An Accurate Method for Measurement of Aldosterone Production

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A method is described for isolation of the acid-hydrolysable metabolite of aldosterone in sufficient purity to measure accurately the daily production rate. Values obtained with six hospital patients were 84–131 $\mu\text{g./day}$ on a daily intake of 100m-equiv. of Na^+ and 227–464 $\mu\text{g./day}$ on a daily intake of 10m-equiv. of Na^+ . Corresponding values for aldosterone excretion were also recorded, but these are a poor index of production rate since they represent from 1.6 to 9.8% of the total daily output of aldosterone.

Nowaczynski, Steyermark, Koiv, Genest & Jones (1956) found that isolation of aldosterone from other urinary steroids was difficult, yet complete separation is essential since no specific biochemical reaction has been described for measurement of the relatively small amount of aldosterone in the urine.

In the present method, t.l.c. and three paper-chromatographic systems were used to isolate the free steroid released by acid hydrolysis of urine. For production rate, ^3H -labelled aldosterone was infused intravenously and the specific radioactivity of the metabolite in the urine measured. Aldosterone excretion was derived from the absolute quantity of steroid released at pH 1 and the recovery of labelled aldosterone added directly to the urine before extraction.

Purity of aldosterone eluted from the final chromatogram was confirmed by the constant specific radioactivity before and after acetylation. Accuracy of the method was assessed by the reproducibility of results and measurements of aldosterone added to urine.

METHODS

Measurements of aldosterone were performed on convalescent patients who had no evidence of endocrine disease or of any condition that might predispose them to oedema. Six patients received a constant dietary and fluid intake for a period of 8 days. The diet contained 100m-equiv. of Na^+ /day for the first 4 days and 10m-equiv. of Na^+ /day for the last 4 days. The K^+ intake was constant at 80m-equiv./day throughout the study. The 24 hr. urine specimens were obtained each day and creatinine excretion was measured to ensure complete collection.

[1,2- ^3H]Aldosterone (specific radioactivity 7800mc/m-mole), supplied by The Radiochemical Centre, Amersham, Bucks., was purified by paper chromatography in the Bush C system (Bush, 1952) before use. A 1 μC sample was infused

intravenously over a period of 2 hr. on both the last day of high-salt intake and on the last day of low-salt intake for measurements of aldosterone production. Aldosterone excretion was measured on the third day of each period and losses were assessed by the addition of a known amount of [1,2- ^3H]aldosterone radioactivity (120000–150000 c.p.m.). Random urine collections were obtained from three other patients on an ordinary ward diet for measurement of recovery of aldosterone added to urine and the specific radioactivities of aldosterone and its diacetate.

All measurements were done in duplicate on two halves of a 24 hr. urine.

Hydrolysis. Urine was adjusted to pH 1 and allowed to stand overnight.

Extraction. The urine, saturated with $(\text{NH}_4)_2\text{SO}_4$ (500 g./l.), was extracted with 1 l. of ether ($3 \times \frac{1}{3}$ vol. of urine). This ether extract was washed repeatedly with 0.1M-NaOH, usually 3×20 ml., until the aqueous layer was clear. The alkaline washes, saturated with $(\text{NH}_4)_2\text{SO}_4$, were back-washed with an equal volume of ether and also the water washes required to adjust the ether extract to pH 7. Water present in the solvent was frozen out overnight and the extract evaporated to dryness.

Thin-layer chromatography. The crude extract and standards of cortisol, cortisone and aldosterone were applied to chromatoplates coated with silica gel GF254 (E. Merck A.-G., Darmstadt, Germany) and run in a tank containing 200 ml. of chloroform-ethanol (99:1, v/v) for 60 min. This procedure was repeated after the plates had been dried. The solvent was then replaced by 200 ml. of ethyl acetate-1,2-dichloroethane-water (90:10:1, by vol.) and the plates were developed in this system for 100 min.

The area of silica gel from 1 cm. proximal to 4 cm. distal to the aldosterone zone was scraped off and the powder was placed in a Universal container. Steroids were extracted from the silica gel with methanol (3×20 ml.) and the mixture was separated by centrifugation. The methanol washes were combined and evaporated to dryness.

Paper chromatography. System 1. This was a modification of the chloroform-formamide system described by Sunderman & Sunderman (1960). A lane 1.5 cm. wide was cut in a 15 cm. strip of Whatman no. 1 paper and the paper was soaked in 30% (v/v) formamide in acetone. The extract

was spread along the origin of the broad lane and standards of cortisol and cortisone were applied to the side lane. After equilibration overnight, the paper was developed for 4 hr. in chloroform–formamide–hexane–water (18:1:2:1, by vol.).

In this and subsequent systems, cortisone precedes cortisol. A portion of paper 4 cm. above to 4 cm. below the cortisone zone was eluted in 80% (v/v) methanol. The aqueous mixture was evaporated to $\frac{1}{4}$ volume, 10 ml. of water was added and the steroids were extracted into chloroform (3 $\times$ 30 ml.). The chloroform was evaporated to dryness and the steroids were redissolved in a small volume of ethanol.

System 2. This extract was run for 2 days in the toluene–propylene–glycol system of Burton, Zaffaroni & Keutmann (1951). Whatman no. 2 paper was used with standards of cortisol and cortisone. The paper was cut 2 cm. above and 2 cm. below the cortisone zone. It was eluted in 80% (v/v) methanol and extracted with chloroform as in system 1.

System 3. The extract and standards of cortisone and cortisol were applied to 1.5 cm. lanes cut in a 15 cm. strip of Whatman no. 1 paper. Some lanes were left blank. After equilibration overnight the paper was run for 4 hr. in the Bush C system (Bush, 1952). An area of paper corresponding exactly to that of cortisol and a paper blank of equal size from an adjacent blank lane were eluted in ethanol for 1 hr. The solvent was evaporated and the steroid and paper blank were each made up to a known volume in fresh ethanol.

Measurement of aldosterone. A small quantity of the final extract (0.1 vol.) was taken for measurement of radioactivity and a portion was also taken for measurement of aldosterone by using 3,3'-dianisole-4,4'-bis-(3,5-diphenyl)tetrazolium chloride (Mader & Buck, 1952).

Fresh solutions of Blue Tetrazolium (50 mg. in 10 ml. of 95% ethanol) and of tetramethylammonium hydroxide (1 ml. of 25% tetramethylammonium hydroxide diluted to 25 ml. with 95% ethanol) were prepared for each measurement.

To develop colour, 0.1 ml. of Blue Tetrazolium solution and 0.1 ml. of dilute tetramethylammonium hydroxide were added to 1 ml. of each sample and paper blank and to standards made up to this volume. The reaction was stopped after exactly 10 min. by adding 0.2 ml. of 0.1 M-HCl and the extinction was read against a reagent blank at 510 nm.

Purity of aldosterone. The purity of the aldosterone obtained after chromatography on the Bush C system was assessed by measurement of its specific radioactivity before and after acetylation. One drop of acetic anhydride and 2 drops of pyridine were added to part of the dry extract. The tube was sealed and set aside overnight. The derivative and standards of aldosterone and aldosterone diacetate were applied to 1.5 cm. lanes cut in Whatman no. 1 paper and run for 4 hr. in the Bush A system (Bush, 1952) after equilibration overnight. The specific radioactivity of the derivative was compared with that of the steroid isolated.

RESULTS

Duplicate measurements of aldosterone production agreed closely; the coefficient of variation was 4.3% (Table 1). Mean values for each patient show that the production rate varied between 84 and 131 $\mu\text{g./day}$ on a daily intake of 100 m-equiv.

Table 1. *Duplicate values for aldosterone production and excretion rate*

Subject	Aldosterone production and excretion ($\mu\text{g./day}$)			
	Na ⁺ intake 100 m-equiv./day		Na ⁺ intake 10 m-equiv./day	
	Production	Excretion	Production	Excretion
G.T.	129	8.0	426	32.6
	125	9.4	400	30.0
N.F.	122	5.0	223	13.8
	130	4.6	230	15.0
T.H.	123	13.0	250	26.3
	135	14.6	252	23.0
A.G.	90	6.2	439	42.0
	89	5.8	489	38.5
H.C.	80	2.1	231	13.5
	88	2.0	244	16.0
G.C.	122	7.5	395	33.6
	141	8.2	342	34.7
Mean	114	7.2	326	26.6

Table 2. *Recovery of aldosterone added to urine*

Subject	Aldosterone measured ($\mu\text{g.}$)	
	Urine	Urine + 10 $\mu\text{g.}$ of aldosterone
T.H.	6.6	17.2
A.G.	6.2	16.2
C.P.	10.0	19.9

Table 3. *Purity of aldosterone*

Subject	Specific radioactivity (c.p.m./ $\mu\text{g.}$)	
	Aldosterone	Aldosterone diacetate
N.S.	21.2	19.2
G.C. (1)	16.5	13.8
(2)	40.0	38.0
C. P.	15.0	18.0

of Na⁺ and from 227 to 464 $\mu\text{g./day}$ on a daily intake of 10 m-equiv. of Na⁺.

Aldosterone excretion was measured in duplicate when the patients were on a daily intake of 100 m-equiv. of Na⁺: mean values of between 2.0 and 13.8 $\mu\text{g./day}$ were obtained. When the patients were on a low-salt intake, mean values rose to between 14.4 and 40.2 $\mu\text{g./day}$. The mean of all paper blanks for measurements recorded in this study was 0.46 ± 0.10 (s.d.) $\mu\text{g.}$

Recovery of [1,2-³H]aldosterone varied from 13 to 68%, but recovery of aldosterone added to urine was 99–106% (Table 2). There was good agreement between the specific radioactivity of the steroid eluted from the Bush C chromatogram and its diacetate (Table 3), indicating that aldosterone was isolated in pure form.

DISCUSSION

For the isolation of aldosterone, Neher & Wettstein (1956) employed two chromatographic systems, but their method was criticized by Nowaczynski, Koiw & Genest (1957) because it did not ensure complete separation of aldosterone from other steroids in the urine. Subsequent workers have used three and sometimes four systems, often with acetylation of the steroid, to facilitate isolation of aldosterone. Kliman & Peterson (1960), for their double-isotope method, thought it necessary to partially oxidize the acetylated compound to improve separation, even though this meant an additional loss of up to 50% of the steroid ester.

In the present method, t.l.c. was used to separate aldosterone from some of the corticoids and their metabolites in urine. We adopted, with modification, the solvent systems described by Benraad & Kloppenborg (1964). Thereafter, isolation of aldosterone was accomplished with three paper-chromatographic systems. This was confirmed by the agreement between duplicate determinations of aldosterone production and excretion rate, recovery of aldosterone added to urine and the constant specific radioactivity of aldosterone and its diacetate. An attempt to shorten the procedure by omitting the toluene-propylene-glycol system proved unsuccessful, since an unidentified steroid, often visible under u.v. light, was present in the final chromatogram. This may have been one of the contaminants described by Nowaczynski *et al.* (1957) in their detailed study of aldosterone.

Values for aldosterone production are similar to those reported by several authors who have measured the specific radioactivity of aldosterone released at pH 1 (Jones *et al.* 1959; Flood *et al.* 1961; Tait, Tait, Little & Laumai, 1961; Siegenthaller, Dowdy & Luetscher, 1962; Espiner, Tucci, Jagger, Pauk & Lauler, 1967) and correspond to values quoted by Ulick, Laragh, Lieberman & Loeb (1958)

and by Cope, Nicholis & Fraser (1961), who isolated the tetrahydro metabolite. There was no direct correlation between aldosterone excretion and production rate, the acid-hydrolysable steroid representing between 1·6 and 9·8% of the absolute output. Cope *et al.* (1961) found an even greater discrepancy with the tetrahydro metabolite, which represented from 3·1 to 31% of the total daily aldosterone output. Measurement of aldosterone production is therefore preferable to determination of aldosterone excretion.

Production rate increased from two- to five-fold on restriction of Na⁺ intake, emphasizing once more the importance of measuring aldosterone output under conditions of strict dietary control.

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