A Rapid and Practical Determination of Aldosterone in Urine by Double Isotope Method

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Synopsis

A double isotope derivative method for urinary aldosterone determination was improved in terms of economy (to one fifth the original cost) and efficiency (to less than half the time) by a combination of several individual improvements. Aldosterone-4-14C was used as an indicator, eliminating the procedure of preparing aldosterone-14C-diacetate. 3H-acetylation was done after, instead of before, dichloromethane extraction and a thin layer-chromatography, to avoid unnecessary acetylation of abundant contaminants. The cost of radioisotope was thus reduced to one tenth. dl-Aldosterone diacetate, added as a carrier, was easily located on thin layers by ultraviolet light. Two dimensional thin layer, instead of repeating one dimensional paper chromatography, was employed for the purification, thereby shortening the time to one twentieth to thirtieth, and enabling to eliminate the oxidation of the diacetate to the monoacetate. The specific radioactivity of 3H-acetic anhydride was determined by acetylation of dl-aldosterone mixed with urine, instead of cortisone or dl-aldosterone, for both economy and calibration. One technician can thus work out with 4–8 samples a week.

Aldosterone excretion in 24 hr, thus determined, was $10.99 \pm 3.83$ (S.D.) μg for normal subjects and $32.12 \pm 8.38$ μg for preoperative primary aldosteronism with a postoperative decrease to $12.03 \pm 4.10$ μg.

Aldosterone plays an important role both in physiology and clinical pathology, yet, because of the strong potency, like many other active substances, the existence in the body fluid is extremely scanty and makes an ordinary approach of chemical determination extremely difficult.

Kliman and Peterson’s double isotope derivatives method (1960) combined both chemical and physical techniques and opened a new way. Despite the excellency in sensitivity and specificity, we found it too long time consuming and expensive. This is especially true if many essential hypertensive patients are to be examined for a possible diagnosis of normokalemic primary aldosteronism (Conn et al., 1965) which is otherwise impossible.

We here rapidified the method to half the time and economized to about one fifth the original cost improving the procedure of extraction, isotope labelling, and purification without sacrificing the precision.

Materials and Apparatus

All reagents used (acetic acid, hydrochloric acid, sodium hydroxide and sodium sulfate) and solvents (dichloromethane, anhydrous benzene, cyclohexane, ethanol, methanol, ethyl acetate, carbon tetrachloride, chloroform and toluene) were of the first grade.

Pyridine was refluxed over barium oxide for 4–6 hr, collected at 115°C by distillation, and stored in a desiccator over calcium chloride until used.

Acetic anhydride was refluxed over calcium
carbite for 4–6 hr, collected at 139°C by distillation, and stored in a desiccator over calcium chloride.

Tetrahydroaldosterone was the gift from Dr. S. Ulick, New York Veterans Administration Hospital. Other various steroids used were either the gift from Takeda Pharmaceutical Co. Ltd., and Teikoku Zoki Co. Ltd., or bought from Merck Co. Ltd., Sigma Co. Ltd., and Mann Research Laboratories.

³H-labelled acetic anhydride with a specific activity of 100 mC/mM (cord number; TMM-36) was bought from CNE-SCK Radioisotopes Department MOL, Belgium. 0.13 ml of 25 mC ³H-acetic anhydride was diluted 10 times to a total volume of 1.3 ml with an addition of 0.22 ml of redistilled acetic anhydride and 0.95 ml of anhydrous benzene. The diluted solution of 10 mC/mM was stored in a glass stoppered tube in a desiccator over calcium chloride in a deep freezer until used. This is sufficient for acetylating 40 samples (0.03 ml of 0.6 mC for each sample).

¹⁴C-labelled aldosterone(d(+)-aldosterone-4-¹⁴C) with a specific activity of 56.7 mC/mM (cord number CFA 365) was bought from the Radiochemical Center, Amersham, England. 1 µC of aldosterone-4-¹⁴C was pipetted into a 10 ml brown glass graduate and adjusted to a total volume of 10 ml with an addition of 10% ethanol in benzene. The graduate, after the air replaced with nitrogen gas, was stoppered tightly and kept below 5°C. 0.025–0.015 ml of the solution (about 2,000 cpm) was added as an indicator to each one sample.

Silica gel thin layer was prepared using Kiesel gel HF 254 from Merck Co. Ltd., in 250 µm thick on a glass plate of 20 × 8, or 20 × 20 cm. The plate was dried in an oven at 100–110°C for 60 min and stored in a desiccator with silica gel desiccant. The following solvent systems (Ibayashi et al., 1964; Gerdes et al., 1965, Suzuki, 1965) were employed

- System A; ethyl acetate
- System B; cyclohexane: chloroform: acetic acid (4:5:1 by vols.) with a drop of water
- System C; chloroform: ethanol (9:1 v/v)
- System D; benzene: methanol: water (4:1:0.05 v/v)
- System E; benzene: ethanol: water (4:1:0.0025 by vols.)

Aloca Thin Layer Chromatogram Scanner TLC-28 was used for the scanning.

Beckman Liquid Scintillation System LS-100 was used for the scintillation counting of ³H and ¹⁴C. As the scintillator 2, 5-diphenyloxazole (Dotite POP) and 1, 4-bis 2-(5-phenyloxazole)-benzene (Dotite POPOP) of Wako Junyaku Co. Ltd., were used. 2 g of the former and 0.02 g of the latter were dissolved in 500 ml of toluene and 8 ml of the solution was used for each vial.

**Experiment and the Method**

An outline of the method is diagramed in Figure 1 and carried out as follows.

**Treatment of urine**

24 hr urine was collected and kept in a refrigerator without any additives. After reading the total volume

1st day: 100 ml of urine sample, taken from a 24 hr urine

   treatment with pH 1.0 overnight

   addition of indicator aldosterone-4-¹⁴C

2nd day: Extraction with dichloromethane

   TLC-I by system A

   elution

3rd day: Acetylation with ³H-acetic anhydride

   addition of carrier dl-aldosterone diacetate

4th day: TLC-II by system A and B (two dimensional)

   elution of aldosterone diacetate spot visualized with UV light

5th day: TLC-III by system C and D (two dimensional)

   elution of aldosterone diacetate spot visualized with UV light

Counting radioactivity of ³H and ¹⁴C

Fig. 1. A schematic diagram of the improved method
and pH of the urine, 100 ml was taken into a plastic bottle and frozen until used. When to determine, it was adjusted to pH 1.0 with approximately 2 ml of 12 N hydrochloric acid. The acidified urine was allowed to stand at room temperature for 24 hr for hydrolysis.

**Addition of indicator**

After hydrolysis, approximately 2,000 cpm of aldosterone-4-\(^{14}\)C was added to it in a 100 ml of separatory funnel as an indicator of losses occurring throughout the procedure.

**Extraction**

The urine was extracted with 30 ml of dichloromethane by vigorous shaking for 1 min. Emulsion was filtered through cotton funnel and the lower layer separated by centrifugation at 2,500 rpm for 10 min. The dichloromethane layer was taken into a 50 ml flask and washed with each one tenth volume of 0.1 N sodium hydroxide, 0.1 M acetic acid, and water, successively. The dichloromethane remained in a lower layer and the washings in the upper layer was removed by glass capillary connected to aspirator. The solution was dried with 2 g of sodium sulfate for 30 min and evaporated down in a 50 ml conical flask by rotary evaporator.

**Thin layer chromatography-I (TLC-I)**

The sample was collected to the bottom of the flask by rinsing with 0.2 ml of 20% ethanol in dichloromethane and applied on a thin layer. An authentic sample of dl-aldosterone was also applied alongside and developed simultaneously with ethyl acetate at room temperature for 30 min. The authentic sample of aldosterone was located by visualization under ultraviolet light (254 m\(\mu\)) and aldosterone-4-\(^{14}\)C in the sample by a chromatoscanner as shown in Figure 2. The Rf value varied from time to time, and that of aldosterone ranged between 0.25-0.5.

The located area of the sample on the thin layer was gently scratched by a spatula and the silica gel of the area was carefully collected on a cotton filter in a small glass column (0.3 \(\times\) 8 cm) by aspirating the other end of the column (Cerny et al., 1961). The column was stood upside down, and from it, the sample was eluted with 1 ml of methanol. The eluate was collected in an acetylation tube and evaporated down with nitrogen stream. It was then dried with calcium chloride in a desiccator under a reduced pressure of 1–10 mmHg for more than one hr.

**Acetylation**

To the tube was added 0.025 ml of anhydrous pyridine and 0.03 ml of \(^3\)H-acetic anhydride quickly with a special precaution to the humidity. With a tight stopper and parafilm sealing, the tube, after the residue dissolved, was incubated at 37\(^{\circ}\)C overnight approximately for 18 hr. The excess acetic anhydride was hydrolyzed with 0.5 ml of 20% ethanol and removed by the extraction with 5 ml of carbon tetrachloride, in a separatory funnel. The carbon tetrachloride layer was washed twice with each 0.5 ml of water and evaporated down to dryness in an air stream at 30–40\(^{\circ}\)C.

**Thin layer chromatography-II (TLC-II)**

The dried sample was mixed with 5 \(\mu\)g of unlabelled carrier dl-aldosterone diacetate for reference marker. Additional reference standards such as 21-deoxycorticosterone and adrenosterone are not needed, but may also be mixed for comparison of the Rf values. After spotting, the thin layer was developed in the first dimension in system A for 25 min and air dried.

![Fig. 2. A scheme of TLC-I of dichloromethane extract of normal urine with a tracer aldosterone-4-\(^{14}\)C as detected by UV light and \(\beta\)-ray scanning (thin layer prepared from Kieselgel HF 254, developed with ethyl acetate at 25\(^{\circ}\)C for 30 min.) See Table 3 for abbreviation.](image-url)
for 15 min or more, at room temperature. A trace of ethyl acetate was further removed in an evacuated desiccator (1–10 mmHg) for another 15 min. The average Rf values of 22 chromatograms, detected by ultraviolet light (254 m,u) were 0.77 for both aldosterone diacetate and adrenosterone, and 0.83 for 21 deoxycorticosterone.

The thin layer was allowed to stand in air for another 15 min to equilibrate with air humidity, and then developed in the second dimension by the system B at room temperature for 30 min. The average Rf values, likewise detected, were 0.31 for aldosterone diacetate, 0.35 for adrenosterone, and 0.18 for 21 deoxycorticosterone.

The small area of aldosterone diacetate, located just below adrenosterone, was carefully scratched by a spatula and the silica gel was collected on a cotton in a small column by aspirating the other end of the column.

The sample was eluted with 0.75 ml of ethyl acetate and further with another 0.25 ml of methanol. The eluate was evaporated down in an air stream at room temperature.

**Thin layer chromatography-III (TLC-III)**

The sample then was again, with or without an addition of each 5 μg of adrenosterone and 21 deoxycorticosterone as reference standards, spotted on a thin layer. It was first developed with system C for 30 min, and then to the second dimension, with the system D for 35 min, both at room temperature.

Rf values in the former system, detected as in TLC-II, were 0.78 for both aldosterone diacetate and adrenosterone and 0.61 for 21-deoxycorticosterone, while those in the latter system were 0.33, 0.34, and 0.24, respectively.

The small area located for aldosterone diacetate was again likewise collected, and eluted with 0.75 ml of ethyl acetate and 0.25 ml of methanol successively into a vial for scintillation counting. The eluate was evaporated down in an air stream at room temperature.

**Counting and calculation**

To the dried residue in a vial was added 8 ml of scintillator solution. The vial was subjected to the assay for 3H and 14C by liquid scintillation counter with two channels (3H wide window, 14C above 3H, gain 134.5). Aldosterone in 24 hr urine was calculated from the following formula:

\[
\text{μg of aldosterone} = \frac{3H \times MW}{SA \times 1,000} \times 24 \text{ hr urine volume} \div \text{sample volume}
\]

where

MW: molecular weight of aldosterone = 360

\[
3H: \text{cpm of } 3H\text{-diacetylated urine aldosterone} \times \frac{1}{2} = (U - u) \cdot \frac{A}{a} + \frac{1}{2} = \frac{U}{u} - \frac{A}{a}
\]

\[
\text{where}
\]

U: total cpm of 3H and 14C of urine sample at 3H wide window

u: cpm of 14C of urine sample at 14C above 3H window

A: cpm of 14C of indicator aldosterone at 3H window

a: cpm of 14C of indicator aldosterone at 14C above 3H window.

SA: specific radioactivity of the 3H-acetic anhydride determined for every new bottle as follows; Each 100 ml of urine, to which 0, 12.5, 25, and 50 μg of authentic dl-aldosterone added respectively, was subjected to the whole procedure of determination of aldosterone in the method described above. The 3H count thus determined represents the sum of urinary intrinsic and the exogenously added aldosterone. The difference of 3H cpm between the added and non-added samples represents the amount of the exogenously added aldosterone. Thus, the SA was given by the formula

\[
\text{cpm of } 3H \text{ representing added aldosterone } 1/2 \text{ MW amount (μg) of the added aldosterone } 1,000
\]

**Results**

**Extracting condition**

The efficiency of extracting aldosterone from urine was checked in 2 samples of 90 ml urine. After an additions of 0.2 ml of aldosterone-4-14C (2160 cpm) and pH 1 treatment for 20 hr, the urine was extracted with each 30 ml (one third volume) of dichloromethane 4 times.

The extraction was done in a 200 ml separatory funnel under vigorous shaking for one min. The dichloromethane layer, after separated by centrifugation when emulsified, was washed with 0.1 N sodium hydroxide, acetic acid and water successively. It was evaporated down in a flask, transferred with 3 portions of 7 ml of dichloromethane to a vial for scintillation, dried under an air stream and counted.

The results of the counting, as shown in Table 1, indicated that more than 95% of the aldosterone was successfully extracted with the first 30 ml dichloromethane, unnessessitating any further extraction.
Table 1. Efficiency of extracting urine aldosterone with each one third volume of dichloromethane as determined by cpm of aldosterone-4-14C added to urine

<table>
<thead>
<tr>
<th>Sample A</th>
<th>cpm</th>
<th>Sample B</th>
<th>cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>amount of added tracer</td>
<td>2160</td>
<td>2160</td>
<td></td>
</tr>
<tr>
<td>1st extract</td>
<td>2097 (97.1%)</td>
<td>1st extract</td>
<td>2042 (95.4%)</td>
</tr>
<tr>
<td>2nd extract</td>
<td>219</td>
<td>2nd extract</td>
<td>180</td>
</tr>
<tr>
<td>3rd extract</td>
<td>25</td>
<td>3rd extract</td>
<td>26</td>
</tr>
<tr>
<td>4th extract</td>
<td>5</td>
<td>4th extract</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 2. Rf value of authentic steroids averaged from 4 thin layer chromatograms (Kieselgel HF 254/ethyl acetate)

<table>
<thead>
<tr>
<th>abbreviation</th>
<th>steroid</th>
<th>migration</th>
<th>Rf for solvent front 15.7 cm</th>
<th>Rf for prog.</th>
<th>method of location</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>H$_2$SO$_4$/EtOH</td>
<td>UV</td>
</tr>
<tr>
<td>1 E$_1$</td>
<td>esterone</td>
<td>14.9</td>
<td>0.95</td>
<td>1.05</td>
<td>r</td>
</tr>
<tr>
<td>2</td>
<td>progestrone</td>
<td>14.9</td>
<td>0.95</td>
<td>1.00</td>
<td>v</td>
</tr>
<tr>
<td>3 DOCA</td>
<td>deoxytocorticosterone acetate</td>
<td>14.1</td>
<td>0.90</td>
<td>0.99</td>
<td>v</td>
</tr>
<tr>
<td>4 E$_2$</td>
<td>17β-estradiol</td>
<td>13.9</td>
<td>0.89</td>
<td>0.98</td>
<td>d</td>
</tr>
<tr>
<td>5 17α-OH prog</td>
<td>17 α-OH progestrone</td>
<td>13.7</td>
<td>0.87</td>
<td>0.97</td>
<td>v</td>
</tr>
<tr>
<td>6 DH epiandr</td>
<td>dehydroepiandrosterone</td>
<td>13.2</td>
<td>0.84</td>
<td>0.94</td>
<td>d</td>
</tr>
<tr>
<td>7 andro</td>
<td>androsterone</td>
<td>13.1</td>
<td>0.83</td>
<td>0.93</td>
<td>d</td>
</tr>
<tr>
<td>8 21 DC</td>
<td>21-desoxycorticosterone</td>
<td>12.1</td>
<td>0.77</td>
<td>0.86</td>
<td>v</td>
</tr>
<tr>
<td>9 S</td>
<td>11-desoxycorticisol</td>
<td>11.7</td>
<td>0.75</td>
<td>0.84</td>
<td>w</td>
</tr>
<tr>
<td>10 T</td>
<td>testosterone</td>
<td>11.5</td>
<td>0.73</td>
<td>0.80</td>
<td>d</td>
</tr>
<tr>
<td>11 DOC</td>
<td>desoxycorticosterone</td>
<td>11.5</td>
<td>0.73</td>
<td>0.80</td>
<td>v</td>
</tr>
<tr>
<td>12 preg 2OH</td>
<td>pregnanediol</td>
<td>11.3</td>
<td>0.72</td>
<td>0.80</td>
<td>w</td>
</tr>
<tr>
<td>13 adr</td>
<td>adrenosterone</td>
<td>11.4</td>
<td>0.66</td>
<td>0.74</td>
<td>d</td>
</tr>
<tr>
<td>14aldo diAc</td>
<td>aldosterone diacetate</td>
<td>10.5</td>
<td>0.67</td>
<td>0.74</td>
<td>v</td>
</tr>
<tr>
<td>15 E</td>
<td>cortisone</td>
<td>9.5</td>
<td>0.60</td>
<td>0.67</td>
<td>w</td>
</tr>
<tr>
<td>16 F</td>
<td>cortisol</td>
<td>9.1</td>
<td>0.58</td>
<td>0.64</td>
<td>v</td>
</tr>
<tr>
<td>17 B</td>
<td>corticosterone</td>
<td>7.8</td>
<td>0.49</td>
<td>0.55</td>
<td>g</td>
</tr>
<tr>
<td>18 THB</td>
<td>tetrahydrocorticosterone</td>
<td>6.7</td>
<td>0.43</td>
<td>0.47</td>
<td>y</td>
</tr>
<tr>
<td>19 THF</td>
<td>tetrahydrocortisol</td>
<td>6.5</td>
<td>0.41</td>
<td>0.46</td>
<td>g</td>
</tr>
<tr>
<td>20 E$_a$</td>
<td>estriol</td>
<td>6.5</td>
<td>0.41</td>
<td>0.46</td>
<td>p</td>
</tr>
<tr>
<td>21 aldo</td>
<td>aldosterone</td>
<td>3.8</td>
<td>0.24</td>
<td>0.27</td>
<td>d</td>
</tr>
<tr>
<td>22 TH aldo</td>
<td>tetrahydroaldosterone</td>
<td>2.8</td>
<td>0.18</td>
<td>0.20</td>
<td>b</td>
</tr>
<tr>
<td>23 cortin</td>
<td>cortrone</td>
<td>1.0</td>
<td>0.06</td>
<td>0.06</td>
<td>b</td>
</tr>
<tr>
<td>24 cortl</td>
<td>cortol</td>
<td>0.8</td>
<td>0.05</td>
<td>0.05</td>
<td>d</td>
</tr>
</tbody>
</table>

DL; day light
UV; ultraviolet light (254 m/λ)
BT; blue tetrazorium reagent
SbCl$_3$; antimonium trichloride

dk; dark
sl; salmon
v; violet
y; yellow
b; blue
bn; brown
gn; green

dk; dark
sl; salmon
v; violet
y; yellow
b; blue
bn; brown
gn; green

*1; Takeda Yakuhin Co.
*2; Teikoku Zoki Co.
*3; Mann Res. Lab
*4; Sigma Co.
*5; Merck Co.
*6; Dr. S. Ulick
Recovering condition of aldosterone from thin layer

Overall recovery through the whole procedures of the present method, determined for 57 urine samples, was 45.92% ± 10.15 (S.D.) ± 1.34 (S.E.).

Recovery was also checked in each main step of thin layer chromatogram. Two urine samples with each 10 µl (1,333 cpm) of the authentic aldosterone were subjected to two thin layer chromatographies by system B and system C upon B. The spot, located by ultraviolet light, was collected and eluted with 3 portions of 1.5 ml methanol into a vial for scintillation counter and the elution repeated four times. 85.8% was recovered from the TLC with system B by the first 1.5 ml of methanol elution, and even after rechromatography by system C upon elution from B, still 66.2% was recovered in the first 1.5 ml of methanol.

Condition of separating other steroids

1) TLC-I

In order to check the efficiency of the thin layer chromatography in differentiating other steroids, each 5 µg of 24 different steroids were spotted, either singly or combinedly, on a thin layer plate (20 × 20 cm) of Kieselgel HF 254 and developed with ethyl acetate.

Each steroid was located either by a) ultraviolet light (254 mµ), b) spraying 50% of sulfuric acid in ethanol, c) blue tetrazolium reagent, or d) antimon trichloride, as shown in Figure 3 and Table 2. The experiment was quadrupled and the average Rf value for
Table 3. Change of $^3$H/$^14$C ratio after each thin layer chromatography, compared with the ratio of the eluate from the thin layer chromatography-II (expressed as 100), in 6 urine samples

<table>
<thead>
<tr>
<th>urine sample</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>eluate from TLC-II 5.68 (100)</td>
<td>33.14 (100)</td>
<td>19.14 (100)</td>
<td>12.95 (100)</td>
<td>15.79 (100)</td>
<td>16.22 (100)</td>
<td></td>
</tr>
<tr>
<td>eluate from TLC-III 6.26 (110)</td>
<td>34.01 (103)</td>
<td>8.57 (45)</td>
<td>9.72 (75)</td>
<td>11.97 (76)</td>
<td>9.81 (60)</td>
<td></td>
</tr>
<tr>
<td>eluate from TLC-IV 5.86 (103)</td>
<td>33.54 (101)</td>
<td>7.70 (40)</td>
<td>9.12 (70)</td>
<td>11.37 (72)</td>
<td>9.30 (57)</td>
<td></td>
</tr>
</tbody>
</table>

Each steroid, either to the solvent front or progesterone, were calculated. As evident from Figure 3, aldosterone was separated by thin layer from most of the steroids except for tetrahydroaldosterone.

2) **TLC-II**

To determine whether the aldosterone can be separated from tetrahydroaldosterone in further treatment of acetylation and chromatography, 10 µg of tetrahydroaldosterone was acetylated with $^3$H-acetic anhydride and subjected to the TLC-II. Aldosterone deacetate (Rf: 0.69 by system A and 0.46 by B) was now completely separated from the tetrahydroaldosterone acetate (Rf: 0.88 and 0.67). An authentic sample of aldosterone monoacetate ran with 21 deoxycorticosterone (Rf: 0.74 and 0.30).

3) **TLC-III**

For further assurance, the tetrahydroaldosterone-$^3$H-diacetate, upon elution from TLC-II, was subjected to TLC-III. Its Rf values were 0.93 by system C and 0.72 by D whereas those of aldosterone monoacetate were 0.93 and 0.43, being separated even more clearly.

4) **Unnecessity of further purification**

In order to determine whether further purification is needed, 6 urine samples were further subjected to one more additional thin layer chromatography by system E. As shown in Table 3, there was some decrease in $^3$H/$^14$C ratio between the TLC-II and -III, while no appreciable difference was noted between the TLC-III and -IV. Based on the ratio, therefore, the amount of aldosterone thus calculated became stable after TLC-III, indicating unnecessity of further purification. Furthermore, an oxidation of 4 samples by chromium trioxide decreased the ratio of $^3$H/$^14$C to 55-47% which is quite close to the calculated value (= 50%) for the monoacetate.

5) **Result**

The amount of aldosterone excreted to

<table>
<thead>
<tr>
<th>Subject</th>
<th>Condition</th>
<th>Number</th>
<th>Range (µg)</th>
<th>Mean (µg)</th>
<th>S.D.</th>
<th>S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal</td>
<td>active daily life, supine</td>
<td>8</td>
<td>5.72 - 17.00</td>
<td>10.99</td>
<td>±3.83</td>
<td>±1.36</td>
</tr>
<tr>
<td>various diseases without known relation to aldo.*</td>
<td>hospitalized</td>
<td>11</td>
<td>1.83 - 14.73</td>
<td>7.15</td>
<td>±3.42</td>
<td>±1.03</td>
</tr>
<tr>
<td>primary aldosteronism</td>
<td>hospitalized, before adrenalectomy</td>
<td>7</td>
<td>21.3 - 46.35</td>
<td>32.12</td>
<td>±8.38</td>
<td>±3.16</td>
</tr>
<tr>
<td></td>
<td>after adrenalectomy</td>
<td>5</td>
<td>5.7 - 17.6</td>
<td>12.03</td>
<td>±4.10</td>
<td>±1.83</td>
</tr>
</tbody>
</table>

* hyper-, hypo-thyroidism, ventricular septal defect, hyperdynamic β-adrenergic circulatory state, bronchial asthma, primary myocardial disease, schizophrenia, and liver cirrhosis without ascites.
urine in 24 hr were determined in various subjects by the present method and the result is given in Table 4.

**Discussion**

To start with, 100 ml of urine was taken as the sample. This is ten times larger volume than in the original method (Kliman and Peterson, 1960), but we found that 95% of aldosterone in it was readily transferred into 30 ml of dichloromethane as shown in Table 1.

As a recovery indicator, aldosterone-4-14C was used which unnecessitated the procedure of preparing aldosterone-14C-diacetate. In addition, it was mixed with the urine sample before extraction to cover the recovery rate of extraction.

The specific radioactivity of 3H-acetic anhydride was originally based on the 3H-acetylation of cortisone (Kliman and Peterson, 1960). We have replaced it with dl-aldosterone added to urine. After purification by the same chromatographies as in the determination of urinary intrinsic aldosterone itself, the count of 3H from the added aldosterone was divided by the weight of the added aldosterone. dl-Aldosterone is economical since it costs one tenth the cost of d-aldosterone, yet behaves exactly similar to d-aldosterone in so far as the chemical procedures employed.

Then, to avoid unnecessary 3H-acetylation of abundant contaminants in urine, most of them were removed before the acetylation. It was most efficiently done, as evidenced by TLC-I shown in Figure 1 and 2.

Despite the ten times larger volume of the starting sample, therefore, even less amount of 3H-acetic anhydride (0.03 ml of 10 mC/mM) was found enough to acetylate the sample resulting the same 3H/14C ratio after final purification as in the original method, thereby reducing the cost of 3H-acetic anhydride to 1/10.

After the 3H-acetylation of the sample, an authentic dl-aldosterone diacetate was added as a carrier and served for easy locating the sample with ultraviolet light in the following chromatographies.

Rf value of steroid varies by thin layer, room temperature, and humidity, and, sometimes resulted in an insufficient separation of aldosterone diacetate from tetrahydroaldosterone acetate in the TLC-II. This is especially true when the lower solvent front in the second dimension is less than 5 cm or a distance between adrenosterone and 21 dioxycorticosterone is less than 2 cm and necessitated a repetition of TLC-II. It is our experience that an addition of one drop of water into the solvent system in the second dimension often helps better separation.

Two dimensional thin layer chromatography was employed twice in place of paper chromatography for the further purification. Tetrahydroaldosterone, which was not removed by the TLC-I (Fig. 3), was now evidently separated by TLC-II and -III after acetylation. The development of TLC completes within 30 min and the spot was successively developed, without elution, to the second dimension. This is the largest factor contributed to save the time with quite a satisfactory recovery.

3H/14C ratio after the TLC-II still varied and became stable only after TLC-III. A further additional chromatographic purification did not influence the 3H/14C ratio, as shown in Table 3. Furthermore, the ratio remained in theoretical value even after oxidation to the monoacetate, indicating the purification was complete by the TLC-III without oxidation as far as the purification is done by the presently improved system. This again not only helped save the time, but also eliminated the danger of 14C gas evolution. In case even if it is oxidized, the monoacetate runs at the same Rf value as that of 21 deoxycorticosterone, and still separated from either aldosterone diacetate or tetrahydroaldosterone acetate.

Of the improvements employed, some of
them have been already introduced individually by different investigators, e.g., an addition of dl-aldosterone diacetate as a carrier by Tait et al. (1961), thin layer chromatography for steroid purification by Lisboa and Diczfalusy (1962) or for aldosterone by Nishikaze and Staudinger (1962), and, Kiesel gel HF 245 by Ibayashi et al. (1964). Brodie et al. (1967), applied these advantages to plasma aldosterone. Upon all these advantages combined, further improvements, e.g., the use of dl-aldosterone for the determination of specific radioactivity of 3H-acetic anhydride, a preliminary thin layer purification before 3H-diacylation of urine sample, and so forth, were added here, to make it far efficient both in economy and time, enabling one technician carry out a simultaneous determination of 4–8 urine samples a week.

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References