DETERMINATION OF PLASMA ALDOSTERONE IN CHILDREN BY THIN LAYER CHROMATOGRAPHY AND RADIOIMMUNOASSAY

By K. Parth, H. Zimprich and Roswitha Brunel

ABSTRACT

An accurate and relatively simple radioimmunoassay for the determination of aldosterone concentration in peripheral plasma has been developed. 0.5–2.0 ml plasma with added [1,2-3H]aldosterone is extracted with dichloromethane. Purification of the extract is achieved by thin layer chromatography in the system benzene-acetone 1:1. Recovery of [1,2-3H] aldosterone is 58 ± 6 (sd) %. Bound and free fractions are separated by dextran-coated charcoal. The intra-assay reproducibility is 8.8 % and the inter-assay reproducibility varies from 11.4–16.1 %. The sensitivity of the assay for a 5 ml plasma sample can be put at 0.2 ng/100 ml. Normal values determined in 52 healthy children of different age groups are presented. Furthermore the aldosterone stimulating effect of low sodium diet (17 children), severe and prolonged vomiting (19 children) and synthetic ACTH (10 children) has been studied by our modified method.

The determination of plasma aldosterone has been described by several investigators with different methods, an effort understandable because of the technical difficulty of detecting this substance occurring in much smaller amounts in blood than other adrenal steroids. Purification of plasma extracts has been achieved by paper chromatography (Bayard et al. 1970; Bizollon et al. 1974; Ito et al. 1972; Mayes et al. 1970; Underwood & Williams 1972).
Sephadex LH 20 column chromatography (Ito et al. 1972), partition and periodate oxidation (Farmer et al. 1973; Varsano-Aharon & Ulick 1973) or immunological (Gomez-Sanchez et al. 1973; Martin & Nugent 1973).

We have used thin layer chromatography as a simple and rapid purification step and have applied our method for the determination of plasma aldosterone levels in children under various conditions with satisfactory results. As a consequence of technical difficulties and the small amounts of blood available for determination in smaller children, clinical data on aldosterone in this field are extremely scanty. Our data should shed light into the aldosterone regulation of normal and sick children of different age groups under different stimulation tests.

MATERIALS AND METHODS

[1,2-3H]d-aldosterone (specific activity 54 Ci/mmol) was obtained from New England Nuclear and purified every 4–6 weeks by paper chromatography in Bush B 5 system. Unlabelled d-aldosterone was purchased from Sigma.

Dichloromethane, acetone, benzene, toluene, 95% ethanol (spectrograde) and gelatine were obtained from Merck and used without further purification.

For preparation of borate buffer pH 8 Titrisol from Merck was used. Other materials were as follows: thin layer plates, 20 × 20 cm, precoated with Silicagel by Merck, dextran molecular weight 500 000 and charcoal (Norit A) by Serva, Triton X 100, PPO and dimethyl-POPOP by Packard.

Sheep serum against aldosterone 18,21 disuccinate coupled to bovine serum albumin (lot 088) in a dilution of 1:100 was kindly supplied by the National Institute of Arthritis and Metabolic Diseases in Bethesda.

The cross-reactivities with cortisol (F), cortisone (E), corticosterone (B), 18-OH-B. deoxycorticosterone (DOC), progesterone, oestradiol-17β and the androgens testosterone and dehydroepiandrosterone were given as less than 1%.

1) Chemical

Extraction and purification of aldosterone from plasma. – After addition of 11 000 CPM [1,2-3H]d-aldosterone in 50 μl ethanol two different sample volumes (usually 1 and 2 ml) are extracted for 15 min with 30 ml dichloromethane (DCM) in glass stoppered test tubes 11 × 2.7 cm. The tubes are centrifuged for few minutes at low speed, the supernatant aqueous layer is aspirated and the protein gel removed by transferring the DCM-phase into another tube. The extract is washed successively with 5 ml 0.2 n acetic acid, 5 ml 0.1 n NaOH and twice 5 ml distilled water and then taken to dryness, the residue dissolved in 0.2 ml ethanol and spotted to the start line of the precoated thin layer plates. TLC is performed in the system benzene-acetone 1:1 (v/v), resulting in an effective separation from the major cross-reacting corticosteroids. The aldosterone area on the plate is detected by radioscanning (Berthold, model LB 2723), scraped off and eluted with ethanol into disposable plastic tubes.

After taking to dryness the residue is re-dissolved in 1 ml ethanol, 0.1 ml is counted for recovery and determination of remaining radioactivity and the remaining taken to dryness again.
Radioimmunoassay. – Solution A: Aldosterone antibody is diluted 1:10^5 by a 0.1 % gelatine solution in borate buffer pH 8.

Solution B: Solution A + 5800 cpm [1,2-3H]aldosterone per ml.

Charcoal suspension: 50 ml 1.2 % gelatine solution in borate buffer pH 8 + 60 mg dextran + 1 g charcoal.

A standard curve is established in duplicate ranging from 0–400 pg aldosterone. Usually 0, 40, 100, 200, 300, 400 pg were used and 1 ml of freshly prepared solution B is added containing approximately the same amount of radioactivity as in the dry samples. 0.5 ml of solution B is counted in duplicate.

To the sample tubes 1 ml freshly prepared solution A is added and the contents of all the tubes are mixed on a Vortex mixer and incubated overnight at 4°C. Next morning the tubes are immersed into melting ice for 15 min and 0.3 ml chilled charcoal suspension is added, mixed and after 10 min centrifuged for 5 min at 2500 r.p.m. Consecutively 0.5 ml of the supernatant is transferred into counting vials with disposable plastic tips and 10 ml scintillation fluid is added.

Liquid scintillation counting. – Our liquid scintillation cocktail consists of 1500 ml Trition X 100, 3000 ml toluene, 22.5 g PPO and 0.45 g dimethyl-POPOP.

Counting is performed in a Packard Scintillation Spectrometer to at least 10 000 counts with an efficiency of about 40 % determined by an internal toluene standard.

Calculation. – The standard curve was constructed by plotting the percentage bound aldosterone against the amount of unlabelled steroid added.

Standards: \( \% \text{ bound} = \frac{A \cdot 10^5}{B} \)

A: number of bound cpm \(^{3} \text{H}\) in standard.
B: initial number of cpm \(^{3} \text{H}\) in 1 ml solution B added.

Samples: \( \% \text{ bound} = \frac{C \cdot 10^5}{9 \cdot D \cdot R} \)

C: number of bound cpm \(^{3} \text{H}\) in sample.
D: number of cpm \(^{3} \text{H}\) added before extraction procedure.
R: \( \% \) recovery after extraction and TLC.

The amount of aldosterone in the unknown plasma samples can be determined directly from the standard curve.

The concentration of plasma aldosterone in ng/100 ml is calculated from the formula:

\[
\text{Concentration of plasma aldosterone (ng/100 ml)} = \frac{(U-L) \cdot 100}{9 \cdot R \cdot S}
\]

U: pg aldosterone in sample read off the standard curve.
R: \( \% \) recovery.
L: blank value (pg).
S: sample volume (ml).

Blank values are determined by analysing 1 ml charcoal pretreated plasma in duplicate within each series and are subtracted without correction for procedural losses.
2) *Clinical*

Plasma aldosterone levels were determined in 52 healthy children, both girls and boys aged from 2-14 years, at 8 a.m. in the supine position. All subjects had unrestricted sodium intakes before the test. In addition to this, the aldosterone stimulating effect of two factors was studied: salt restriction and synthetic ACTH (Synacthen). In a group of 17 healthy children, aged from 5-15 years, sodium restriction tests were performed as follows: the children got a low sodium diet (10-20 mEq. Na⁺/24 h) for 4 days. Blood samples were drawn at the beginning of the test, on the 3rd and 4th day at 8 a.m. in the supine position.

Ten children, 5-14 years old, were stimulated with a single dose of 0.25 mg Tetra-cosactid (Synacthen). Before and 20 min after the iv injection blood samples were taken.

Furthermore of 19 children with different, but non-endocrine diseases causing severe vomiting for several days, blood samples were taken on admission in the supine position.

**RESULTS**

1) *Chemical*

Recovery. – The mean recovery of [1,2-³H]aldosterone added to plasma was 58 ± 6 (sd) % (N = 80). The samples were corrected for losses during extraction and TLC.

Standard curve. – In Fig. 1 the mean ± SEM for 10 standard curves obtained during several months is presented.

![Standard curve for the radioimmunoassay of aldosterone. [1,2-³H]aldosterone bound (%) vs. unlabelled aldosterone (pg/tube). The points represent the mean values ± SEM of 10 assays.](image-url)

**Fig. 1.**

Standard curve for the radioimmunoassay of aldosterone. [1,2-³H]aldosterone bound (%) vs. unlabelled aldosterone (pg/tube). The points represent the mean values ± SEM of 10 assays.
Table 1.

<table>
<thead>
<tr>
<th></th>
<th>Intra-assay var. coeff.</th>
<th>Inter-assay var. coeff.</th>
<th>Number of determinations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pool 1</td>
<td>10.6 ± 0.9 ng/100 ml</td>
<td>8.8</td>
<td>24</td>
</tr>
<tr>
<td>Pool 2</td>
<td>7.7 ± 1.2 ng/100 ml</td>
<td>16.1%</td>
<td>20</td>
</tr>
<tr>
<td>Pool 3</td>
<td>12.6 ± 1.4 ng/100 ml</td>
<td>11.4%</td>
<td>9</td>
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</table>

**Precision.** – Precision was estimated by determining the intra- and inter-assay variation coefficients on plasma pools with different concentrations of aldosterone. The results are presented in Table 1.

**Sensitivity and method blanks.** – Charcoal pretreated plasma and distilled water was used for the determination of the blank values. Thirty 1 ml samples showed apparent "aldosterone" levels of 11 ± 5 (sd) pg or 0.21 ± 0.10 (sd) ng/100 ml when corrected for procedural losses. Only very slight sample volume dependence was noted up to 5 ml.

Regarding the standard deviation of the blank as the limiting factor, the sensitivity of the method for a 5 ml sample can be put at about 0.2 ng/100 ml.

**Accuracy.** – Accuracy was determined by adding known amounts of unlabelled aldosterone up to 400 pg to charcoal pretreated plasma. Least square regression analysis for 36 points gave the following equation (pg, Fig. 3):

\[ y = 1.037x - 0.946 \quad r = 0.995 \]

Thus no significant systematic error can be detected.

**Specificity.** – The specificity of our method is determined by the efficiency of the TLC-purification. This was checked by using two alternative purification procedures, paper chromatography in Bush B 5 system and Sephadex LH 20 column chromatography as described by Ito et al. (1972) and comparing the results of 15 plasma samples with the results obtained by our method. In only 5 cases did the difference exceed 10% the highest value reaching 17%, and no systematic direction of the deviation could be detected.

In addition to this, plasma samples of known aldosterone content with about 10-fold physiological concentrations in E, F and B were measured. The mean value of 6 samples was only 4% higher than before.

2) **Clinical**

a) **Basal levels.** – In the age group 2–6 years the mean value of plasma aldosterone concentration in our normal children at 8 a.m. in the supine position was 5.7 ± 3.6 (sd) ng/100 ml (N = 16), from 6–10 years 8.5 ± 5 (sd) ng/100 ml
Plasma aldosterone concentrations (mean values ± 1 sd are represented by horizontal lines) of normal children of different age groups, of subjects under sodium restriction or ACTH stimulation and of vomiting children.

(N = 15) and from 10–14 years 7.8 ± 5.0 (sd) ng/100 ml (N = 21). There is no significant difference between age groups (P > 0.1 by t-test).

b) Stimulation-tests. – Only 20 min after injection of synthetic ACTH, plasma aldosterone levels were elevated from a baseline value of 6.1 ± 3.6 (sd) ng/100 ml to 29.8 ± 11.9 (sd) ng/100 ml in 10 normal children. The statistical significance by paired t-test is P < 0.001.

Stimulation of aldosterone production by low sodium diet gave results as follows: baseline levels in 17 healthy children were 7.0 ± 4.3 (sd) ng/100 ml in agreement with our data mentioned above. On the 3rd and 4th day of low sodium intake we observed an elevation of the plasma aldosterone concentration to 21.3 ± 18.0 (sd) ng/100 ml and 20.6 ± 16.1 (sd) ng/100 ml, respectively. In spite of finding a widespread range of stimulation values from 3.2–69.8 ng/100 ml the stimulation effect is statistically significant (P < 0.005 by paired t-test).

Vomiting causes increased aldosterone production by a similar biochemical mechanism. As a consequence of water and electrolyte losses, the observed effects are even more distinct than in sodium restriction alone. In 19 children
Correlation of aldosterone recovered and aldosterone added to 1 ml samples of charcoal pretreated plasma.

without endocrine diseases but suffering from more or less severe vomiting during several days, we found plasma aldosterone levels from 19.7 to 170 ng/100 ml with a mean of $53.7 \pm 39.8$ (sd) ng/100 ml. Again this elevation is statistically highly significant ($P < 0.001$). Our results are summarized in Fig. 2 and Table 2.

**DISCUSSION**

The described method for the determination of aldosterone in peripheral plasma has been shown to be accurate and relatively simple. In our laboratory a skilled technician processes 40–60 samples in 5 working days excluding time for liquid scintillation counting.

In spite of using a relatively specific antibody against aldosterone we were forced to introduce some kind of purification step. Up to the present time only one antibody is known of unique specificity making purification steps unnecessary (*Mc Kenzie & Clements* 1974). Purification was achieved by thin layer chromatography, separating aldosterone easily from competing steroids, especially E, F and B, TLC being less time consuming than other methods.
Table 2.
Plasma aldosterone concentrations (ng/100 ml) of normal children of different age groups, of subjects under sodium restriction or ACTH stimulation and of vomiting children.

<table>
<thead>
<tr>
<th>Age groups</th>
<th>Low sodium diet</th>
<th>ACTH-stimulation</th>
<th>Vomiting children</th>
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<tr>
<td></td>
<td>Baseline</td>
<td>3rd day</td>
<td>4th day</td>
</tr>
<tr>
<td>2-6 years</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-10 years</td>
<td>2.9</td>
<td>12.6</td>
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<td>10-14 years</td>
<td>6.4</td>
<td>3.8</td>
<td>2.9</td>
</tr>
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<tr>
<td>18.7</td>
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<td>11.5</td>
<td>11.0</td>
</tr>
</tbody>
</table>

Precision and accuracy are satisfactory. In our experience, the blank value is essentially the result of the elution of the thin layer silicagel. It possibly might be decreased by washing the TLC-plates several times with polar solvents (ethanol) resulting in improved sensitivity of the method.

Using two alternative purification steps, no evidence for non-specificity was found. The addition of competing corticosteroids in 10-fold physiological concentrations did not alter the measured aldosterone concentration.

Accordingly to our knowledge up to the present time, there is only one published study dealing with plasma aldosterone levels in children (Kowarski et al. 1974) aged from 1-15 years but probably determined in an active state causing higher concentrations than observed by us. Compared with published
results in adults in the supine position for several hours (Bayard et al. 1970; Bizollon et al. 1974; Corvol et al. 1972; Farmer et al. 1973; Mayes et al. 1970; Underwood & Williams 1972; Varsano-Aharon & Ulick 1973) there is good agreement with our findings in children indicating that in the supine position there is no significant difference in plasma aldosterone levels between children aged from 2–14 years and adults. The same has been shown to be valid for plasma levels in an active state (Kowarski et al. 1974).

The response of aldosterone levels to restricted sodium intake governed by the renin-angiotensin system was found to be less pronounced than described by Underwood & Williams (1972) in adults. However, a statistically significant \( P < 0.005 \) by paired t-test elevation was seen, but there are great quantitative differences in the degree of response in our 17 examined children. Further investigations will be necessary including controlled potassium intake and varying parameters like sodium intake and duration of diet to get a better understanding of these individual differences.

The combined effects of electrolyte and water loss in vomiting children leads to a dramatic elevation of plasma aldosterone concentration by stimulation of the renin-angiotensin-system. The highest value found in this group of children was 170 ng/100 ml with a mean elevation of aldosterone levels by a factor of 10.

The direct stimulation of the cells in the zona glomerulosa by Tetracosactid is clearly shown by our results in 10 children. Only 20 min after injection of synthetic ACTH there was a rise in the plasma aldosterone concentrations to about a 5-fold baseline level. Similar results in adults have been reported (Varsano-Aharon & Ulick 1973), even though there has been a lot of discussion about the mode of ACTH-stimulation of aldosterone-production and its time limited effect.

ACKNOWLEDGMENTS

The authors wish to express their gratitude to the National Institute of Arthritis and Metabolic Diseases for the gift of aldosterone antibody and to Miss Arzberger for expert technical assistance.

REFERENCES


Received on February 3rd, 1975.