RADIOIMMUNOASSAY OF ALDOSTERONE IN PLASMA AND URINE: VALIDATION OF A NOVEL SEPARATION TECHNIQUE AND A RAPID URINE ASSAY

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SUMMARY

1. A rapid, robust, accurate and sensitive radioimmunoassay procedure is described for the measurement of plasma and urinary aldosterone.

2. A new, simple method is validated for the separation of free from bound moieties which has general applicability and which involves the measurement of free steroid by direct extraction into toluene-based liquid scintillator.

3. A new method, of validated specificity, is described for the measurement of the acid-labile metabolite of aldosterone in urine which avoids the necessity for preliminary chromatographic purification.

Key words: aldosterone, radioimmunoassay, plasma, urine.

The frequency of curable arterial hypertension caused by hyperplasia or an adenoma of the adrenal zona glomerulosa and the inadequacy of plasma potassium and sodium measurements as a screening procedure, necessitates the assay of aldosterone if patients with hypertension are to be assessed adequately.

However, estimation of the very small quantities of aldosterone present in plasma and urine relative to other structurally-similar, C-21 adrenocorticosteroids presents a considerable analytical challenge so that highly specific micro-analytical methods are essential. Isotope derivative techniques and gas–liquid chromatography have been used but, owing to the non-specificity of the mass measuring systems, tedious purification involving multiple chromatographic steps is usually necessary. In addition, the volume of blood required generally precludes repetitive sampling during short-term physiological manoeuvres.

The application of the principles of saturation analysis (Ekins, 1960; Yalow & Berson, 1960) offers an attractive alternative method. The use of a naturally occurring protein is theoretically promising, but aldosterone is not bound specifically to any circulating plasma protein and renal receptor proteins for aldosterone (Edelman, Bogoroch & Porter, 1963; Sharp, Komach & Leaf, 1966), although adequately energetic and stereospecific (Herman,
Fimognari & Edelman, 1968), are too unstable for routine use (Vyzantiades, Slater & Ekins, 1970; Robinson & Fanestil, 1970). However, many steroids can be rendered antigenic by coupling their 3-oxime or 21-hemisuccinate derivatives to bovine serum albumin (Erlanger, Borek, Beiser & Lieberman, 1957). In this study, we report the production of useful antibodies to aldosterone-3-carboxymethoxime-albumin conjugate and describe its application to the measurement of aldosterone in urine and plasma by a novel, simple and rapid technique. Some of the material of this paper has already been described in summarized form (Banks, Ekins & Slater, 1971; Ekins, Newman, Piyasena, Banks & Slater, 1972; Piyasena, 1972).

MATERIALS AND METHODS

Reagents

[1,2-3H]17β-aldosterone (New England Nuclear Corp., or The Radiochemical Centre, Amersham, 50 Ci/mmol) was purified by chromatography. D-Aldosterone was donated by Ciba; organic solvents were Analar grade and dichloromethane was redistilled twice. All chromatography was carried out on a Bush 5 system (benzene-methanol-water, 2:1:1, by vol.) with silica-gel impregnated paper (Whatman SG 81).

Incubation and radioactive assay

Incubations for radioimmunoassay were conducted in 10 ml conical stoppered glass tubes at 4°C by using Sørenson’s phosphate buffer (66 mmol/l) at pH 7.4. 3H was assayed with an efficiency of about 50% by liquid-scintillation spectrometry (Packard, Model 3320) by using a phosphor containing 2,5-diphenyloxazole (PPO) 4 g, 1,4 bis-(4-methyl-5-phenyloxazol-2-yl)-benzene (Dimethyl POPOP) 40 mg in 1 litre of toluene (scintillation grade, British Drug Houses). A ‘Zippette’ [Jencons (Scientific) Ltd, Mk. II] was used to add the phosphor to the assay tubes. 3H was located on paper chromatography strips by radio-scanning (Packard, Model 7200).

Antibody production

Aldosterone 3-carboxymethoxime was prepared and conjugated to bovine serum albumin as described by Erlanger et al. (1957). After dialysis against water for 3–4 days at 4°C the final yield of conjugate, as assessed by the recovery of 3H marker attached to protein, gave a molar ratio of aldosterone-3-carboxymethoxime/albumin of approximately 12:1.

The antigen (1 mg) was injected into eight lop-eared rabbits either subcutaneously and intramuscularly or intradermally (ten to twenty sites) after emulsification of 0.5 ml of antigen (25 mg/ml) in 0.5 ml of Freund’s complete adjuvant to produce a water-in-oil mixture. Booster injections (0.5–1 mg of antigen in complete Freund’s adjuvant) were given at approximately monthly intervals for 3 months and thereafter quarterly. Paired antisera dilution curves were prepared by using a trace quantity (4–8 pg) of 3H-labelled aldosterone with and without an excess of aldosterone (40–80 pg). With this criterion, antibodies to aldosterone were observed in every animal within 6 weeks; two of the five animals injected subcutaneously and intramuscularly and all of the three animals injected intradermally were found within 3 months to provide antisera with acceptable standard response curves at 1/300–1/750 final dilution in an incubation volume of 300 μl. The antisera showed equilibrium constants of 2.7–3.3 x 10^10 litre mol^{-1} at 4°C as measured by the techniques of both Scatchard and Sips
Radioimmunoassay of aldosterone

(Pinckard & Weir, 1967). Three antisera from a single rabbit on the subcutaneous/intramuscular schedule were used in this report, collected 5, 15 and 20 months after primary inoculation.

Preparation of plasma or urine for aldosterone assay

(a) With chromatography. (i) Urine. The aldosterone released from 2 ml of urine after incubation at pH 1 for 16 h was extracted with dichloromethane (2 x 6 ml) after adding 17 pg and 5 x 10^3 d.p.m. of ^3H-labelled aldosterone. The extracts were then washed with 2 x 1 ml of 0.05 mol/l of NaOH followed by 1 ml of 0.1 mol/l acetic acid, and finally, 1 ml of distilled water.

For the measurement of the secretion rate of aldosterone (when the patient is given about 3 μCi of ^3H-labelled aldosterone) 10-30 ml of urine was extracted with dichloromethane (1:3, v/v) after being acidified overnight.

(ii) Plasma. Aldosterone was extracted at 4°C from 2 ml of plasma (diluted 1:1 with deionized water) with dichloromethane (1 x 10 ml) after adding 34 pg and 10^4 d.p.m. of ^3H-labelled aldosterone. This technique minimizes emulsion formation. Washing the extract (as described above) was found to be unnecessary.

After drying under reduced pressure and redissolving in a few drops of ethanol, the extracts of both urine and plasma were developed chromatographically for 16 h. ^3H-labelled aldosterone was located by radioscanning and the observed peak was eluted (about 2 cm of paper cut into small pieces) with 1-2 ml of phosphate buffer for 15-30 min.

(b) Without chromatography. (i) Urine (5 ml), without pH adjustment, was pre-extracted with dichloromethane (2 x 15 ml) and the extracts were discarded. The pre-extracted urine (2 x 2 ml) was incubated overnight at pH 1, and, after the addition of ^3H-labelled aldosterone (5 x 10^3 d.p.m.), re-extracted with dichloromethane (2 x 6 ml). The extracts were washed (as described above), dried and redissolved in 2 ml of phosphate buffer.

All manipulations were carried out at room temperature unless otherwise specified.

Water blanks were assayed by substituting 2 ml of water instead of the unknown sample.

Incubation

Eluates or extracts were incubated with 100 μl of antiserum diluted 1/100-1/250 with phosphate buffer. In the urine assay (with or without chromatography) portions of eluate or extract were diluted with phosphate buffer so that 100 μl would contain about 5-40 pg of aldosterone. ^3H-labelled aldosterone 100 μl (1.2 x 10^3 d.p.m. and 4 pg) in phosphate buffer was then added to make a final incubation volume of 300 μl.

In the plasma assay, 100 μl of eluate was mixed with 100 μl of buffer, but no extra ^3H-labelled aldosterone was added. This is appropriate for a range of plasma concentrations of about 2-30 ng/100 ml.

The method allows considerable flexibility, the only limitation being that no more than one-fifth of the paper eluate is used.

A standard response curve was set up by incubating 100 μl of buffer containing 0-40 pg of aldosterone with 100 μl of antiserum and 100 μl of ^3H-labelled aldosterone.

After mixing and preincubation for 1 h at 37°C, the tubes were incubated at 4°C for 16 h. The assays were carried out in duplicate and the two tubes arranged in the rack symmetrically about the centre, i.e. the duplicates were separated and, whatever the order of the first set of tubes, the order of the second set was reversed.
A separate sample (one-fifth of the eluate or extract) was taken for the determination of recovery by the radioassay of $^3$H to within $\pm 2\%$ (SD).

Separation of free and bound aldosterone

Separation of free from bound aldosterone was carried out by direct extraction of the free moiety into toluene scintillator. After incubation, 5 ml of toluene scintillator at 4°C was added to each tube. The tubes were stoppered and shaken vigorously for 20 s. The tubes were left to stand for at least 15 min at 4°C (see the Results section) the scintillator was decanted into a counting vial and the water phase shaken again with a further 5 ml of cold scintillator for 5 s. This was added to the first 5 ml of scintillator.

The whole procedure for the extraction and radioimmunoassay of aldosterone in plasma and urine is summarized in Fig. 1.

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**Fig. 1.** Flow diagram for extraction of aldosterone from urine (a and b) or plasma (c) and the radioimmunoassay procedure. (a) With chromatography and (b) without chromatography.
Calculation of aldosterone content

Each assay run contained, apart from background and blank tubes, two or four tubes incubated with the same dilution of nonimmune rabbit serum as that of immune rabbit serum. The radioactivity extracted from these tubes (Cn, see below) provided a reference standard by which the 'percentage free' aldosterone was calculated in the tubes containing antiserum.

The assay of aldosterone in urine involves making a rough assessment of the likely aldosterone content, since this can vary considerably. Hence, a relatively wide range of dilution or sample factors is essential. By using 2 ml of urine, dilutions of 1/40 to 1/1000 or more should be anticipated. Therefore, a standard quantity of 3H-labelled aldosterone was added to each assay tube before incubation, i.e. the same amount which was added to each of the tubes used to construct the standard response curve.

The assay of aldosterone in plasma does not generally involve such a wide range of dilutions. Hence, enough radioactivity was added to the plasma initially and no further addition of 3H-labelled aldosterone was made.

The observed radioactivity extracted into the scintillator was expressed as a percentage of the total radioactivity present in each tube. The latter was calculated from the measured recovery plus, in the case of the urine assay, Cn.

The general formula for calculation of aldosterone concentrations (pg/ml) is

\[
R_1/V[1/SA_2 - 1/SA_1 (1 + R_2/R_p)]
\]

where \(R_1\) is radioactivity added to original sample, \(R_2\) is radioactivity added to incubation tube, \(SA_2\) is radioactivity carried through from the purification procedure (Rr)/pg measured, \(SA_1\) is specific radioactivity of tracer used, \(V\) is amount (ml) of sample used.

This formula is applicable to both urine and plasma providing the abscissa of the standard-response curve is expressed in total picograms (i.e. both radioactive and inert). For plasma \(R_2\) is 0, so the equation simplifies to

\[
R_1/V(1/SA_2 - 1/SA_1)
\]

This simplified formula is also applicable to the urine assay when the abscissa of the standard-response curve is expressed as picograms of inert aldosterone, as shown in Fig. 6.

RESULTS

Separation of free from bound aldosterone by direct extraction into toluene scintillator

Fig. 2 shows the steps involved in the use of this technique. Early in this study it was found that errors were minimized by the use of two separate shaking steps. The assay tubes can be shaken simultaneously or, more conveniently, in pairs.

Holding other variables constant, variations in the vigour of scintillator addition \(t_1\) led to significant differences of counts in the scintillator phase but after shake 1 these differences were abolished (Table 1).

Experiments designed to detect the effect of varying standing time \(t_1\), from 0 to 15 min, failed to show a significant difference (Table 1). Similarly variations of \(t_2\) from 15 to 105 min (Fig. 3) were without effect whether shake 1 was carried in pairs immediately after addition 1 (i.e. \(t_1 = 0\)) or whether all the tubes were shaken simultaneously \((t_1 > 0\) but variable). If \(t_2\) was less than 15 min, there was a small but significant decrease in the counts extracted and the
errors were increased \((P<0.02)\) when the tubes were shaken simultaneously but not if they were shaken in pairs. At a concentration of 10 pg, the coefficient of variation was 7.5\% \((n=10)\) and 4.1\% \((n=20)\) respectively. Varying \(t_3\) from 15 to 180 min was without effect (Fig. 4). This indicates that, provided a minimum of 15 min is allowed after shake 1, the limitation on the number of tubes which can be assayed simultaneously without decreased precision is governed only by \(t_2\) (105 min). We estimate that 100 tubes can be processed comfortably in a single assay run (about twenty unknown samples in duplicate) and that the time for separation would be about 2 1/2 h.

Finally, the effect of variations of the duration of shake 1 are shown in Fig. 5, also at different levels of response. The counts extracted varied little beyond a shaking time of 20 s.

The duration of shake 2 was held constant at 5 s throughout and all the variations depicted in Figs. 3–5 lay within the 95\% confidence limits of the assay, except for the points beyond 105 min in Fig. 3.

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**TABLE 1.** Effect of the vigour of addition 1 and the effect of prolongation of standing time, \(t_1\) (see Fig. 2). Values are \% free aldosterone in the presence of 10 pg added aldosterone. All values with shake 1 lie within the 95\% confidence limits of the method.

<table>
<thead>
<tr>
<th>Addition of scintillator</th>
<th>(t_1) (min)</th>
<th>Careful</th>
<th>Vigorous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without shake 1</td>
<td>0</td>
<td>0.6</td>
<td>15.9</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.9</td>
<td>14.9</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>2.0</td>
<td>11.5</td>
</tr>
<tr>
<td>With shake 1</td>
<td>0</td>
<td>47.6</td>
<td>48.2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>48.2</td>
<td>51.7</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>50.1</td>
<td>48.0</td>
</tr>
</tbody>
</table>
Radioimmunoassay of aldosterone

Fig. 3. Effect of varying the duration of standing time \( t_2 \) at two different response levels (see Fig. 2); \( t_1 = 0, t_3 = 15 \) min.

Fig. 4. Effect of varying the duration of standing time \( t_3 \) at two different response levels (see Fig. 2); \( t_1 = 0, t_2 = 15 \) min.
With the assay system described here, the protein concentration in the incubation tube is about 250 µg/ml or less. If it exceeds about 2 mg/ml, separation of the aqueous and organic phases is difficult.

![Graph](image)

**Fig. 5.** Effect of varying the duration of shake 1 at three different response levels obtained by the addition of aldosterone as indicated (see Fig. 2); shake 2 = 5 s.

**Sensitivity and error**

Fig. 6 shows that a useful response or standard curve was obtained within the range 0–40 pg. The error of the measurement as determined by the coefficient of variation of replicate estimations showed a pattern depicted in Fig. 7.

The standard curve gave a detection limit (±2 standard deviations of the zero point) of 1·7 pg. This corresponded to 0·64 ng/100 ml for the plasma aldosterone method when 2 ml of plasma was used.

**Specificity**

Table 2 shows the cross reactivity with other steroids which might be found in the plasma or urine of patients. The data presented were obtained with antiserum collected 20 months after primary inoculation (see the Methods section). Antisera collected 15 months after inoculation showed similar cross-reactivity and both were marginally superior to the cross-reactivity seen at 5 months. When the concentration of other steroids in plasma or urine, such as cortisol and corticosterone, is considered, the observed cross-reactivity is too high to obviate the need for further purification. However, even without preliminary purification, it is unlikely that the presence of prednisone, prednisolone or dexamethazone will interfere, even if large amounts have been given.
FIG. 6. (a) Composite standard response curve with 95% confidence limits (shaded). (b) effect of the addition of paper blank eluate at different points on the response curve. Results obtained by using 1/5 (v), 1/10 (■, ●) and 1/20 (●) of the eluate in two different laboratories (v or ■, ●) by three different workers (v, ■, and ●).

FIG. 7. Mean error from seven response curves at specified ranges of the response.
Table 2. Cross-reactivity (%) of specified steroids with antibody raised against aldosterone-3-carboxymethyl-oxime conjugate. Note change of relative potency according to the magnitude of the response.

<table>
<thead>
<tr>
<th>Response (% free aldosterone)</th>
<th>30</th>
<th>50</th>
<th>75</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol</td>
<td>0.03</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>Cortisone</td>
<td>0.14</td>
<td>0.08</td>
<td>0.04</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>1.48</td>
<td>0.82</td>
<td>0.39</td>
</tr>
<tr>
<td>Deoxycorticosterone</td>
<td>3.04</td>
<td>1.69</td>
<td>0.78</td>
</tr>
<tr>
<td>Progesterone</td>
<td>1.69</td>
<td>0.98</td>
<td>0.50</td>
</tr>
<tr>
<td>17 OH-Progesterone</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>0.003</td>
</tr>
<tr>
<td>Δ1,2-Cortisol</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Δ1,2-Cortisone</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Dexamethazone</td>
<td>&lt;0.15</td>
<td>&lt;0.15</td>
<td>&lt;0.15</td>
</tr>
<tr>
<td>Testosterone</td>
<td>2.10</td>
<td>0.60</td>
<td>0.12</td>
</tr>
<tr>
<td>Spironolactone</td>
<td>0.22</td>
<td>0.07</td>
<td>0.02</td>
</tr>
<tr>
<td>Aldadiene</td>
<td>1.40</td>
<td>0.43</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Accuracy

Formal experiments to test the recovery of added inert aldosterone were carried out for the plasma assay and for the urine assay without chromatography; the results are shown in Table 3.

Table 3. Accuracy of aldosterone assay in urine (no chromatography) and plasma (Bush 5 chromatography). Values in parentheses in left-hand column relate to number of estimates made.

<table>
<thead>
<tr>
<th>Added (pg)</th>
<th>Recovered (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Urine</td>
</tr>
<tr>
<td>5 (2)</td>
<td>95.6</td>
</tr>
<tr>
<td>10 (4)</td>
<td>103.4</td>
</tr>
<tr>
<td>20 (2)</td>
<td>101.7</td>
</tr>
<tr>
<td>50 (8)</td>
<td>—</td>
</tr>
<tr>
<td>100 (10)</td>
<td>100.3</td>
</tr>
<tr>
<td>200 (2)</td>
<td>96.1</td>
</tr>
<tr>
<td>Mean±1 SD</td>
<td>99.7±5.4</td>
</tr>
</tbody>
</table>

For plasma, agreement with added aldosterone lay within the confidence limits of the estimates. For urine without chromatography, experiments performed with a wider range of added aldosterone, were still within the confidence limits.
Correlation of the urine aldosterone assay, with and without chromatography

Fifteen urine samples covering a wide range of aldosterone concentration were assayed by both methods and failed to reveal any important systematic error. The results in thirteen samples are shown in Fig. 8. Two urine samples with a very high aldosterone content are not shown for convenience of presentation; paired values with/without chromatography were 68·0/72·8 and 72·9/78·3 ng/ml (correlation coefficient, $r = 0.999$ when these values are included). Eleven samples were collected from patients receiving drugs [$\alpha$-methyl dopa (five), spironolactone (one), frusemide (three), frusemide and spironolactone (one) and postoperative analgesics (one)].

![Fig. 8. Correlation of urine aldosterone assay with and without chromatographic separation ($r = 0.99$). The error in drawing the confidence limits as parallel lines is less than 3% at the extremes of the range depicted. $y = 1.13x + 0.09$; SE of estimate of slope is $\pm 0.06$.](image)

Two further urine samples (also not shown in Fig. 8) were collected from patients with high circulating levels of cortisol, and with consequent suppression of aldosterone secretion. Paired values with/without chromatography were 0·06/0·22 $\mu$g/day in a patient with a grossly abnormal steroid excretion pattern owing to a disseminated adrenal carcinoma and 1·7/3·1 $\mu$g/day in a patient receiving 120 mg of cortisol daily.

Loss of $^3$H-labelled aldosterone during purification

The recovery of $^3$H-labelled aldosterone added to urine after acidification averaged 87·5% $\pm 3·5$ ($n = 28$), without chromatography. Recovery of $^3$H-labelled aldosterone added to plasma averaged 64·0% $\pm 7·4$ ($n = 40$), with chromatography.
Reproducibility

This was calculated from duplicate estimates involving either plasma or urine as starting material. The relationship between error and the magnitude of the response shown in Fig. 7 indicates the need to express intra-assay and inter-assay variability as a function of response (or pg/assay tube). Intra-assay variability over the ranges 0–10, 10–20 and 20–40 pg/assay tube was 6.6%, 4.2% and 4.4% respectively. Interassay variability over the range 7–25 pg/assay tube was 7.3%.

Blanks

In the measurement of plasma aldosterone, variable blank values create a major problem which threatens the reliability of the method. Blanks are often difficult to detect unless adequate replicate assays are performed, but careful experiment with quadruplicate measurements established that the dominant cause of significant blank effects was limited to the chromatography step. The use of Whatman SG 81 appears to have successfully eliminated significant blank values (Fig. 6), by using either charcoal-stripped plasma or water, throughout the range of concentrations used in the response curve, provided that one-fifth of the paper eluate (or less) was used. Higher quantities regularly produced blank values which were positive. Charcoal-stripped plasma gave a mean value of 0.52 ng/100 ml ±0.06 (n = 8) which is not significantly different from the detection limit described above. It must be stressed that even when the assay system is running well, significant blank values do appear periodically, probably owing to variations within the chromatography paper itself; hence regular monitoring for blanks on at least two points on the standard curve is essential.

For the routine estimation of urine aldosterone with chromatography, a dilution factor of at least 1/100 is used so that blank problems are obviated.

For the determination of blank values when urine aldosterone was measured without chromatography, the urine from two adrenalectomized patients was used. Slightly positive values of less than 200 pg/ml were obtained. This represented a value of less than 0.2 μg/24 h.

Dissociation time

The rate of dissociation of the 3H-labelled aldosterone–antibody complex at 4°C was determined by preincubation for 41 h and 64 h (two separate experiments) followed by the addition of either buffer alone or buffer containing 80 pg of aldosterone to alternate tubes. Extraction with toluene scintillator at 4°C after 30 min to 5 h allowed the determination of the rate of displacement of the bound 3H-labelled aldosterone by the added inert aldosterone. When the results were plotted semilogarithmically the values fell along a straight line (within the 95% confidence limits of the estimate) which indicates a first-order reaction. Extrapolation gave figures of 7.02 and 7.5 h, respectively, for the half-time of dissociation.

Illustrative physiological changes of aldosterone

Examples of the use of the aldosterone assay to assess changes of plasma aldosterone concentration and the rate of adrenal secretion of aldosterone are shown in Tables 4 and 5. These reflect well the results obtained under similar conditions when aldosterone is measured by double isotope-derivative procedures.
Radioimmunoassay of aldosterone

Table 4. The rate of aldosterone secretion (µg/24 h) in hypertensive patients measured by radioimmunoassay from the specific activity of the urinary acidlabile conjugate

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign essential hypertension</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild sodium deprivation</td>
<td>7</td>
<td>252</td>
<td>117-379</td>
</tr>
<tr>
<td>Mild sodium loading</td>
<td>7</td>
<td>72</td>
<td>30-123</td>
</tr>
<tr>
<td>Proven glomerulosa adenoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild sodium deprivation</td>
<td>2</td>
<td>290584</td>
<td>--</td>
</tr>
<tr>
<td>Mild sodium loading</td>
<td>2</td>
<td>235332</td>
<td>--</td>
</tr>
</tbody>
</table>

Table 5. Plasma aldosterone concentration measured by radioimmunoassay in chosen normal people on an unrestricted diet; response to a diuretic and to orthostasis

<table>
<thead>
<tr>
<th>Physiological context</th>
<th>n</th>
<th>Mean (ng/100 ml)</th>
<th>Range (ng/100 ml)</th>
<th>Mean increase (%)</th>
<th>Range (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30-60 min after lying down at 14.00-17.00 hours</td>
<td>16</td>
<td>7.5</td>
<td>3.3-14.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>90 min after i.v. frusemide</td>
<td>6</td>
<td>18.6</td>
<td>10.4-29.9</td>
<td>+211</td>
<td>+86-+510</td>
</tr>
<tr>
<td>45 min after resumption of upright posture</td>
<td>10</td>
<td>16.8</td>
<td>5.9-26.0</td>
<td>+104</td>
<td>+43-+296</td>
</tr>
</tbody>
</table>

Discussion

Consideration of the relative merits of radioimmunoassay procedures for a particular substance, such as aldosterone, involves two main problems: (a) the extent of the preliminary purification procedure necessary to minimize the difference in the incubation conditions for unknowns and standards, and (b) the convenience and reliability of the procedure designed for separating the free from the bound moieties.

The method of separation described here relies on the measurement of the free moiety by direct extraction into toluene-based liquid scintillator. The theoretical implications of measuring the free as opposed to the bound moiety have been described already (Ekins et al., 1972). As mentioned above, the technique appears to be relatively robust and reproducible but it is necessarily limited to those substances which have a water-toluene scintillator partition coefficient sharply in favour of the organic phase. It might be thought that the success of the technique depends upon the production of an antibody with a very high affinity for aldosterone. However, our separation procedure will also operate when the equilibrium constant for the antibody–ligand reaction is relatively low and when the rate of dissociation of the antibody–ligand complex is relatively rapid. For example, Fig. 9 shows that a useful response curve is obtained when, for the measurement of cortisol, plasma corticosteroid-binding globulin \((K = 5 \times 10^8 \text{ litre mol}^{-1})\), halftime of dissociation \(= 23\ \text{min, at} \ 4^\circ\text{C}\) is used instead of anti-
body (for the antibody described here, $K = 3 \times 10^{10}$ litre mol$^{-1}$, halftime of dissociation = 7.2 h, at 4°C). Hence, the method is likely to have general applicability for relatively non-polar substances, measurable by liquid scintillation spectrometry. From the values quoted above, it appears that the energy of the antibody–ligand reaction is unlikely to be an important determinant for the success of the separation technique. The physical characteristics of the ligand alone may be important but, since the technique itself is likely to cause protein-denaturation (a precipitate at the phase interface is detectable after shaking with toluene scintillator), it is probable that changes in the characteristics of the antibody–ligand complex are dominant. For example, a diffusion barrier may form at the interface of the aqueous and organic phase and/or the half-time of dissociation of the ligand–antibody complex may greatly increase during denaturation.

![Response curve for cortisol](image)

**Fig. 9.** Response curve for cortisol by using a 1/30 dilution of charcoaled pregnancy plasma as the cortisol binding agent and $^{3}$H-labelled cortisol to determine distribution.

The preliminary purification procedure has, in previously reported plasma aldosterone radioimmunoassays, always involved the use of a chromatographic step which is both time-consuming and prone to produce blank values (Ito, Woo, Haning & Horton, 1972; Mayes, Furuyama, Kem & Nugent, 1970; Bayard, Beitins, Kowarski & Migeon, 1970; Underwood & Williams, 1972). The method for plasma aldosterone assay described here is no exception but by the use of silica-gel impregnated chromatography paper and by the careful statistical assessment of blank values over the whole range of the standard-response curve (Ekins et al., 1972) the problem has been contained and the limits defined.
Radioimmunoassay of aldosterone

In contrast, our method for the measurement of aldosterone 18-glucuronide in urine appears to obviate the need for chromatographic purification. The method depends for its specificity on the fact that the acid-lability of the 18-glucuronide is unique for aldosterone and that the pre-extraction technique for the removal of likely cross-reacting steroids (Table 2) is adequate. As a general rule, this will be so, particularly when the method is used for screening hypertensive patients. However, when the aldosterone/cross-reactant concentration ratio is unusually low (as, for example, after adrenalectomy; when the steroid excretion pattern is grossly disordered; or when very large quantities of extraneous steroids such as cortisol, cortisone or spironolactone are administered) the method will be likely to produce a modest over-estimate of aldosterone content. If the clinical context is adequately described, then the pre-extraction procedure can be modified appropriately, e.g. by increasing the number of extraction steps. Our results show that with our pre-extraction procedure over 99% of cortisol is removed; less polar steroids are likely to be extracted even more efficiently. It should be pointed out that without chromatography, the free unconjugated aldosterone in the urine is pre-extracted and discarded. Since this is only one-fiftieth of the concentration of the 18-glucuronide (Ekins et al., 1972) the difference from the value obtained with chromatography is negligible.

An elegant method for the measurement, without chromatography, of urine aldosterone 18-glucuronide by a radioimmunoassay technique has been described which depends upon the production of an antibody to aldosterone γ-lactone (Farmer, Roup, Pellizzeri & Fabre, 1972). Aldosterone 18-glucuronide is converted into the γ-lactone by periodic acid oxidation. However, the sensitivity of the method (±100 pg) demands the use of inconveniently large quantities of urine (25 ml).

One of the more surprising aspects of the development of radioimmunoassay techniques for aldosterone is that, contrary to experience with testosterone (Niswender & Midgley, 1970), specificity is not improved when a hapten attached to carrier protein through a part of the steroid molecule remote from the main site of chemical specificity is used. For example, the relative potency of the antibody raised against the 3-carboxymethoxime (which should maximize the ability of the specific 18-aldehyde radical to operate as a specific antigen) is not significantly different from the antibody raised against the 18,21-disuccinate (which should minimize the ability of the 18-aldehyde radical to operate as a specific antigen). Relative potency for cortisol (at 50% binding) ranges from 1% (Mayes et al., 1970) to 0.01% (Bayard et al., 1970) when the antibody is raised against the 3-carboxymethoxime and yet is only 0.01% when the antibody is raised against the 18,21-disuccinate (Underwood & Williams, 1972). It is possible that in the preparation of the oxime hapten, a mixture of mono- and disubstituted oximes (at the ketone groups at both C-3 and C-20 of the steroid nucleus) is, in fact, produced which may explain the similarity of relative potency. However, Africa & Haber (1971) have prepared an aldosterone hydrazone substituted in the C-3 position only. When this is linked to bovine serum albumin, the antibody produced still shows a comparable relative potency to cortisol (about 0.25% at 50% binding). It should be pointed out that the relative potency of antibodies raised against either the 18,21-disuccinate or the 3-carboxymethoxime are probably comparable to those described for peptide hormones when considerable structural similarity exists. In the context of aldosterone assay, it is only the 100–1000-fold difference of the concentration of likely cross-reacting steroids in biological fluids which makes the problem poignant, if specificity is to depend on antibody characteristics rather than chemical purification techniques.
Another limitation of the technique, as described for plasma aldosterone, is sensitivity. Defining this as twice the standard deviation of the zero point on the response curve, our method appears to be rather more sensitive (1.7 pg) than the best reported hitherto (4–5 pg; Underwood & Williams, 1972). However, 2 ml of plasma (which, in practice, represents about 5 ml of blood) is a relatively large amount if repetitive sampling is required without serious perturbation of the physiological context. Although the method was set up largely empirically, a sensitivity of 1.7 pg corresponds to 0.64 ng/100 ml which is also the value derived from a theoretical analysis of the maximum sensitivity which could pertain at zero hormone concentration using this particular assay system, i.e. apparent $K_{3.3} \times 10^{10}$ litre mol$^{-1}$ counting time 20 min, tracer specific radioactivity 50 Ci/mmol, incubation volume of 300 µl (Ekins et al., 1972). This value is at the lower limit of the normal range for plasma as determined by double isotope-derivative techniques (Brodie, Shimuzu, Tait & Tait, 1967; Coghlan & Scoggins, 1967).

Comparison with derivative analysis shows that our normal values for plasma aldosterone concentration (mean 7.5 ng/100 ml) in people on an unrestricted diet 30–60 min after lying down in the afternoon are rather higher than those quoted by Brodie et al. (1967) in supine men on an unrestricted diet after overnight recumbency (mean values from four groups 3.3–4.6 ng/100 ml). However, they are closer to the values (mean 5.8 ng/100 ml) given by Coghlan & Scoggins (1967) for normal people consuming 80–120 mmol of sodium daily after a minimum of 12 h overnight recumbency. These differences are small and they probably largely reflect the different physiological contexts.

In conclusion, the reliability of the antibody production technique, the robustness of the novel separation procedure and, for the urine acid-labile metabolite, the specificity, speed and simplicity of the preliminary purification appear to provide a practical technique for the measurement of urinary and plasma aldosterone. The data also suggest that other relatively non-polar substances can be measured by this technique when the radioassay involves liquid scintillation spectrometry.

Note added in proof

Since going to press, the report by Castanier & Scholler [Castanier, M. & Scholler, R. (1970) Comptes Rendus de l'Academie des Sciences (Paris), 271, 1787–1789] has been brought to our notice. They describe a similar direct extraction technique for the measurement of free oestrone and oestradial-17β but no validation data are available.

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Radioimmunoassay of aldosterone


