Estimation of urinary aldosterone using thin-layer chromatography and fluorimetry

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Abstract

Aims—To develop a fluorimetric method for the estimation of urinary aldosterone; to establish a normal range in 24 hour and overnight urine samples; and to investigate the use of overnight urines for detecting hyperaldosteronism.

Methods—Essential steps include hydrolysis of the 18 conjugate to release aldosterone and its oxidation with Benedict's solution, followed by thin-layer chromatography on silica gel and development of fluorescence on the plate with sulphuric acid.

Results—There was a linear correlation between the amount of aldosterone and the area under the peak on the chromatogram. The mean intra-assay and interassay coefficients of variation were 4.4% and 6.8%, respectively. The mean aldosterone excretion in 67 adults was 15.7 (SD 8.1) nmol/24 hours. The mean overnight excretion in 65 adults was 2.6 (1.4) nmol/8 hours. The method detected raised concentrations in patients with primary and secondary aldosteronism.

Conclusions—This technique provides an accurate means of assaying urinary aldosterone. Overnight estimations seem to be as effective as 24 hour assays for identifying patients with hyperaldosteronism.

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Primary aldosteronism (Conn's syndrome) may be missed because definitive diagnostic procedures are complicated and screening tests using serum electrolytes or plasma renin activity are also unreliable.¹ Many laboratories rely on serum aldosterone and plasma renin activity measurements to confirm the diagnosis, but 24 hour urinary estimations have the advantage of providing an integrated reflection of the daily adrenal output of this hormone.^{2,3}

Free aldosterone in urine is present only in trace amounts, but one of its major metabolites, the 18 glucuronide conjugate, is present in much larger quantities. Hydrolysis of this conjugate, followed by purification and measurement of the released aldosterone, has formed the basis of most clinical assays.

Nowadays, this released aldosterone is usually measured by radioimmunoassay (RIA) methods, but such techniques are subject to inaccuracies caused by the cross-

reactivity of the antibodies with other steroids.⁴ In the United Kingdom these estimations are largely confined to a few supraregional laboratories.⁵

In this paper we describe a fluorimetric assay for urinary aldosterone which is a development of a method published by Whigham.⁶ It is highly specific and can be used where facilities for radioimmunoassays are not available. A major disadvantage of using 24 hour urine samples is that they are difficult to collect accurately. For this reason we measured aldosterone in overnight collections as well, because we felt that this might provide a more convenient test for primary aldosteronism.

Methods

Glass-distilled water was used for the aqueous reagents. Glassware was cleaned with the non-fluorescent detergent Decon 90 (Decon Laboratories Ltd, Hove, Sussex, England), and was treated with a silicone solution (Repelcote; BDH, Poole, Dorset) to prevent absorption of steroids on to glass. Pipettes were plugged with cotton wool to eliminate possible contamination from the pipette fillers.

All the chemicals and solvents were of AR grade (BDH). Dichloromethane was redistilled before use. A glycine buffer was made by dissolving 7.5 g glycine in about 200 ml of water which was taken to pH 3.5 with 6 M hydrochloric acid; the mixture was then diluted to 250 ml. Pure d-aldosterone was purchased from Sigma Chemicals, Poole, Dorset, and an accurate working standard of about 10 pmol/ml was prepared.

SPECIAL EQUIPMENT

Glass tubes with ground-glass stoppers (Quickfit, UK) Volume 35 ml.

Glass plates (20 × 5 × 0.3 cm)

These were spread with silica gel (MN Kieselgel G-HR, Camlab, Cambridge, England), using a Shandon Unoplan Spreader (Shandon Scientific Co Ltd, London, England) to produce a layer with a thickness of 200 µm. Six lanes 4 mm wide, separated by blank areas, were scribed into the layer along the length of the plate so that five specimens and a standard could be run together.

Spectrophotofluorimeter with 150 W xenon lamp and TLC scanning attachment (Aminco-Bowman 4-8911E, VA Howes, London, England). We adapted this to

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Figure 1 A typical chromatogram. Upper trace: urine from a normal adult; lower trace: aldosterone standard.

increase the sensitivity.⁷ The wavelength of the exciting light was 455 nm, and that of the emitted light was 520 nm.

Chart recorder (Bryan, model No. 24000 A3, VA Howes London, England).

Planimeter (Albrit, WF Stanley, London, England)

For measuring the area under the peaks on the chromatogram in cm².

Twenty four hour and overnight urine specimens were collected without preservative into polythene containers that had been washed with Decon. The overnight specimens were collected over 8 hour periods. Aliquots of about 30 ml were stored at -18°C. Five urine samples can be assayed in a single run.

ACID HYDROLYSIS

Aliquots (15 ml) of the urine samples and the working standard were transferred to glass tubes, taken to pH 1 with 6 M hydrochloric acid, and left for 22 hours in the dark at room temperature. We have confirmed that for hydrolysis times between 18 and 30 hours the amount of aldosterone released is constant.

FIRST EXTRACTION

Dichloromethane (15 ml) was added to each tube and these were rotated horizontally for 30 minutes. The upper aqueous layers were aspirated and discarded, and the dichloromethane extracts were washed with 5 ml of 0.5 M sodium carbonate by agitating with a vortex mixer for 1 minute. Any emulsion formed was broken by this wash. The upper aqueous layers were aspirated and discarded, and the wash was repeated using 3 ml of water.

REMOVAL OF INTERFERING SUBSTANCES

A 10 ml aliquot of each extract was transferred to a clean tube and evaporated to dryness in a water bath at 56°C under a stream of nitrogen (99.9% pure, British Oxygen Company, England). The residue was dissolved by vortex in 3 ml of ethyl acetate. A water:ethanol mixture (9:1) was added (2 ml), followed by 20 ml of n-heptane, and the stoppered tubes were shaken vigorously on a flask shaker for 20 minutes. The upper n-heptane layers were aspirated and discarded.

OXIDATION OF ALDOSTERONE

Benedict's solution (2 ml) was added and the tubes were heated in a water bath at 56°C for 30 minutes. One millilitre of 6 M hydrochloric acid was then added slowly, the solution changing colour from blue to green.

SECOND EXTRACTION

Dichloromethane (20 ml) was added to each tube and the oxidised aldosterone was extracted by horizontal rotation for 30 minutes. The upper aqueous layers were aspirated and discarded.

REMOVAL OF INTERFERING OXIDATION PRODUCTS

The glycine buffer (5 ml) was added and the tubes were shaken vigorously for 5 minutes using a flask shaker. The upper aqueous layers were aspirated and discarded, and the glycine wash was repeated. Aliquots (15 ml) were transferred to clean tubes and evaporated to dryness in a water bath at 56°C under a stream of nitrogen.

THIN-LAYER CHROMATOGRAPHY

Each residue was transferred quantitatively to the plate using successive volumes of 150, 100, and 50 µl of dichloromethane. During this procedure the plate was placed on a warmed metal surface to accelerate evaporation of the spotting solvent.

The lower end of the plate was immersed in a mixture of acetone: methanol: water (25:4:4) in a chromatography jar; the solvent front took about 1 hour to reach the top. The plate was then dried in a current of air at room temperature.

FLUORIMETRY

Fluorescence was induced by spraying the plate with a fine mist of 3.7 M (20%) sulphuric acid and heating in a hot air oven at 80°C for 30 minutes. The plate was then transferred to the thin-layer chromatography scanning compartment and left for 15 minutes to ensure temperature equilibration, before scanning layer downwards. Oxidised aldosterone has a relative fraction (RF) value of 0.25 in this system, and there is usually no other significant peak between the origin and an RF of 0.7. Most urine specimens produce some fluorescence beyond this point (fig 1). The areas under the peaks on the resulting chromatograms were measured using the planimeter, and the amount of aldosterone (nmol) in each urine specimen was determined as follows:

$$\frac{\text{Sample area (cm}^2\text{)}}{\text{Standard area (cm}^2\text{)}} \times \frac{\text{Urine vol(ml)}}{15} \times \frac{\text{Standard (pmol)}}{1000}$$

OTHER SUBSTANCES TAKEN THROUGH THE METHOD

Cortisol, corticosterone, desoxycorticosterone, fludrocortisone, 18-hydroxycorticosterone, 18-hydroxydesoxycorticosterone, oestrone, oestradiol, oestriol, prednisolone, progesterone, spironolactone, testosterone, and the anti-malarial drugs chloroquine sulphate, proguanil hydrochloride, and quinine sulphate were

Figure 2 Dose-response curve.

tested. None produced a peak on the chromatogram below an RF of 0.7.

Urine was also assayed from patients receiving antihypertensive treatment: thiazide diuretics ($n = 11$); spironolactone ($n = 7$); β -adrenoceptor blocking drugs ($n = 5$); calcium-channel blockers ($n = 4$); angiotensin converting enzyme inhibitors ($n = 3$). In no case was there any interfering peak on the chromatogram.

Results

Known amounts of aldosterone were taken through the method. The largest of these represents a concentration in a 24 hour urine about seven times the upper limit of normal. There was a linear correlation between the amount of aldosterone used and the area under the peak on the chromatogram (fig 2).

Areas as small as 0.5 cm² can be measured accurately on the chromatogram. This corresponds to about 6 pmol of aldosterone in the 15 ml urine sample. Thus the lower limit of sensitivity in a 24 hour urine sample with an average volume of 1.5 litres is about 0.6 nmol/24 hours, and in an overnight sample of 500 ml is 0.2 nmol/8 hours.

The mean recovery of 66.5 pmol of aldosterone added to urine was 94.2%, with a range of 77.8 to 106.0% on 10 estimations. When a sample was assayed five times on a single plate, the mean intra-assay coefficient of variation was 4.4% for five separate runs. When another sample was measured on 20 occasions on different days, the interassay coefficient of variation was 6.8%.

Table 1 Urinary aldosterone excretion (nmol) in different age groups

Age (years)	No	24 hour urine	Overnight urine	% of 24 hour excretion
		Mean (SD)	Mean (SD)	
20-29	11	15.5 (5.0)	2.7 (1.7)	17.4
30-39	12	15.8 (9.7)	3.0 (1.6)	18.6
40-49	12	14.1 (7.8)	2.4 (1.7)	19.2
50-59	14	17.4 (7.8)	2.8 (1.1)	15.3
60-69	11	15.0 (7.5)	2.4 (0.6)	16.0

Conversion: nmols $\times$ 0.361 = μ g.

Figure 3 SA = secondary aldosteronism attributable to diuretic treatment. C = Cushing's syndrome; PA = primary aldosteronism. The upper limit of the normal range is indicated by the horizontal broken lines.

NORMAL RANGES

Normal values for 24 hour and overnight aldosterone excretions were obtained from a group of healthy adults and ambulant patients on unrestricted diets. The latter were normotensive and not taking any medication. There were roughly equal numbers of men and women, and their ages ranged from 17 to 75 years (mean 43 years). There was no significant difference between the excretion in men or women, nor between the healthy individuals and the patients, so that the subjects were regarded as a single group. The mean (SD) value for the 24 hour aldosterone excretion in 67 adults was 15.7 (8.1) nmol/24 hours with an upper limit of 38.5 nmol/24 hours. The mean overnight concentration in 65 adults was 2.6 (1.4) nmol/8 hours, the upper limit being 6.9 nmol/8 hours.

When ranked according to age, there were no significant differences in the mean 24 hour or overnight concentrations from the third to the seventh decade of life (table 1). The average amount of aldosterone excreted at night was less than 20% of the total 24 hour output.

ABNORMAL ADRENAL FUNCTION

To test the validity of the method, specimens of urine were collected from patients with abnormal adrenal function. Low concentrations were found in seven patients with adrenal insufficiency resulting from Addison's disease ($n = 1$), congenital adrenal hyperplasia ($n = 1$), hypopituitarism ($n = 3$), and following bilateral adrenalectomy ($n = 2$). The mean (SD) concentration in this group was only 3.0 (1.4) nmol/24 hours.

Increased concentrations were found in three patients with primary aldosteronism, in four patients with secondary aldosteronism attributed to diuretic treatment, and in two patients with Cushing's syndrome who presented with hypokalaemia (fig 3). In the seven patients with raised overnight concentrations

the proportion excreted at night ranged from 17% to 46% (mean 34%) of the total 24 hour output.

Discussion

The major advantages of the method described in this paper are its good specificity and accuracy. The use of thin-layer chromatography before fluorimetric detection ensures that the identity of the peak produced by the oxidised aldosterone can be verified. Up to five urine samples may be assayed in a single run, but the method could be modified to accommodate a larger number of samples by increasing the width of the chromatography plate. Results can be obtained within two working days, and the method takes about three hours of a technician's time.

Although antihypertensive drugs do not interfere with the assay, they may alter aldosterone production. It has been suggested that patients being investigated for primary aldosteronism should stop taking these drugs for at least two weeks beforehand, and in the case of spironolactone for at least six weeks.⁸

The normal values for 24 hour urinary aldosterone concentrations established by our method are comparable to those obtained by other workers (table 2). Whigham,⁶ using his fluorimetric method, obtained higher figures than ours, but he did not separate the oxidised aldosterone from other fluorescent material still present in the urinary extracts. Gas chromatography methods have been little used, and in a recent study gave a much larger normal range than other techniques.¹⁵ The slightly higher figures obtained by the radioimmunoassay methods may be attributable to cross-reactivity with other steroids.

It has been suggested that the output of aldosterone declines with advancing age.¹⁶ Another study, however, found no significant change in aldosterone excretion between the ages of 20 and 60,¹³ and our experience confirms this.

Twenty four hour urine specimens are inconvenient to collect and may be incomplete. Some years ago, when investigating patients for Cushing's syndrome, we found that the 11-hydroxycorticosteroid values in overnight urine samples could be used as an initial screening test.¹⁷ This suggested to us

that such an approach might also be feasible in patients with suspected hyperaldosteronism.

Although there is a delay between the secretion of aldosterone and the urinary excretion of its 18 glucuronide conjugate, this time lag is only about 3 hours.¹⁸ Thus what is excreted in an overnight specimen is largely representative of the aldosterone secreted during sleep, and is a reliable estimate of the total daily output in normal subjects.¹⁹

Our patients with increased overnight concentrations had increased amounts of aldosterone in their 24 hour urine collections. Thus it would appear that overnight estimations may be as effective as 24 hour assays in differentiating between normal subjects and those with hyperaldosteronism.

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Table 2 Urinary aldosterone values in normal adults on unrestricted diets (nmol/24 hours)

Reference	Year	Method	No	Mean (SD)	Range
Mattox & Lewbart ⁹	1959	C	34	15.5	5.5-44.3
Benraad & Kloppenborg ¹⁰	1965	DID	10	21.3	12.2-29.9
Langan <i>et al</i> ¹¹	1974	RIA	21	20.5 (9.1)	5.5-33.2
Whigham ⁶	1976	F	25	30.7 (18.8)	0-60.9
Abdelhamid <i>et al</i> ¹²	1979	RIA	59	22.2 (10.0)	9.7-48.5
Gomez-Sanchez & Holland ¹³	1981	RIA	43	19.1 (8.3)	
Mattox & Nelson ¹⁴	1981	RIA	72	24.7 (11.6)	5.5-41.6
Bravo <i>et al</i> ¹	1983	RIA	47	19.4 (5.5)	
Koopman <i>et al</i> ¹⁵	1986	GC	20	40.0 (22.0)	0-84.0
Present study		F	67	15.7 (8.1)	3.6-38.5

C = colorimetric; DID = double isotope dilution; RIA = radioimmunoassay; GC = gas chromatography; F = fluorimetric conversion: nmol $\times$ 0.361 = μ g.