Simplification of urinary aldosterone measurement by radioimmunoassay

C. L. COPE and S. LOIZOU

From the Department of Medicine, Royal Postgraduate Medical School, London

SYNOPSIS An anti-aldosterone serum has been used to measure urinary aldosterone concentration. A single stage of paper chromatography provides an extract of sufficient purity. Further purification by additional chromatography in a different solvent system only improves the purity by 3% ± 8 (SD). Blanks are negligible and recovery of added aldosterone is 104% ± 10 (SD).

A simplified rapid screening assay capable of revealing gross deviations from the normal is also suggested. The diagnostic value of the assay is indicated.

Estimation of aldosterone, in either plasma or urine, is recognized to have considerable diagnostic value in the investigation of hypertensive patients and of those suffering from hypokalaemia. But its application has hitherto been handicapped by the complexity of the analysis and the time needed to complete it.

The corticosteroid-binding globulin in normal plasma binds aldosterone much less strongly than it does cortisol, and is therefore not well suited to the development of a competitive binding assay comparable to that used for cortisol.

The use of tissue binding sites has been explored by Robinson and Fanestil (1970) and by Vyzantiades, Ekins, and Slater (1970) but has not proved suitable for routine clinical use.

The recently introduced specific anti-aldosterone sera (Haning, McCracken, St Cyr, Underwood, Williams, and Abraham, 1972) have effectively removed these disadvantages and have reduced the complexity of the analysis. Such sera may be expected to become increasingly widely available.

We describe here a method for using this antisera for the routine clinical estimation of urinary aldosterone. It should be feasible in any laboratory having access to tritium counting facilities and to a refrigerated centrifuge. It does not make undue demands on technical skill.

Materials

Methylene chloride (Hopkins and Williams, GPR)

Simplified rapid screening assay

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National Institutes of Health, Bethesda, in a 1:100 dilution. Part of this was further diluted 100 times with 0.5% crystalline bovine serum albumin (Armour) in 0.1 M borate buffer at pH 8.0. This 1:10 000 dilution was dispensed into 0.5 ml aliquots which were stored frozen at −20°C.

GLASSWARE
All glassware used to contain solutions before chromatography was washed in mild detergent, then in tap water and distilled water, and dried. Glassware used for eluates after chromatography was soaked in 20% hydrochloric acid overnight before being washed and dried.

REAGENTS
Three reagents are prepared for stock.

1. Kinard scintillator solution
To make 2 litres dissolve naphthalene 160 g, PPO 10 g, and alpha-NPO 0.1 g in a mixture of xylene 770 ml, dioxane 770 ml, and ethanol 460 ml.

2. Borate buffer
Dissolve 8.25 g of boric acid and 2.7 g of sodium hydroxide in 500 ml of distilled water. Add hydrochloric acid to bring the pH to 8.0 and make up to 1 litre with distilled water.

3. 9:1 benzene/ethanol
Five reagents need to be prepared fresh for each assay run.

4. Antiserum reagent
One hundred mg of crystalline bovine serum albumin is dissolved with stirring in 20 ml of borate buffer at pH 8.0. To this is added one 0.5 ml tube of the 1 in 10 000 antiserum. Centrifuge for five minutes at 2000 rpm before use. The final dilution of the antiserum is thus 1 in 400 000.

5. Aldosterone standard
For each assay curve 40 μl of the stock solution of aldosterone is freshly diluted with 9:1 benzene/ethanol to a final concentration of 1 ng per ml.

6. 3H-aldosterone dilution
Eighty μl of the stock 3H-aldosterone solution is diluted to 4 ml with 9:1 benzene/ethanol.

7. Methylene chloride
Sufficient for the day’s extractions is well shaken with two successive equal volumes of distilled water.

8. Dextran-charcoal suspension
Twenty-five mg of either Dextran 80 or Dextran T-70 (Pharmacia) and 25 mg of Norit ‘A’ charcoal are dissolved together with stirring for at least 10 minutes in 10 ml of borate buffer. The stirred solution is maintained at 0°C in an ice bath.

Method

Urine is collected for 24 hours or other suitable period with the addition of 10 ml of 20% acetic acid. It may be stored until required in a deep freeze at −20°C. A sample of the urine equivalent to 1/100 of the 24-hour sample is brought to pH 1.0 with concentrated hydrochloric acid, and stood at room temperature for 24 hours. It is then transferred to a separating funnel and extracted with two successive equal volumes of freshly washed methylene chloride. To the combined extracts is added 25 μl of the stock 3H-aldosterone solution as an internal standard. This represents about 150 000 dpm. The well mixed extract is washed twice with 1/5 volume of N/10 sodium hydroxide, once with 1/5 volume of N/10 acetic acid, and once with 1/5 volume of distilled water. The extract is then dried by passing through anhydrous sodium sulphate retained in a filter funnel by a glass wool plug. After evaporation to dryness under reduced pressure on a water bath at 50°C the residue is dissolved in 5 ml of 9:1 benzene/ethanol.

This solution is transferred to a small conical tube and evaporated under a gentle jet of filtered air, to a volume of about 60 μl. This concentrate is applied to the starting line of a paper chromatogram and is run in the Bush B5 system till the mobile phase reaches the bottom of the paper. Fifteen μg of cortisol and of cortisone are run as markers in an adjoining lane. After development and drying the marker spots are located by viewing under ultraviolet light of wavelength 254 λ. The zone from 0.5 cm below the cortisol spot to 0.5 cm above the cortisone spot is cut out for elution. Elution is best made with ethanol, and the eluate diluted to 5 ml with benzene.

One ml of this eluate is removed and dried in a phial for counting of tritium recovery. Part of the remainder of the eluate is diluted 25 times with 9:1 benzene/ethanol for the assay.

The assay
Round bottom glass tubes 50 mm by 12.5 mm are most suitable for the assay. For the standard curve a series of these tubes is set up in duplicate or triplicate containing zero, 0.1, 0.2, 0.5, and 1.0 ng of aldosterone. The dilution of the stock aldosterone is made with 9:1 benzene/ethanol. The total volume of each tube is made up to 1.0 ml with this solvent. Duplicate tubes are set up containing 0.16, 0.4, and...
1·0 ml of the diluted eluates for assay. These also are made up to 1·0 ml with benzene/ethanol. To each standard and unknown tube is added 0·2 ml of the diluted \(^3\)H-aldosterone solution. After gentle mixing all tubes are evaporated to dryness in a stream of filtered air on a water bath at 45°C.

When dry, 0·5 ml of the antiserum reagent is added to each tube. All are covered with parafilm, mixed for 10 seconds on a vortex mixer, and left in a refrigerator at 4°C overnight.

Next morning the racks of tubes are transferred to an iced water bath, and to each tube is added 0·5 ml of the already well chilled and stirred dextran charcoal suspension. After mixing for 10 seconds on the vortex mixer the tubes are left in the ice water bath for a further 10 minutes. While still cold they are then transferred to the previously chilled cups of a refrigerated centrifuge at 4°C. Ten minutes centrifuging at 2000 rpm has proved adequate for our charcoal, but other samples may require longer. After centrifuging the tubes are returned to the ice water bath and 0·5 ml from each is transferred into 10 ml of Kinard reagent in a counting phial. The phial contents are mixed, and are counted in a suitable scintillation spectrometer for tritium to an accuracy of 1%, i.e., more than 10 000 counts.

In addition to these assay tubes a separate tube is set up to determine the total tritium content of the reacting mixture, the 'total activity' This contains no aldosterone, 1 ml of 9:1 benzene/ethanol, and 0·2 ml of the diluted \(^3\)H-aldosterone reagent. After evaporation to dryness 0·5 ml of the antiserum reagent is added and the tube incubated with the others overnight. But 0·5 ml of borate buffer replaces the dextran charcoal suspension. Then 0·5 ml is removed for counting of the 'total activity'.

**Calculation**

The percentage of the 'total activity' which remains in the supernatant fluid in the assay tube represents the percentage of aldosterone bound. The standard curve is drawn from the known dilutions and the unknowns are read from this. If \(D\) is the final dilution of the sample in the assay tube, and \(N\) the number of picograms found from the standard curve, and if \(R\) is the percentage recovery of the internal standard, then the aldosterone content of the extracted urine sample will be \(N \times D \times \frac{100}{R}\) pg. And if 1/100 of the 24-hour urine output has been extracted then the daily aldosterone excretion will be one hundred times this.

With the quantities advocated here the additional aldosterone added in the internal standard will be less than 0·5% of the total, and no correction for this need be applied. The method blank is also negligible.

**Notes on the Method**

A sample representing 1/100 of a 24-hour urine collection, or about 1/4 hour of excretion, may be expected to contain from 30 ng to 1 \(\mu\)g of aldosterone. Even the lower figure provides plenty for this very sensitive assay. By processing a generous excess through the chromatography stage and subsequently diluting the eluate, the problem of blanks derived from the paper becomes negligible.

The \(^3\)H-aldosterone internal standard may be added directly to the urine before hydrolysis, but if it has been stored in benzene solution it will be found more convenient to add it to the methylene chloride extract with which it is more readily miscible. This extract will contain more than 99% of the urinary aldosterone.

Aldosterone itself is best avoided as a marker on the paper chromatograms. Not only is it expensive, but there is serious risk of contamination of the nearby unknown lane. In contrast cross reactivity with either cortisol or cortisone is low (see later). Location of the tritiated aldosterone can, of course, be made by a chromatogram scanner adjusted for tritium, but we have not found this necessary.

We find the simplest and most convenient method of elution to be as follows. The cut-out paper is folded with forceps several times lengthwise to form a strip and this is inserted into a suitable small phial containing 1·0 ml of ethanol. The strip acts as a wick, and if the ethanol is allowed to evaporate overnight the steroids will be concentrated in the top few millimetres of the strip. This is then cut off and eluted with 0·5 ml of fresh ethanol in a stoppered tube. After allowing an hour or more for the elution 4·5 ml of pure benzene is added to the tube. The resultant solution is then ready for assay after further appropriate dilution.

The dilutions suggested for the assay tubes of unknown samples are chosen to provide the maximum chance of two of these dilutions falling within the optimum range of the assay curve, from 100 to 700 pg per tube. Allowing for a 50% recovery, the 1·0 ml tube will cover the range from 2·5 to 17 \(\mu\)g per day, the 0·4 ml tube from 6·2 to 42 \(\mu\)g per day, and the 0·16 ml the range from 15·5 to 105 \(\mu\)g per day.

It has been found very desirable to use 9:1 benzene/ethanol as the solvent throughout. When nanogram quantities of aldosterone were dried from pure ethanol onto glass at 45°C decomposition commenced soon after drying. More than 30% can be lost in two hours at this temperature. Neither siliconing of the tubes, nor replacement of the air stream by nitrogen, nor special purification of the ethanol has prevented this. But it can be prevented completely by the addition of a trace of the anti-
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oxidant 2-mercaptoethanol, and is therefore presumably an oxidation (cf, Doerr, 1971). Such decomposition is very greatly reduced if the aldosterone is dried from 9:1 benzene/ethanol solution.

The assay curve obtained after only one to three hours of incubation at 4°C is almost the same as that after 15 hours. The incubation time may therefore be adjusted within these limits to suit the routine of the laboratory.

It is important to maintain the incubated assay tubes at 0°C throughout the remainder of the assay, either in an ice bath or in a cold room. The dextran charcoal reagent must also be chilled to 0°C before being added. Great deviations from the proper assay curve may result if these precautions are not carefully observed (see also Mikhail, Wu, Ferin, and Vande Wiele, 1970).

Counting of the supernatant tritium may be done by decanting the whole of this into 20 ml of scintillator solution. Results are likely to be somewhat less accurate than when a measured 0.5 ml is removed with an appropriate pipette such as an Eppendorf microlitrepipette.

The 'total activity' measured in this way will differ from the total tritium counts added to the tube. In our hands it has been consistently 80% ± 1.5 (SD) of the added counts. The difference is presumably due to adsorption of the steroid onto the glass wall of the tube during drying (Kandel and Gornall, 1964). We have expressed binding percentage in terms of the actual measured 'total activity' in solution, and not as a percentage of the total tritium added to the tube. Since the ratio between the two is constant no reduction of accuracy results from this absorption.

Results

PURITY OF ³H-ALDOSTERONE

The reliability of the assay is dependent on the degree of purity of the tritiated aldosterone used as internal standard being known. Purity was determined at intervals by running a sample of the tritiated aldosterone with from 10 to 1000 ng of carrier aldosterone on the Bush B5 system, and determining the tritium and the aldosterone content of the eluate from the aldosterone zone. Mean purity determined in this way was 98.7% ± 11 (SD) (N = 9).

RECOVERY OF ADDED TRITIUM

The mean recovery of internal tritium standard in the washed and dried methylene chloride extract before chromatography was 97% (N = 15). Mean recovery of tritium in the eluates from the Bush B5 chromatograms was 59.2% ± 6.7 (SD) (N = 35). After two successive chromatographies the mean tritium recovery was 40.5% ± 7.2 (SD) (N = 11).

ASSAY CURVE

No deterioration has been detected in the activity or properties of the antiserum over more than one year, during which time it has been stored in borate buffer at pH 8-0, containing 0.5% of bovine serum albumin and kept at −20°C. Variations in the assay curves over several months, together with standard deviations, are shown in the figure. With zero aldosterone the mean binding has been 44% ± 1.9, with 100 pg it was 33% ± 1.6, with 200 pg it was 25% ± 1.2, with 500 pg it was 14.8% ± 0.9, and with 1.0 ng it was 9.2% ± 0.17 (SD) (N = 18).

Fig. The standard assay curve showing the standard deviations of the variations occurring during six months.

SENSIVITY

If sensitivity is estimated from twice the SD of the zero point, then the theoretical limit of detection is about 35 pg per tube.

PRECISION

Replicate analyses of a sample of urine gave within-assay variation of ± 5% and between-assay variation of ± 8.4% (N = 12). Agreement between assays of urine samples made at two or more concentrations depends on the area of the assay curve on which the points fall. When both assays showed less than 30% binding, and therefore contained more than 100 pg of aldosterone, the mean difference between the two assays was 5.7% ± 2.9 (N = 25). When one or both
assays were less than 100 pg, so yielding more than 30% binding, the mean difference between assays rose sharply to 23% (N = 8).

**SPECIFICITY**
The specificity of the assay derives in part from the properties of the antiserum itself, and in part from the preparation of a sufficiently purified extract for assay.

The cross reactivity of this antiserum is small but could become significant if inadequately purified extracts are used. Its extent is very dependent on the amount of alternative steroid present, because assay curves with other steroids do not run parallel to those of aldosterone. When tested at a concentration of 10 ng per tube cross reactivity has been found to be as follows:

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldosterone</td>
<td>100 (100)</td>
</tr>
<tr>
<td>Adrenosterone</td>
<td>1-5</td>
</tr>
<tr>
<td>Cortisone</td>
<td>0-7 (0-3)</td>
</tr>
<tr>
<td>Deoxycorticosterone (DOC)</td>
<td>0-7 (0-1)</td>
</tr>
<tr>
<td>Progesterone</td>
<td>0-5 (0-1)</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>0-2 (0-1)</td>
</tr>
<tr>
<td>Tetrahydro DOC</td>
<td>0-15</td>
</tr>
<tr>
<td>Cortisol</td>
<td>0-0 (0-1)</td>
</tr>
<tr>
<td>Tetrahydro aldosterone (3 alpha, 5 beta)</td>
<td>0-0</td>
</tr>
<tr>
<td>Tetrahydro cortisone</td>
<td>0-0</td>
</tr>
<tr>
<td>18 Hydroxy DOC</td>
<td>0-0</td>
</tr>
<tr>
<td>18 Hydroxy corticosterone</td>
<td>0-0</td>
</tr>
<tr>
<td>Aldosterone gamma lactone</td>
<td>28-0 (12-34)</td>
</tr>
<tr>
<td>Aldosterone 21-acetate</td>
<td>260 (tested at 0-5 ng per tube)</td>
</tr>
</tbody>
</table>

The figures in brackets are, for comparison, the values found by Haning et al (1972) who prepared this antiserum.

Adequate purity of the eluate from the Bush B5 chromatogram has been demonstrated by showing constancy of specific activity of the eluted aldosterone when this is further purified by chromatography in a second and different solvent system. When Bush B5 eluates were further purified by chromatography on the Bush C (Bush, 1952) system, the mean change in specific activity was only 3% ± 8 (SD) (N = 8).

**BLANK VALUES**
By extracting and purifying an amount of the urinary aldosterone which is greatly in excess of the amount needed for the assay, a high dilution of the extract with inert solvent is required. As a result blank values have been negligible even with unwashed Whatman chromatography paper.

Twenty-five ml volumes of water extracted and put through the whole analytical procedure gave a 'water blank' equivalent to only 1-1 ng. Even for urines of very low aldosterone content (2 µg per day) this would only represent about 5% of the total.

Eluates made from areas above and below the aldosterone zone of the paper chromatogram of urine extracts gave a 'urine blank' equivalent to only 100 ng per day. This represents about 1% of the average normal aldosterone excretion.

**ACCURACY**
When known amounts of aldosterone varying from 5 to 1000 ng have been added to 50 to 200 ml of water or urine, and have been processed through the assay, the mean recovery of the added steroid has been 104% ± 10 (SD) (N = 14).

**NORMAL VALUES**
Normal urinary aldosterone excretions have fallen within the generally accepted normal range of 5 to 20 µg per day. The mean of a series of 16 urine samples from normal laboratory staff or convalescent hospital patients without metabolic disorder has been 14-3 µg per day ± 6-7 (SD). The mean for the ambulant laboratory staff has been higher (18-1) than for the less active convalescent patients (10 µg).

**DIAGNOSTIC VALUE**
Excretions above 30 µg daily are strong evidence of a state of hyperaldosteronism, but this may be either primary or secondary. In both these conditions we have encountered values up to 140 µg daily.

Distinction between the two is sometimes difficult, and may require plasma renin assays for its elucidation. Plasma renin is usually low in primary and raised in secondary hyperaldosteronism.

But if normal reactivity of the aldosterone-secreting system can be demonstrated by measuring urinary aldosterone during high and low salt intake periods, this is in favour of a secondary hyperaldosteronism, whereas an absent or sluggish response to high and low salt intakes is suggestive of a primary disorder of the adrenal cortex.

Two representative examples may be given. An hypokalaemic patient with an aldosterone excretion of 70 µg daily was suspected of primary hyperaldosteronism. This diagnosis was rendered unlikely by the demonstration that aldosterone excretion rose to 137 µg on a 10 m-equiv sodium intake, and fell rapidly to 7 µg daily on a high intake of 160 m-equiv sodium, indicating a normal reactivity of the aldosterone-secreting system. The cause of his hypokalaemia was subsequently revealed as self-administered diuretics. In contrast another hypokalaemic patient with an aldosterone excretion on a normal diet of 19 µg daily, showed a rise to only 44 µg on a 10 m-equiv sodium intake, and no subsequent suppression below 29 µg on a high sodium intake, indicating abnormality of the reacting system with a
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largely autonomous aldosterone secretion suggestive of adrenal cortical abnormality.

Normal reactivity of the aldosterone-secreting system may often be demonstrated by analysing the overnight urine separately from that excreted during the day. In a series of tests the day-time aldosterone excretion rate has been from 20 to 280% above the overnight aldosterone excretion rate, whereas in five tests on hypokalaemic hypertensive subjects the day-time aldosterone rate has been lower than the recumbent excretion rate by 18 to 33% indicating abnormality of adrenal response.

RAPID SCREENING METHOD

A rough approximation of the true aldosterone excretion can be obtained relatively rapidly by omitting the paper chromatography and carrying out the assay directly on the washed and dried methylene chloride extract. Since the tritium and aldosterone recovery in this extract is nearly 100% compared with only 59% in eluates from chromatograms, only half the recommended amount of extract will be needed in each assay tube. Preliminary dilution of the extract with benzene/ethanol should therefore be 1 in 50.

A series of extracts of urines with aldosterone contents ranging from 10 to 120 μg daily has been assayed with and without chromatography. The rough direct assay always yields a higher result than does the regular chromatographic method. The mean apparent aldosterone content of the unchromatographed extracts was 125% + 14 (SD) (N = 19) of the true value obtained after chromatography, the range being from 103 to 140%.

Some reduction of the discrepancy between the two can be achieved by a preliminary extraction of the urine sample with methylene chloride before the pH 1-0 hydrolysis. Extracts of such pre-extracted urines, when assayed directly, gave a mean of 118% + 10 (SD) of the value obtained after chromatography (N = 8).

Thus a simplified assay, performed on an unchromatographed extract, may be expected to give a result averaging 25% above the true value. This has proved equally true at all levels of aldosterone excretion. The small improvement gained by preliminary extraction with methylene chloride scarcely justifies the inclusion of this extra stage in a routine method.

Discussion

The great advantage possessed by the anti-aldosterone serum is a combination of an exquisite sensitivity with a relatively high degree of specificity. The former is adequate greatly to simplify the hitherto very laborious and difficult assay of aldosterone in plasma (Mayes, Furuyama, Kem, and Nugent, 1970; Bayard, Beitins, Kowarski, and Migeon, 1970), but the latter, the high specificity, is a property greatly simplifying the assay of aldosterone in urine. Urine has several advantages over plasma in clinical investigation. More is known about the behaviour of aldosterone in urine in disease states than of changes in plasma aldosterone. Furthermore urine provides an estimate of the integrated mean aldosterone activity over 24 hours, and this can have greater clinical relevance than the plasma level, since the latter only represents the situation at a moment in time and may fluctuate considerably during the day (Katz, Romfh, and Smith, 1972).

The sensitivity of the aldosterone reaction with antiserum is such that an assay could be carried out on one thousandth part of the daily output, but greater technical care is needed to handle satisfactorily the very small quantities of steroid (2 to 4 ng) contained in such small volumes.

The method presented here makes full use of the high specificity but not fully of the great sensitivity of this antiserum. The fact that we have found unchromatographed urine extracts to give assay values with this antiserum averaging about 25% above the true reading obtained in adequately purified extracts shows that the amount of interfering substances requiring removal by chromatography is relatively small. For some clinical purposes an error of this magnitude may not be a serious disadvantage. The rough test will, for example, permit rapid detection of an aldosterone excretion elevated well above the normal range or the demonstration of an aldosterone-secreting system fully responsive to high and low salt intakes. But for full reliability these findings should be confirmed by repeating the assay on further portions of the urine extracts after chromatography on the B5 system. Any 18-hydroxy corticosterone or 18-hydroxy DOC which may be present in the urine will not interfere with the rapid screening assay since the antiserum reacts to neither steroid. But chromatography on the B5 system will completely remove both these 18-hydroxylated steroids, the former running at 25% and the latter at 210% of the rate of aldosterone.

In most clinical situations urinary aldosterone assay will give as much information as the aldosterone secretion rate.

Since different batches of anti-aldosterone serum will certainly differ considerably in specificity and in other properties, careful testing will be necessary with each batch before it can be used in a rapid screening method without chromatography.

Bayard et al (1970) considered that, with their own antiserum, greatest reliability called for two successive stages of paper chromatography, first on Bush
C and then on Bush B5. This has not proved necessary with the NIH serum 088.

Farmer, Roup, Pellizzari, and Fabre (1972) have used a different approach. They have increased the specificity of their antiserum sufficiently to eliminate the need for any chromatography, by converting the urinary aldosterone to its gamma-lactone by periodate oxidation, and then assaying this with an antiserum raised against the gamma lactone of aldosterone. By this method they found that the introduction of a purification stage by thin-layer chromatography only changed the values found by a mean of 4%.

Our experience with the NIH serum 088 thus lies between that of Bayard et al (1970) and that of Farmer et al (1972). Comparisons in several different laboratories will be needed to establish the relative advantages and disadvantages of these two types of anti-aldosterone serum.

But both types, by simplifying the analysis, make repeated aldosterone assays on individual patients much more feasible than in the past. This will facilitate the observation of response to varying conditions and thereby aid the diagnosis of disorders of aldosterone production.

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References
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