Simple Fluorometric Determination of Aldosterone in Urine without Use of Isotopes or Chromatography

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Aldosterone 18-glucuronide in urine is hydrolyzed by adjusting the pH to 1.0 and allowing the mixture to stand overnight at room temperature. The free aldosterone is then extracted into dichloromethane, which is washed with carbonate to remove acidic compounds and evaporated. The residue is partitioned between a nonpolar organic phase and an aqueous phase, and the aldosterone oxidized to the 13-carboxylic acid derivative with Benedict's qualitative glucose reagent. Neutral compounds are extracted from this oxidation mixture with dichloromethane at pH 7.5, the mixture is acidified, and the oxidized aldosterone extracted into dichloromethane. After washing with pH 3.5 buffer, this extract is evaporated and the oxidized aldosterone determined fluorometrically via a two-stage reaction with sulfuric acid/water (85/15 by vol) and methanol containing ferric chloride.

Many clinical laboratories routinely determine 17-ketosteroids, 17-hydroxycorticosteroids, serum cortisol, and perhaps other steroids. To offer a more complete service in steroids, it is desirable for them to also offer measurements of urinary aldosterone. However, most methods for doing so require a liquid-scintillation counter and expertise in chromatography. Recently, simple nonchromatographic radioimmunoassays have appeared for aldosterone determination (1–4), but these methods also require a liquid-scintillation counter and must depend on the purchase of expensive kits or antibody.

The method presented here requires no isotopes or chromatography, and multiple specimens may be run in one day (after overnight hydrolysis).

Materials and Methods

Apparatus

This includes screw-cap vials, 40 ml, such as Kimble No. 60957; polyethylene-lined caps for these vials, such as Poly- Seal 24-400; a Kahn shaker such as the two-speed model of Eberbach with utility carrier; an air-blowing apparatus constructed of copper tubing to fit vial supports such as Scientific Products S9215, and a compressed-air source; and a spectrofluorometer such as the Perkin-Elmer Model 204.

Reagents

All reagents are reagent grade, used without further purification, De-ionized water is used for all aqueous reagents.

Dichloromethane. Reagent grade is acceptable.

Anhydrous sodium sulfate, granular.

Sodium carbonate, 50 g/liter. Dissolve 10 g of anhydrous Na2CO3 in 200 ml of water.

Ethyl acetate. Reagent grade is acceptable.

Ethanol/water, 10 ml of absolute ethanol plus 90 ml of water.

Benedict's reagent. Dissolve 3.5 g of CuSO4·5H2O in 20 ml water in a flask (may require a little heat). Dissolve 35 g of sodium citrate dihydrate and 20 g of anhydrous sodium carbonate in 150 ml of water (may also require warming). Cool the two solutions and combine them.

Glycine buffer, pH 3.5. Dissolve 7.5 g of glycine in water, add 1.4 ml of HCl (6 mol/liter), and dilute to 250 ml. Check the pH and adjust if necessary.

Sulfuric acid, dilute. Mix cautiously, in an ice bath, 85 ml of concentrated H2SO4 and 15 ml of water.

Methanol containing FeCl3·6H2O. Dissolve 100 mg of FeCl3·6H2O in 100 ml of methanol. Dilute 5 ml of this with 95 ml of methanol.

Standards: d-Aldosterone (Sigma Chemical Co. St. Louis, Mo. 63178; cat. No. A6628) is stored overnight in a desiccator before weighing.
"A" standard, 0.4 g/liter. Dissolve 40 mg of aldosterone in isopropanol and dilute to 100 ml. Store in a dark bottle in the refrigerator. The solution is stable longer than six months.

"B" standard, 4 mg/liter. Dilute 1.0 ml of "A" standard to 100 ml with isopropanol. Store in a dark bottle in the refrigerator. The solution is stable longer than six months.

Procedure

Place 20 ml of urine into a 40-ml screw-capped vial, adjust the pH to 1 with HCl (6 mol/liter), cap, and let stand for 20 h or more at room temperature in the dark. Add 20 ml of dichloromethane, shake slowly for 10 min or longer, centrifuge (1500 rpm) for 2 min or longer, and aspirate the urine (upper) phase, leaving behind any emulsion that may have formed.

Add about 2 g of anhydrous Na2SO4 to each vial and shake to break up the emulsion, centrifuge, and pour the dichloromethane into clean vials, leaving the Na2SO4 behind. Add 5 ml of the Na2CO3 solution to the dichloromethane, shake vigorously for 2 min, centrifuge, aspirate the carbonate (upper) phase, and wash the dichloromethane with 3 ml of water.

Transfer 17 ml of the dichloromethane into a clean vial, add a boiling chip, and evaporate at 56°C in a water bath under a gentle stream of air.

Evaporate 0.2 ml (0.8 μg) of aldosterone "B" standard in each of two tubes, for duplicate standards. Label a clean tube "B" for blank. Treat standards and blank exactly like unknowns from this point on.

Add 3 ml of ethyl acetate to all tubes, mix to dissolve the residue, add 2 ml of the ethanol/water mixture and 20 ml of n-heptane, and shake the mixture for 3 min. Centrifuge, and aspirate as much of the heptane (upper) layer as possible without removing any of the aqueous layer.

Add 2 ml of Benedict's reagent, mix well, cap, and incubate for 30 min at 56°C. This oxidizes aldosterone to its 13-carboxylic acid derivative.

Add 3 ml of NaH2PO4·H2O (100 g/liter), mix, add 20 ml of dichloromethane, shake the mixture for 3 min, centrifuge, and carefully aspirate the dichloromethane (lower) layer.

Add 1 ml of HCl (6 mol/liter) to each tube dropwise with vigorous mixing, to remove CO2. Add 20 ml of dichloromethane, shake for 3 min, centrifuge, and aspirate the aqueous (upper) layer as completely as possible.

Add 5 ml of the glycine buffer to the organic phase, shake for 2 min, centrifuge, and aspirate the aqueous (upper) layer as completely as possible. Repeat this wash with glycine buffer one more time.

Transfer 17 ml of the dichloromethane to clean tubes and evaporate it at 56°C in a water bath, under a gentle stream of air. Add 5 ml of the dilute H2SO4 to each tube and mix vigorously to dissolve the steroid, cap, and heat for exactly 15 min at 56°C. Place the tubes in an ice bath for 5 min. When the tubes are cold, add 2 ml of the methanol/FeCl3·6H2O to each and mix vigorously for 5 s. Return to the ice bath. (It is best to do just two tubes at a time, adding the methanol, mixing immediately, and returning to the ice bath.) After methanol has been added and mixed, cap the tubes and place them in water at room temperature for 2 min, then in the dark at room temperature for exactly 25 min.

Read the fluorescence within the next 5 min, because it reaches a peak and then fades rapidly. The excitation wavelength is 580 nm, the fluorescence wavelength is 610 nm. Average the readings for the two standards.

Calculation

(Unknown fluorescence – blank fluorescence/standard fluorescence – blank fluorescence) × 47 = aldosterone, μg/liter

To correct for an average 6% loss of aldosterone in the first extraction with dichloromethane and its washing, the factor (μg/liter)/0.94 is used. Then: (μg/liter (corr.)) × (liters/24 h) = μg/24 h.

The 24-h urines used were collected without preservative (refrigerated during collection) or contained either 10 ml of glacial acetic acid or 10 ml of toluene that had been put into the collection jug. They were refrigerated soon after collection.

Results and Discussion

Method Development

Pre-extraction. Pre-extraction as described by Drewes et al. (1) did not alter values significantly, so was not done.

Hydrolysis. The specimens are hydrolyzed in the usual manner (1, 5) by incubation overnight at pH 1 and room temperature, which hydrolyzes aldosterone 18-glucuronide. Incubation at 56°C for 2 h at pH 1 gave similar values, but pigment formation was increased considerably, so the overnight hydrolysis was preferred.

Initial extraction. At first, we washed the original dichloromethane extract with 0.1 mol/liter NaOH. The resulting analytical recovery of aldosterone was poor and inconsistent. This problem was corrected by substituting the Na2CO3 solution for the NaOH solution. Analytical recovery averaged 94% through these steps, and is corrected for in the calculations. After this correction, analytical recovery for urine taken through the entire procedure averaged very nearly 100%.

Water taken through the entire procedure gave a blank identical to the blank started after the initial dichloromethane evaporation. Thus, introduction of the blank at this stage is acceptable. Introduction of the standard at this stage is compensated for by the recovery correction factor.

Partition between ethyl acetate/heptane and water/ethanol. Running the procedure without this partition results in values that are about 65% higher than with this partition. In addition, the final fluorescent solution has a faint brown color, which varies from specimen to specimen. Experiments were done to study aldosterone partition between 20 ml of n-heptane containing various amounts of ethyl acetate and 2 ml of water containing 0, 10, 20, or 30 ml of ethanol per deciliter. Those conditions chosen gave good recovery of aldosterone in the aqueous phase and removed considerable interfering material.

Oxidation. Oxidation of aldosterone is essentially complete in 20 min at 56°C with half-strength Benedict's reagent. Routinely, 30-min incubation was used.

Addition of Na2HPO4·H2O (100 g/liter) after oxidation lowers the pH to about 7.5, still well above the expected pKa of the aldosterone oxidation product, which is retained in the aqueous phase at this step.

Wash with pH 3.5 glycine buffer. Cortisol standards were run through the entire procedure initially without this wash. It was found that there was no fluorescence when I omitted heating of the Benedict's reagent. However, when heating was included, cortisol gave a fluorescence equivalent to 2% of that of an equal amount of aldosterone in the method. A reasonable explanation is that cortisol is being oxidized to some extent by the Benedict's solution; most likely to the 17-carboxylic acid derivative. Because this derivative would be expected to have a pKα near 3.5, while
that of aldosterone derivative should be near 5, a double wash with pH 3.5 buffer was included in the procedure. This diminished the interference of cortisol from 2% to about 0.2% without causing undue loss of aldosterone oxidation product.

Development of fluorescence. I thought that, after optimization of the oxidation step, a one-stage reaction to develop fluorescence similar to that of cortisol used by Clark and Rubin (6) or perhaps a two-stage reaction similar to that for dehydroisoandrosterone used by Allen et al. (7) might be satisfactory for the aldosterone oxidation product. Thus sulfuric acid concentrations, amounts of methanol or ethanol, and heating times were varied in one-stage and two-stage reactions to obtain maximum color (assuming that the colored product would fluoresce). Initially, the reaction was optimized without including iron in the methanol. A yellow product was formed with absorbance and excitation peaks at 480 nm and a fluorescence peak at 525 nm. I attempted to intensify the color by adding Fe3+ to the methanol, because it is used in some sensitive cholesterol methods (8). When this was done, the second-stage reaction occurred rapidly at room temperature, but instead of a more intense yellow color, the yellow color was decreased by about half and a red color was formed with absorbance and excitation peak at 586 nm and a fluorescence emission peak at 610 nm. I found that the red fluor gave a relatively lower blank than did the yellow fluor (although the fluorescence was not as great) and that the red fluor gave lower values with partially purified urine extracts than did the yellow fluor, so I considered the red fluor the better of the two for measurement in the procedure.

Analytical Variables

Standardization. Aldosterone standards from 0.4 to 4.0 µg, representing 25 to 250 µg of aldosterone per liter of urine, were taken through the procedure. There was no significant deviation from linearity over this range.

Blank. The blank value averaged the equivalent of about 2.5 µg of aldosterone per liter, but was very consistent, so that blank-corrected aldosterone concentrations even lower than this can probably be considered significantly different from zero (see sensitivity).

Day-to-day precision. Ten control urines run on different days by the same technologist gave values ranging from 5.0 to 6.7 µg/liter, with a mean of 5.87 µg/liter and a standard deviation of 0.58 µg/liter.

Sensitivity. A value two standard deviations above zero could be considered significantly different from zero, such that the sensitivity could be considered to be 1.16 µg/liter. This assumes that the method standard deviation is the same at zero aldosterone concentration as 5.87 µg/liter (the control value). Actually, the method may be a little more sensitive than this.

Accuracy and specificity. A manual scan of excitation and fluorescence peaks at 5-nm intervals of the standard and of control urine from 565 to 590 nm and from 610 to 635 nm indicated that after subtracting the blank, the control spectra were essentially identical to those of the standard over these wavelengths. The values for urine have been corrected to yield an apparent 100% recovery through the entire procedure. The mean obtained for 25 randomly selected urine specimens (11.1 µg/24 h) was not significantly different from that obtained by other accepted procedures for aldosterone (1, 2, 5).

Ten steroids and the drug spironolactone were tested for interference when run through the entire assay. The percent fluorescence compared to aldosterone is shown in Table 1.

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldosterone</td>
<td>100.00</td>
</tr>
<tr>
<td>Cortisol</td>
<td>0.18</td>
</tr>
<tr>
<td>Cortisone</td>
<td>0.00</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>1.40</td>
</tr>
<tr>
<td>11-Deoxycorticosterone</td>
<td>0.10</td>
</tr>
<tr>
<td>11-Deoxycortisol</td>
<td>0.33</td>
</tr>
<tr>
<td>Tetrahydrocortisol</td>
<td>0.02</td>
</tr>
<tr>
<td>Tetrahydrocortisone</td>
<td>0.00</td>
</tr>
<tr>
<td>Tetrahydro 11-deoxycorticosterol</td>
<td>0.02</td>
</tr>
<tr>
<td>β-Cortol</td>
<td>0.02</td>
</tr>
<tr>
<td>β-Cortolone</td>
<td>0.06</td>
</tr>
<tr>
<td>Spironolactone</td>
<td>0.00</td>
</tr>
</tbody>
</table>

The method has been used routinely for several months in this laboratory. During this time, three specimens have had abnormally high aldosterone values: 32, 46, and 48 µg/24 h. All three were from patients hospitalized with severe hypertension. On the last two of these plasma renin values were above normal at about the same time as the urines were collected, confirming a diagnosis of secondary hyperaldosteronism.

Additional clinical studies still remain to be done, as well as comparisons with other methods. It may be that the fluorescence reaction can be improved. In all probability though, the method is acceptable for clinical use, and the suggestion of Cope and Loizou (5) that aldosterone excretions exceeding 30 µg/24 h be considered strong evidence for a diagnosis of hyperaldosteronism can be applied to this procedure, because 30 µg/24 h is nearly three standard deviations above the mean of 11.1 µg/24 h I found for 25 normal specimens.

A technologist can conveniently run one blank, two standards, a control, and four unknown specimens in about 6 h, with actual bench time a little less than this. Thus, this is a very economical assay for aldosterone.

Normal Range

Twenty-five 24-h urines for which a normal 17-hydroxycorticosteroid value had been obtained were assayed for aldosterone by this method. They ranged from 0 to 22 µg/24 h, with a mean of 11.1 µg/24 h and a standard deviation of 6.8 µg/24 h. A statistical normal range of 0–25 µg/24 h therefore was accepted for patients whose diets and activity are not being controlled.

I thank Trini Addis for assaying the normal specimens and the patients' specimens and Dr. Dale Nabb for allowing ample time from regular duties for development of this method.

References


Glucose Oxidase Chemiluminescence Measurement of Glucose in Urine Compared with the Hexokinase Method

David C. Williams,1 Glenn F. Huff, and W. Rudolf Seltz

This method is more sensitive and specific than an enzymatic (hexokinase) technique with which it was compared, has a greater range of linearity, and presents no problems of background correction.

Because of the presence of other reducing substances in urine (primarily uric acid), it is difficult to quantitate the glucose in urine. These reducing substances interfere with nonenzymatic methods for glucose, making them unsuitable for quantitation of glucose in urine samples. The hexokinase (EC 2.7.1.1) method requires that three measurements of absorbance be made because there is a significant absorbance by both the reagents and the sample (1). Bostick and Hercules reported a chemiluminescent (CL) method for glucose in blood, but they were unable to apply the method to urine samples because uric acid interfered by reducing the peroxide (2-4). We have developed and evaluated a method for removing the interferences from urine by using the Somogyi precipitate obtained by adding equimolar portions of Ba(OH)2 and ZnSO4. The centrifugate from the above mixture reportedly is free of reducing substances other than glucose (5).

Materials and Methods

Instrumentation

The apparatus we used was very similar to that reported by Bostick and Hercules (2-4). An infusion pump drives three flow lines containing $10^{-2}$ mol/liter K$_3$Fe(CN)$_6$, $2 \times 10^{-4}$ mol/liter luminol in 0.1 mol/liter KOH/H$_2$B$_4$O$_7$ buffer, and $10^{-2}$ mol/liter pH 5.6 acetate buffer, respectively. The ferricyanide and luminol are mixed before they enter the CL cell. The acetate line flows over a column of immobilized glucose oxidase before entering the CL cell. A sampling valve is used to introduce the glucose sample to the acetate flow line. As the glucose passes over the enzyme column it is converted to H$_2$O$_2$, which then mixes directly with the Fe(CN)$_6^{3-}$/luminol in the CL cell. The CL cell is positioned directly in front of a photomultiplier that measures CL intensity. For more detail, see reference 2.

One major change from the previous study was to use glucose oxidase (EC 1.1.3.4) immobilized on porous glass rather than on the Sepharose support used in the previous study. The glass (CPG-10-500, 200-400 mesh; Electro-Nucleonics, Inc., Fairfield, N. J. 07006) was aminated by the procedure of Robinson et al. (6). The aminated glass was then coupled to the enzyme by a standard procedure for azo linkage (7). This change substantially reduced the back pressure of the enzyme column, making it possible to use a Model 975 infusion pump (Harvard Apparatus Co., Inc., Millis, Mass. 02054) to drive the flow system rather than the more powerful pump required in the previous study. Sensitivity and linear range were not affected by changing the enzyme support.

Procedures

To remove the interfering substances from urine, we added 1 ml of 0.1 mol/liter Ba(OH)$_2$ and 1 ml of 0.1 mol/liter ZnSO$_4$ to 0.1 ml of urine. A 0.25 ml aliquot of the centrifugate from this solution was diluted with 4.0 ml of the acetate buffer (8). One milliliter of the diluted sample was then aspirated into the sample loop of the CL flow system.

Hexokinase kits (cat. No. UV15-10; Sigma Chemical Co., St. Louis, Mo. 63178) were used in a correlation study. The reagent was diluted according to the manufacturer's directions. A 3.0-ml aliquot of the reagent was used to determine reagent blank absorbance at 340 nm. The urine blank absorbance was determined by diluting 0.1 ml of urine with 3.0 ml of NaCl solution (8.5 g/liter). The absorbance of the reaction mixture was determined after about 10 min of incubation, and the glucose concentration was determined from standard curves.

For the correlation study, 100 μl of each urine sample was added to three different test tubes: one containing the hexokinase reagent, another containing NaCl solution, and a third in which the precipitation was done for the CL


