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# RADIOIMMUNOASSAY FOR ALDOSTERONE AND NORMAL VALUES UNDER VARIOUS PHYSIOLOGICAL CONDITIONS*

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## SUMMARY

A rapid, specific, precise and reproducible radioimmunoassay for plasma and urinary aldosterone has been developed using sheep antibody preparation. Two millilitres of plasma were usually extracted with dichloromethane and the dry residue chromatographed on a blank-free sephadex LH-20: water column. Separation of free from bound hormone was achieved by dextran-coated charcoal suspension. The recovery of [$^3$H]-aldosterone ranged from 69 to 80%. The sensitivity in measuring the unknown samples was 15 pg. The plasma levels of aldosterone were measured in men and women under various physiological conditions. In 45 male and female controls on a diet containing 135 m-equiv of Na and 90 m-equiv of K, the mean recumbent plasma aldosterone, at 0800 h, was 8·0 ± 4·2 S.D. ng/100 ml and at noon after 4 h upright, ($n$ = 11) 18·3 ± 9·5 ng/100 ml, as compared to 7·9 ± 4·1 S.D. ng/100 ml recumbent; the recumbent mean was 7·7 ± 4·1 in 25 males and 8·4 ± 4·4 in 20 females (blood sampling, always within 6 days of the onset of the menstruation). The mean recumbent (0800 h) plasma aldosterone in 22 similar controls on random diet was 6·6 ± 3·5 ng/100 ml. Plasma aldosterone in 15 female controls on random diet after the 6th day of the menstrual cycle gave a higher mean of 9·9 ± 5·4 ng/100 ml.

Plasma aldosterone (0800 h in recumbent posture) showed two peaks in two experiments involving two ovulatory menstrual cycles—one in midfollicular and one in mid-luteal phase (11·1 and 8·5 ng/100 ml). The second peak was present in a third anovulatory cycle (12·7 ng/100 ml) as well. Plasma aldosterone in all three experiments ranged from 2 to 12·7 ng/100 ml.

Excretion of the 18-hydroxyhemiacetal glucuronoside of aldosterone in 11 controls gave a mean of 9·1 μg/24 h ± 4·8 S.D.

## INTRODUCTION

This paper describes a radioimmunoassay for plasma aldosterone and for the acid-labile urinary 18-hydroxy-hemiacetal glucuronoside of aldosterone (18-oxo-conjugate), as well as an assessment of the reliability of the complete method. This procedure utilizes a high-titer antiserum and provides a higher degree of precision than those already described [1, 2]. Our method consists of extracting plasma or acidified urine with dichloromethane, purifying the aldosterone by blank-free sephadex column chromatography, and by reaction with aldosterone antiserum forming a complex that is excluded from dextran gel while allowing the adsorption of the free aldosterone. Radioactive determination of the percentage bound and comparison with a standard curve gives the amount of aldosterone in the sample.

## MATERIALS AND METHODS

### *Reagents*

(a) All solvents were of reagent grade and were further purified before use [3, 4].

(b) Crystalline *d*-aldosterone (Ciba) was used without further purification.

(c) The purity of 1,2-[$^3$H]-aldosterone (53·0 Ci/m-mol, Amersham) was verified by paper chromatography in cyclohexane–benzene–methanol–water 1:9:6:4.

### *Buffer solutions*

(d) A borate buffer was prepared by dissolving 8·25 g of boric acid (Fisher reagent grade, powder) and 2·7 g

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of sodium hydroxide in about 500 ml of distilled water. After the addition of 3 ml of concentrated HCl, the total volume was brought to 1 litre and the pH adjusted to 8 with 5 N HCl.

A 1/15 molar phosphate buffer at pH 7 was prepared by mixing solutions A and B in proportion 41·3:58·7 respectively. Solution A: monopotassium phosphate ($KH_2PO_4$) 9·073 g/l. Solution B: disodium phosphate ($Na_2HPO_4.2H_2O$) 11·87 g/l.

(e) All steroids except aldosterone were dissolved in purified methanol (i.e. methanol purified as in ref. [4] and redistilled once more). Aldosterone was dissolved in the 1/15 M phosphate buffer at pH 7·0.

(f) A dextran-coated charcoal suspension (in 1:1 proportion) was prepared every month by adding 250 mg of Dextran T 70 (Pharmacia, Uppsala, Sweden) and 250 mg of Darco g 60 charcoal (Matheson Colman and Bell) to 100 ml of the phosphate buffer at pH 7·0.

(g) Sephadex LH-20 and Blue Dextran 2000 (Pharmacia) were used without purification.

(h) Automatic micropipettes (samplers of 250, 500 and 1000 $\mu$l capacity) were purchased from Oxford Laboratories, San Mateo, California.

(i) Chromatographic columns consisted of a borosilicate glass tube, 65 cm long, i.d. 100 mm. The bottom of the column was equipped with a Teflon no. 2 stopcock and the top with an outer 10/30 clear seal joint. An inlet tube was fused into the top of the inner joint; the bottom of the inlet tube serving as a drip tip.

(j) All the water used throughout this procedure was bi-distilled in all quartz apparatus obtained from Heraeus-Schott Quartzschmeltze CMBH, Hanau am Main, Germany with 50 mg/l of sodium azide (Fisher) added as preservative.

(k) Bovine serum albumin fraction V (Nutritional Biochemicals Co.).

*Antibody*

(l) The antialdosterone serum used was kindly supplied by Dr. Robert W. Bates, Hormone Distribution Officer, National Institute of Arthritis and Metabolic Diseases. This antiserum (sheep 088) was prepared by Dr. Ray Haning by the procedure of Erlanger *et al.* [5] in the laboratories of Drs. J. F. and Sylvia A. S. Tait at the Worcester Foundation for Experimental Biology, and was characterized as described [6]. This antibody was produced by injecting the albumin conjugate of aldosterone-18,21-dichemisuccinate to sheep. The antiserum was diluted 1/1000 with the borate buffer and stored in sealed glass ampoules in 0·2 ml aliquots at $-10°C$. The antiserum stored in this way appears to be stable over a period of 2·5 yr.

Aliquots of the antiserum diluted with the borate buffer from 1/50,000 to 1/500,000 were tested for titer. In the assay system used (see below) the dilution 1/500,000 could bind about 54–60% of the [$^3$H]-aldosterone in the absence of the unlabeled hormone. For each determination, a 1/250,000 dilution of antiserum was prepared in the phosphate buffer containing 2% of bovine serum albumin fraction V (BSA) as carrier protein to minimize adsorption of the antigen to glass, and [$^3$H]-aldosterone to give a concentration of about 10,000 d.p.m./ml. This solution was ready to use after standing for three days in the refrigerator and was usually prepared every 3 weeks. It becomes unstable when stored for longer periods of time.

*Counting*

Bray's solution (5 g of Permablend II, Packard and 120 g of Naphthalene, Baker's reagent grade, dissolved in dioxane to make 1 l. of solution) was used for counting. Fifteen millilitres of Bray's solution was added to a vial containing 0·5 ml of phosphate buffer (background), to two vials containing 0·5 ml of the solution containing the antibody plus [$^3$H]-aldosterone (total count), and to the vials containing the supernatant from the tubes containing the standard and the blank and left for 4 h at room temperature. Then, all the vials were counted 10 times for 10 min in a Packard Liquid Scintillation Spectrometer Model 3375 with an efficiency of 48·6%. Counts were corrected for quenching by the addition of known amounts of [$^3$H]-aldosterone and the use of channels ratio technique.

*Extraction and purification of plasma aldosterone (flow-sheet)*

*(a) Extraction.* A solution of 0·5 ml of the phosphate buffer, containing about 1500 d.p.m. of [$^3$H]-labeled aldosterone as internal standard, was mixed with 2 ml of heparinized plasma and 0·1 ml of 1N NaOH in a 25 ml cylinder fitted with a ground glass stopper. The sample was equilibrated for 5 min at room temperature and the mixture extracted once with 10 vol. of water-washed dichloromethane as described before [3]. After clear separation of the two phases, the aqueous layer was discarded by aspiration and the dichloromethane was washed successively with 1/10 vol of 0·01 N NaOH, 0·1 N acetic acid, and distilled water. Biogel P-10 (0·5 g) was added and the extract was thoroughly shaken for several min.* Two hours

* It is often difficult to solubilize in small vol. of water or organic solvents the dry residue obtained after the evaporation of dichloromethane rendering the transfer to the chromatographic column less complete. The treatment with Biogel P-10 removes the humidity and some impurities without causing any loss of the aldosterone marker counts and allows a nearly quantitative transfer of the sample.

later it was filtered over a plug of glass-wool and evaporated to dryness in a 20 ml elution tube [Fig. 1 of ref. 3] on a Buchler Evapo-Mix.

(*b*) *Column chromatography.* A small plug of glass wool, previously washed with purified methanol and water, was first tamped down to the bottom of the column and then covered with about 2 g of washed and ignited sea sand. Sephadex LH-20 gel previously swollen overnight in water was allowed to settle and excess water removed until a fairly thick slury was formed. This gel swells in water to give a bed volume of 4–4·4 from 1 g dry gel. The slury was poured carefully down the wall of a vertically mounted column to a height of 55 cm. To obtain an even sedimentation and to stabilize and equilibrate the gel bed, the flow of water was started immediately after filling the column and continued for 24 h. The flow was maintained by gravity feed from a 5000 ml Erlenmayer flask suspended 50 in. above the top of the column, connected to the inlet tube of the inner joint with Teflon tubing, and with the stopcock opened. Before starting any experiments, the homogeneity of the bed was checked by running a small sample of blue dextran 2000 (Pharmacia) dissolved in 0·2 ml of water through the column, which ran with the liquid front. Only columns with perfect homogeneity and without any air bubbles were used.

Each sample was applied with disposable Pasteur pipettes on a drained bed surface in 0·2 ml of water and they were drained again into the bed. The tube containing the sample, and the column wall in contact with the sample were washed once with 0·2 ml of water which was drained into the bed before starting the elution. A narrow starting zone is important to obtain good separation. The elution of all the columns with water in a given series (usually 12), was started at the same time. First 45 ml of water were collected from each column followed by seven 2·3 ml fractions collected with an LKB circular Radi-Rac automatic fraction collector. Aliquots of 0·2 ml of these fractions were counted to detect the radioactivity, and the water fractions containing aldosterone were pooled and evaporated to dryness in a 20 ml elution tube [Fig. 1 of ref. 3] on a Buchler Evapo-Mix.

The columns were washed between runs overnight with water containing 0·02% sodium azide (Fisher Scientific Co. Ltd.).

The dry residue resulting from the evaporation of the combined water phases was dissolved in 0·7 ml of phosphate buffer, out of which 0·1 ml was counted in a Packard liquid scintillation counter to give the correction factor for incomplete recovery and 0·5 ml was pipetted into a 12 × 75 mm disposable test tube for the binding assay.

*Binding assay (flowsheet)*

A standard curve was established for each assay by pipetting duplicate aliquots of 0·5 ml of the phosphate buffer containing 15, 25, 50, 75, 100, 150, 200, 250, 500, 750, 1000, 2000 and 3000 pg of unlabeled aldosterone solution into 12 × 75 mm test tubes. In addition, there were two tubes to which no aldosterone was added to give the initial counts bound, (I) as well as two tubes to which 10 ng of aldosterone had been added to determine the amount of radioactivity that is bound in an irreversible way to other components than the antibody [non-specific count (Bn) of the assay procedure] and is not participating in the binding assay. Aldosterone is not bound to BSA, in any other way, except for the small portion (about 200 d.p.m.) which forms a stable complex, not dissociating on addition of dextran coated charcoal, probably with a denatured part of BSA preparation which may vary slightly from batch to batch. The non-specific counts (Bn) have averaged 200 d.p.m. and were subtracted from all numbers prior to calculating the standard curve and the unknowns (Fig. 1).

One-half ml of the antiserum solution (dilution 1/250,000) described above was added to each of the standard and sample tubes, and the tubes were gently agitated for 15 s by means of a vortex mixer (Fisher). Final concentration of this solution is 1 g/100 ml for

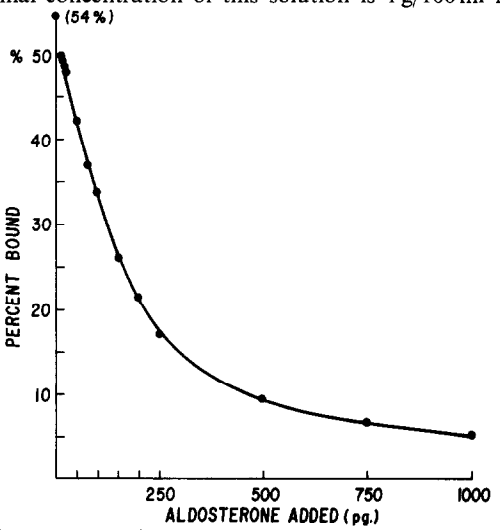
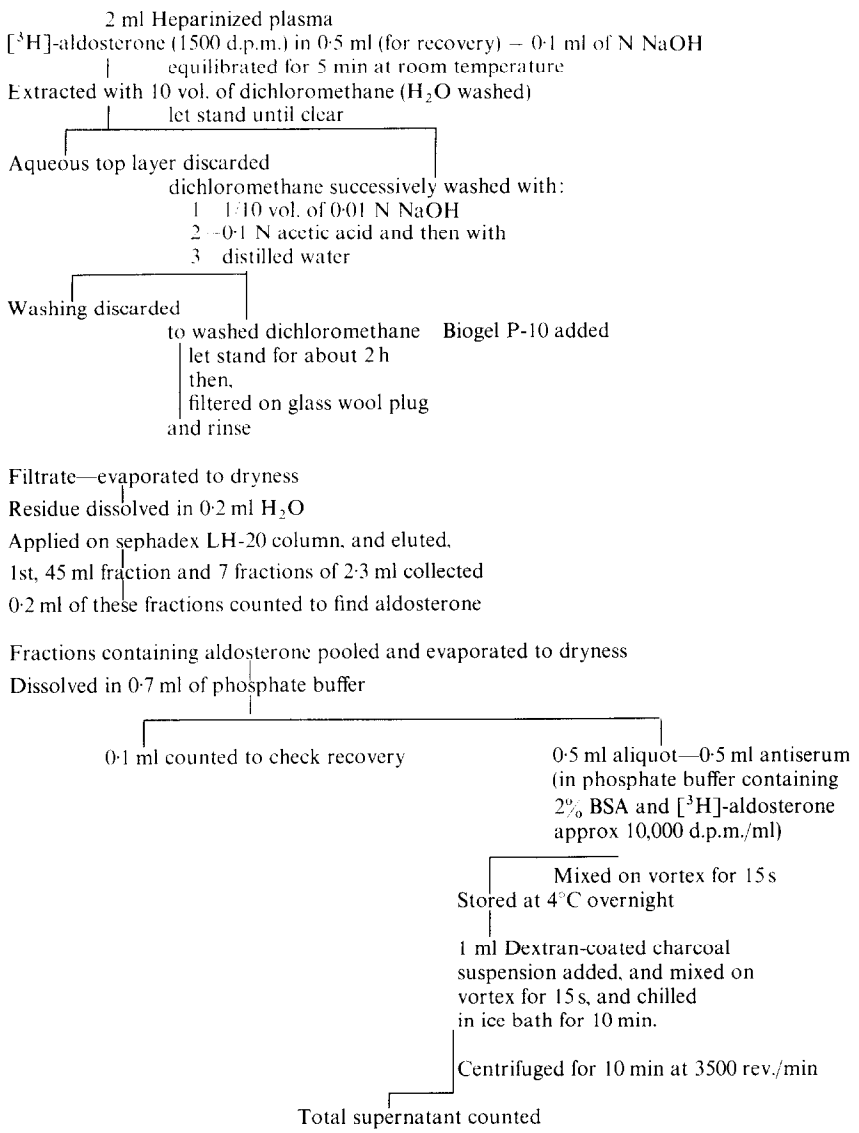



Fig. 1. A typical standard curve for aldosterone assay. The percentage of [$^3$H]-aldosterone bound to the antibody and therefore excluded from dextran coated charcoal (Ordinate) is plotted against the amount of [$^3$H]-aldosterone present (Abcissa). Values for plasma samples are read off from the curve after a similar determination of the percentage of added [$^3$H]-aldosterone bound. The per cent of bound counts is obtained according to the formula $(B - Bn)/(I - Bn) \times 100$. Where B are the bound counts, I the initial counts and Bn the non-specific blank (usually 200 d.p.m.).




BSA and 5000 d.p.m./ml for aldosterone, while the dilution of the antiserum is 1/500,000.

The tubes were covered with Parafilm (American Can Co.) and stored in a cold room at 4°C overnight. Free aldosterone was separated from bound aldosterone by adding 1 ml of the dextran-coated charcoal suspension to each tube. During the addition, the particles were kept suspended by slow rotation of a magnetic stirrer. The contents were gently swirled on a vortex mixer for 10–15 s, chilled for 10 min in an ice bath and centrifuged for 10 min at 3500 rev./min in a refrigerated centrifuge. The resulting supernatants (bound fraction) were decanted directly into counting vials (following the centrifugation of 3500 rev./min the decantation of the total supernatant does not disturb the charcoal) containing 15 ml of Bray's solution and the vials were shaken, kept in the dark for 4 h at room temperature, and counted in a liquid scintillation spectrometer. The 4 h wait is necessary for effective dissociation of the aldosterone–antibody complex, causing an increase in counts of approximately 3%.

*Extraction and purification of urinary aldosterone (acid–labile conjugate)*

A solution of 0·5 ml of the phosphate buffer, containing about 1500 d.p.m. of [$^3$H]-labeled aldosterone as internal standard, was mixed with a 1/500th aliquot of a 24 h urine collection, previously adjusted to pH 1

with hydrochloric acid, and was extracted 20 min later with 10 vol. of dichloromethane in a 25 ml cylinder fitted with a ground glass stopper. The aqueous layer was discarded by aspiration and the organic phase was washed successively with 1/10th vol. of 0·1 N NaOH, 0·1 N acetic acid, and distilled water.

The column chromatography was carried out as above. The resulting dry residue was redissolved in 2 ml of phosphate buffer, out of which 0·5 ml was counted by liquid scintillation to give the correction factor for incomplete recovery. Two aliquots of 0·25 ml and 0·5 ml were pipetted into 12 × 75 mm disposable test tubes for the binding assay. The 0·25 ml aliquot was first made up to a 0·5 ml vol. using the phosphate buffer.

*Calculations*

The standard dose–response curve was established by plotting the percentage of bound aldosterone against the amount of added unlabeled steroid (Fig. 1).

The concentration of aldosterone in plasma samples was read off from the above standard curve and the results in ng/100 ml were calculated by means of the equation:

$$\text{ngAl/100 ml} = \text{pgAl} \times \frac{0{\cdot}7}{\text{Ea}} \times \frac{100}{\%} \times \frac{1}{1000} \times \frac{100}{\text{Pa}} = \frac{\text{pgAl} \times 0{\cdot}7}{\text{Ea} \times \text{Pa} \times \%},$$

ngAl/100 ml—aldosterone nanograms/100 ml plasma; pgAl—picograms of aldosterone, read from the standard curve; Ea—eluate aliquot taken for binding assay (usually 0·5 ml); Pa—plasma aliquot used for analysis (usually 2 ml); %—per cent of label recovered.

The concentration of aldosterone in urine samples was read on the same standard curve and the results (in μg/24 h) were calculated from a similar equation after multiplication by a factor related to the aliquot used for analysis.

## RESULTS

The recovery of 1,2-[$^3$H]-aldosterone added to the plasma samples and recovered at the end of the extraction and purification procedure in the analysis of 50 plasma samples was 69–80%.

It should be pointed out that batches of bovine serum albumin (Fraction V) vary in their ability to stabilize the reaction and at the same time to produce a minimal non-specific binding blank. Equally good results were obtained when lysozym (Muramidase) grade I (Sigma) at a concentration of 100 mg/100 ml was used as carrier protein (non-specific blank about 100 d.p.m.), while gelatine, at the same concentration, produced too high non-specific blanks in the binding assay. The concentration of bovine serum albumin to be used, therefore, needs to be determined for each batch.

Binding reactions using various concentrations of BSA as carrier protein indicate that concentrations of 0·5 g/100 ml and 1 g/100 ml BSA showed a consistent decrease in bound fraction when unlabeled aldosterone was increased. Higher BSA concentrations resulted in excessive non-specific blanks while at concentrations below 0·5 g/100 ml unlabeled aldosterone binding was considerably lower, indicating the disappearance of the antibody by adsorption on the walls of of the assay tube.

In 30 experiments the standard curves, in duplicate, provided the following coefficients of variation for different amounts of aldosterone: 20 pg—4%, 50 pg—3·5%, 100 pg—3·8%, 250 pg—3·3% and 500 pg—4·1%.

## ASSESSMENT OF THE METHOD

*Column chromatography*

To determine where various steroids were eluted from the sephadex LH-20 column about 10,000 d.p.m. (tritium labeled) of each steroid tested were applied to the column and sequentially eluted with 2 ml aliquots of water. Each fraction was dried directly in a counting vial and determined by liquid scintillation. The steroids tested and the results are given in Fig. 2.

As shown on Fig. 2, 18-hydroxydeoxycorticosterone migrates on this column under its two forms [7]. The 18–20 hemiketalic form constituting only about 6% of the total amount, migrates slightly more slowly than aldosterone, while the less polar form, which is probably a dimer of the same hemiketal [7] has a migration slower than that of cortisone.

This method offers the advantage over all similar previously described procedures in that the total procedural blank cannot be distinguished from 0. Blanks obtained with 4 ml of water or plasma samples of totally bilaterally adrenalectomized patients or charcoal treated plasma put throughout the entire procedure gave blank values which consistently could not be distinguished from 0.

The separation with water as the only eluting solvent indicates that a liquid–gel reversed-phase partition system is obtained, water serving as the polar (mobile) phase and sephadex LH-20 with its lipophilic hydrophobic properties as the non-polar (stationary) phase. Corticosteroids of highest polarity such as aldosterone are eluted first and the less polar compounds later. The solubility in water is important for the polarity in this system. The solubility of aldosterone and 18-hydroxydeoxycorticosterone in water at

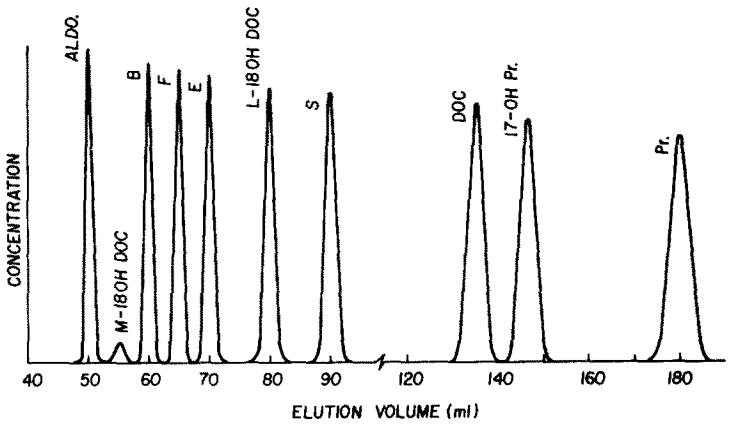



Fig. 2. Elution diagram of a mixture of steroids on Sephadex LH-20 in water. Bed dimensions: 1 × 55 cm. Sample 0·2 ml water containing a mixture of tritiated steroids (10,000 d.p.m. of each: aldosterone (aldo), 18-hydroxydeoxycorticosterone (18 OH-DOC)*, corticosterone (B), cortisone (E), cortisol (F), 11-deoxycortisol (S), deoxycorticosterone (DOC), 17α-hydroxyprogesterone (17–OH Pr) and progesterone (Pr). Aldosterone peak is at about 4·5 bed vol. of the column (or about 52 ml).
* 18–OH-DOC is eluted under its two forms M and L. [7]

23°C is 72 mg per 100 ml and 6·9 mg per 100 ml respectively, while all the other steroids tested are less soluble.

The columns are suitable for repeated use over long periods of time. After use, packed columns are immediately washed with water containing sodium azide to prevent microbial growth and stored until the next run. Sodium azide does not interfere with any step of the procedure including chromatography or the binding assay.

A series of 12–15 columns can be run all at once by one individual under the conditions described above.

*Specificity*

The antiserum used in this assay is highly specific for aldosterone, having a cross reaction of less than 1% with steroids, such as cortisol, cortisone, corticosterone, testosterone, dehydroepiandrosterone, progesterone, estradiol, 18-hydroxycorticosterone. The degree of cross reactivity was of 0·3% with 18-hydroxydeoxycorticosterone. Tetrahydroaldosterone and aldosterone γ-lactone cross-reacted with this antiserum to an extent of 2% and 1% respectively. All the above steroids were examined for interference with binding to the antibody at a level of 55% of [$^3$H]-aldosterone bound following addition of 15,000 pg of each respective compound. However, because some of these steroids, especially cortisol and cortisone are present in much higher concentrations than aldosterone in plasma and urinary samples, chromatographic purification of extracts prior to the binding assay is needed. In addition, corticosterone, 18-hydroxydeoxycorticosterone and corticosterone production may be markedly increased in some pathological conditions [8].

A dietary, dichloromethane soluble factor designated as compound III, has been previously isolated from human urine and citrus fruit juices [9–11], where it is present under free form, as well as conjugated as a β-glucuronoside. In various paper and thin-layer chromatographic systems, the compound has a mobility identical to that of aldosterone.

None of the related steroids tested, including urinary compound III, was found which would remain associated with aldosterone following the extraction, chromatographic purification and partition in amounts which would interfere with the binding assay.

The contribution of the mass of the internal standard to the assay was negligible (less than 1 pg at 53 Ci/mmol), and no correction was made.

Accuracy was evaluated by adding unlabeled aldosterone in known amounts ranging from 0 to 1000 pg to 2 ml samples of a plasma pool that had been previously extracted four times with an equal vol of water-saturated diethyl ether. The ether was removed on a rotary flask evaporator at room temperature. After correction for procedural losses, recovery was found to be 95–105% for all quantities of aldosterone added.

*Sensitivity*

The useful range of the standard curve was from 20 to 700 pg. Below 15 pg, the variability is too high. With a recovery of 60% in the assay tube, the method can measure as little as 2·0 ng/100 ml of aldosterone in a 2 ml plasma sample. Consequently for lower levels, larger plasma aliquots are needed.

*Precision*

Ten 2 ml samples from a pool of plasma containing 2 ng/100 ml from normal subjects, on a high sodium intake, afforded a coefficient of variation of 5%. When a pool of normal plasma containing 8 ng/100 ml was assayed in 10 experiments, the coefficient of variation was 4·5%.

Plasma concentrations of aldosterone obtained by this method in normal recumbent subjects were identical to those previously reported by our double-isotope dilution procedure [3].

All urine analysis were performed in duplicate; each sample representing a different dilution, usually 1/2 and 1/4 of 3 ml of the original urine sample.

*Normal range under various physiological conditions*

Plasma aldosterone was determined in 45 normal controls on the 4th day of a balanced diet containing 135 m-equiv. of Na and 90 m-equiv. of K. All subjects under study, unless stated otherwise, were on the above diet. The blood samples were obtained at 0800 h with the subjects recumbent since the night before

Table 1. Recumbent plasma aldosterone in control subjects at 0800 h, on a diet containing 135 m-equiv. Na and 90 m-equiv. K

| Samples | Males | Females |
|---|---|---|
| 1 | 5·1 | 6·6 |
| 2 | 6·0 | 9·2 |
| 3 | 6·3 | 2·1 |
| 4 | 10·8 | 7·6 |
| 5 | 12·0 | 5·0 |
| 6 | 5·9 | 5·8 |
| 7 | 7·8 | 4·6 |
| 8 | 11·4 | 9·4 |
| 9 | 17·7 | 3·5 |
| 10 | 8·6 | 12·7 |
| 11 | 7·3 | 9·4 |
| 12 | 17·5 | 15·3 |
| 13 | 15·1 | 4·0 |
| 14 | 6·0 | 10·9 |
| 15 | 2·0 | 6·6 |
| 16 | 3·9 | 2·0 |
| 17 | 7·8 | 10·7 |
| 18 | 3·8 | 3·6 |
| 19 | 6·5 | 11·6 |
| 20 | 6·0 | 7·7 |
| 21 | | 5·1 |
| 22 | | 17·6 |
| 23 | | 13·2 |
| 24 | | 4·4 |
| 25 | | 4·3 |
| | ($n$ = 20) | ($n$ = 25) |
| Mean | 8·37 | 7·71 |
| S.D. | 4·40 | 4·17 |
| S.E. | 0·98 | 0·83 |

M = 8·0; S.D. = 4·24; S.E. = 0·63; ($n$ = 45).

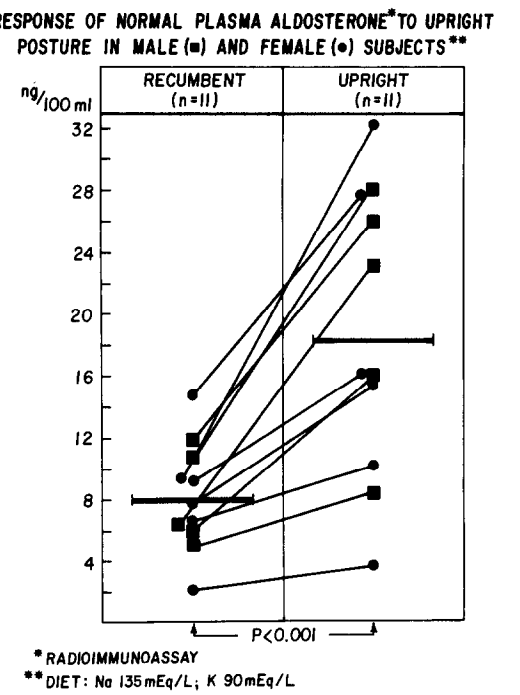



Fig. 3. Plasma aldosterone in control subjects, in recumbent posture at noon and again on the next day at noon after 4 h in upright posture (diet 135 m-equiv. Na and 90 m-equiv. K).

(Table 1), and, in some of them, at noon after recumbency since the night before and again the following day at the same time after 4 h in the upright posture (standing and quiet walking). The mean recumbent plasma aldosterone ($n$ = 45) was 8·0 ± 4·2 S.D. ng/100 ml and after 4 h, upright ($n$ = 11) 18·3 ± 9·5 ng/100 ml as compared to 7·9 ± 4·1 S.D. recumbent (Fig. 3). There is no significant difference in the recumbent plasma aldosterone between the mean in 25 male (7·7 ± 4·1 ng/100 ml) and 20 female (8·4 ± 4·4 ng/100 ml) controls.

The mean recumbent plasma aldosterone in 22 normal subjects on random diet, was 6·6 ± 3·6 ng/100 ml (Table 2). The plasma samples in all female subjects in Tables 1 and 2, as well as Fig. 1, were drawn within 6 days from the onset of menstruation. In addition, plasma aldosterone was determined in 15 female controls on a random diet later than the 6th day of the menstrual cycle and gave a higher mean of 9·9 ± 5·4 ng/100 ml (Table 2).

Table 3 summarizes the results obtained in recumbent plasma aldosterone measured through three menstrual cycles (two ovulatory and one anovulatory) with plasma samples drawn at 0800 h on days 3, 8, 12, 15, 18, 22 and 27 starting from the onset of menstruation. The ovulation estimated by basal temperature

Table 2. Plasma aldosterone (ng/100 ml) in recumbent normal controls on random diet

| Subjects | A Males | A Females | B Females |
|---|---|---|---|
| 1 | 7·0 | | 18·9 |
| 2 | 7·4 | | 8·0 |
| 3 | 7·9 | | 12·8 |
| 4 | | 4·3 | 2·3 |
| 5 | | 8·1 | 18·0 |
| 6 | | 2·4 | 12·4 |
| 7 | | 5·7 | 12·3 |
| 8 | 11·6 | | 10·8 |
| 9 | | 6·4 | 9·5 |
| 10 | 3·3 | | 6·4 |
| 11 | | 2·0 | 5·0 |
| 12 | 4·6 | | 6·9 |
| 13 | | 6·9 | 4·9 |
| 14 | 7·2 | | 3·4 |
| 15 | | 9·6 | 17·6 |
| 16 | | 4·4 | |
| 17 | | 8·4 | ($n$ = 15) |
| 18 | | 9·5 | M = 9·94 |
| 19 | | 4·9 | S.D. = 5·35 |
| 20 | | 2·1 | S.E. = 1·38 |
| 21 | | 3·4 | |
| 22 | | 17·5 | |
| | ($n$ = 7) | ($n$ = 15) | |
| | M = 7·0 | M = 6·37 | |
| | S.D. = 2·63 | S.D. = 4·00 | |
| | S.E. = 0·99 | S.E. = 1·03 | |
| | ($n$ = 22) | | |
| | M = 6·57 | | |
| | S.D. = 3·57 | | |
| | S.E. = 0·76 | | |

A: blood sampling in female subjects within 6 days from the onset of menstruation.

B: samples obtained in female subjects later than the 6th day from the onset of the menstruation.

occurred on days 16 and 21 in subjects I and II respectively. Subject III had an anovulatory cycle.

The variations in plasma aldosterone are reflected by expected changes in urinary Na/K concentration in subjects I and II.

Excretion of the 18-oxo-conjugate of aldosterone in 11 control subjects gave a mean of 9·1 $\mu$g/24 h $\pm$ 4·8 S.D. ranging from 2·0 to 15·0 $\mu$g/24 h.

## DISCUSSION

The main advantage of this procedure is the complete absence of any procedural blank related to the extraction or purification steps. This blank in methods using paper or column chromatography is unavoidable and usually not reproducible from sample to sample. The only blank, which is constant, present in our method is the non-specific and irreversible binding of some aldosterone counts by the antiserum preparation (about 20 d.p.m.) and bovine serum albumin fraction V. These counts (about 200 d.p.m.) do not, therefore, participate in the binding assay; their estimations, however, can be reproduced with high accuracy. This non-specific binding, only detectable at high aldosterone concentration, was nearly the same in the assay tube with and without the antibody. In the absence of the antibody, only about 180 d.p.m. of aldosterone were bound and no displacement occurred upon the addition of increasing amounts of unlabeled aldosterone.

The secretion and plasma concentration of aldosterone in normal subjects is markedly influenced by a number of factors such as diet, postural adaptation [12–16] and circadian periodicity [17]. In addition, fluctuations of aldosterone excretion [18–20], secretion [21] and plasma concentration [22] have been

Table 3. Cyclicity in plasma aldosterone (ng/100 ml) during normal menstrual cycle

| | Subject I | | Subject II | | Subject III |
|---|---|---|---|---|---|
| Days* | Aldosterone | Urinary Na/K m-equiv/l | Aldosterone | Urinary Na/K m-equiv/l | Aldosterone |
| 3 | 2·0 | 1·8 | 5·0 | 1·5 | 11·6 |
| 8 | 2·0 | 1·8 | 3·4 | 2·1 | 8·5 |
| 12 | 5·5 | 1·2 | 2·0 | 1·5 | 7·4 |
| 15 | 3·7 | 1·7 | 3·1 | 1·9 | 8·8 |
| 18 | 4·1 | 1·1 | 4·0 | 0·9 | 12·7 |
| 22 | 11·1 | 0·8 | 8·5 | 1·1 | 12·7 |
| 27 | 3·7 | 1·4 | 6·2 | 1·7 | 7·7 |

* Days from the onset of menstruation.

Ovulation: subject I, day 16; subject II, day 21; subject III, anovulatory cycle.

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reported during the course of the normal menstrual cycle.

For these reasons, normal values must be established under controlled conditions giving consideration to all of the above parameters.

In female subjects, the urinary [18] and plasma concentrations [Table 3 and Ref. 20, 23] showed, sometimes but not always, a moderate increase in the mid-follicular phase and invariably a more marked increase in the mid or late luteal phase of the cycle [23]. Both peaks were reflected by variations in the urinary sodium and potassium concentration, indicating that both peaks are of physiological importance; the second peak occurring in an anovulatory cycle as well (Table 3). This latter observation indicates that normal values in female subjects should be obtained in the first half of the follicular phase and also explains the higher mean obtained in female subjects when the blood sampling was done after the 6th day from the onset of the menstruation (Table 2).

With the method described, the sum of both the free and protein bound [24] plasma aldosterone is measured.

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