma aldosterone: primary and secondary aldosteronism,4 upright posture,16 accelerated hypertension,13 diuretics,18 estrogens and oral contraceptives,13 potassium loading,10 pregnancy,17 salt depletion,13 and volume expansion.10 It is important that these factors are known and that the posture, salt intake, and medication be controlled to obtain a clearer understanding of the variables which might contribute to the elevated concentrations. Factors such as primary adrenal defects, renin deficiency,16 sodium loading,10 and administration of mineral-corticoids are known to decrease the quantity of aldosterone secreted by the adrenal gland.

Resumé of Clinical Investigation

Although elevated levels of aldosterone are observed in both primary and secondary aldosteronism, the major use of the assay is for the diagnosis of primary aldosteronism, as defined by Conn4: (1) high aldosterone secretion which cannot be suppressed by increased sodium intake or the administration of mineral-cortisols; (2) low levels of plasma renin activity; and (3) hypokalemia and elevated excretion of potassium.

Although the incidence of primary aldosteronism as a cause of hypertension is not as high as originally reported by Conn, the determination of aldosterone levels should be considered in the majority of patients with hypertension, particularly those patients with unexplained hypokalemia associated with hypertension.

Low aldosterone concentrations may occur in patients with primary adrenal de-
fects, infants with enzymatic defects in aldosterone biosynthesis, and adults with renin deficiency. Patients with hypoaldosteronism may be recognized by hypokalemia which is not related to significant kidney failure.

References