


Chemiluminescence Immunoassay of Aldosterone in Serum

T. V. Stabler and A. L. Siegel

A chemiluminescence immunoassay (CLI) for the direct measurement of aldosterone in serum was developed with aminobutyethyl isoluminol (ABEI) as the label. In this competitive assay the samples are incubated with sample, antibody, aldosterone–carboxymethyl oxime–ABEI, and paramagnetic particles coated with second antibody. After magnetic separation and washing, the samples are incubated with 200 \( \mu \)L of NaOH (2 mol/L) at 60 °C for 30 min. In the luminometer the chemiluminescence is produced by the serial injection of 150 \( \mu \)L each of microperoxidase and \( \text{H}_2\text{O}_2 \) solutions. Comparison of results with an RIA method showed excellent agreement: CLI = 1.001 RIA + 0.020 (r = 0.99, n = 93). The method is simple and avoids the hazards and costs associated with isotopic waste. The label has a shelf life of at least two years.

Additional Keyphrases: isoluminol; paramagnetic separation of bound and free label

Chemiluminescent immunoassays (CLI) involving isoluminol labels have now been reported for several steroids (1-5) but no such assay for serum aldosterone has yet been described. In the main advantages these labels offer a sensitivity comparable with that realized by RIA, simplicity of label synthesis, and a reading time of several seconds. A shelf life of several years for the labeled steroids enhances the robust nature of this method.

As with other nonisotopic immunoassays, the use of CLI spares the laboratory the hazard and expenses associated with the use and disposal of radioactive waste.

Here we describe and characterize the direct serum aldosterone CLI method and compare it with an RIA kit procedure (Biotecx Labs., Inc., Friendswood, TX 77546) that uses the same highly specific polyclonal antibody.

Materials and Methods

Materials

For the RIA procedure we used a Model 20/20 gamma counter (Iso-Data, Rolling Meadows, IL 60008). Chemiluminescence was measured with the Magic Lite luminometer (Ciba Corning Diagnostics Corp., Houston, TX 77060).

The RIA kits and rabbit anti-aldosterone antibody were kindly supplied by Biotecx Labs., Inc. The cross-reactivity of this antibody with steroids that could be present in patients' samples is <0.001%. Bovine serum albumin (BSA), \( N,N \)-dicyclohexylcarbodiimide (DCC), \( N \)-hydroxysuccinimide, microperoxidase (MP-I), Tween 20, and ABEI were obtained from Sigma Chemical Co., St. Louis, MO 63178. Goat anti-rabbit IgG coated on paramagnetic particles was from Advanced Magnetics, Inc., Cambridge, MA 02138. Thin-layer chromatography plates (Silica Gel-60) were from Merck, Darmstadt, F.R.G. Quality-control material was purchased from Bio-Rad Labs., Anaheim, CA 92806.

For the RIA and CLI procedures we prepared the aldosterone calibrants (range 0 to 2.78 nmol/L) in charcoal-stripped serum. All solutions were prepared in de-ionized, glass-distilled water.

**Assay buffer:** This was phosphate-buffered saline, 50 mmol/L, pH 8.0, containing 9 g of NaCl, 0.1 g of BSA, and 1 g of sodium azide per liter.

**Rabbit anti-aldosterone antibody:** The material from Biotecx Labs. was diluted 40-fold with assay buffer, and 50 \( \mu \)L was used for each assay tube. The final dilution in each assay tube was 1:640.

**Wash solution:** Per liter, 9 g of NaCl, 1 g of sodium azide, and 0.5 mL of Tween 20.

**Microperoxidase stock solution:** This contained 0.1 g of microperoxidase per 100 mL of Tris·HCl (0.01 mmol/L, pH 7.4). The solution is stable for one month when stored at 4 °C. Fresh working solution is prepared daily by adding 0.1 mL of stock to 9.9 mL of distilled water.

**\( \text{H}_2\text{O}_2 \) solution:** Add 0.1 mL of 300 mL/L solution to 15 mL of distilled water.

**Sodium hydroxide:** 2 mol/L.

**Stock aldosterone–ABEI:** This was an ethanolic...
solution of the conjugate, 2 mg/25 mL. When refrigerated and shielded from light, this reagent is stable for at least two years.

**Working solution of aldosterone-CMO-ABEI:** The conjugate was diluted with assay buffer to yield 180 000 RLU (relative light units) for the zero tube in our antibody/antigen system. The refrigerated solution was stable for at least a week. Each 0.1 mL of solution contained about 10 pg of conjugate.

**Procedures**

**Preparation of aldosterone-CMO-ABEI:** We synthesized the label according to the procedure described by Kim et al. (3). Briefly, this involved two steps. First, 10 mg of aldosterone-CMO was activated with 5 mg of N-hydroxysuccinimide and 10 mg of DCC in 0.5 mL of dry dimethylformamide. After a 24-h incubation in the dark, the urea spicules were removed by centrifugation. To the supernate remaining we added 0.5 mL of a solution of 5 mg of ABEI in 0.13 mol/L NaHCO₃ reagent and let this react at room temperature for 2 h with stirring. The solution was acidified to pH 2 and the precipitate that formed was dissolved in a small volume of methanol. The product was purified on thin-layer chromatographic plates developed in chloroform/methanol (80/20 by vol). The sky-blue fluorescent material at Rₜ 0.45, visible under short-wavelength illumination, was removed, weighed, and dissolved in ethanol. Shielded from light and refrigerated, the conjugate is stable for at least two years.

**Assays:** The Biotecx RIA procedure for serum aldosterone was performed according to the instructions supplied with the kit.

The CLI assay was performed as follows: To 400 μL of assay buffer add 200 μL of patient’s serum (or calibrator), 100 μL of label, and 50 μL each of antibody and paramagnetic particles coated with second antibody. Place the samples on a shaker and incubate for 2 h at room temperature. After magnetic separation, wash the magnetic particles twice with wash solution, then add 200 μL of NaOH (2 mol/L) to each tube and incubate at 60 °C for 30 min. This step releases the label from its binding to the antibody. Place the samples in the luminometer and initiate the chemiluminescence by the consecutive injection of 150 μL each of the microperoxidase and H₂O₂ solutions; measure RLU for 5 s. The instrument performs the data reduction according to the log of RLU vs the log of the concentrations fitted by a spline function.

**Results**

Figure 1 illustrates a typical standard curve (B/B₀ vs concentration).

**Correlation method:** The agreement of the CLI assay with RIA results from 0 to ~2 nmol/L was excellent: CLI = 1.001 RIA + 0.020 nmol/L (r = 0.99, n = 93, Sₓₓ = 2.58 nmol/L).

**Sensitivity:** The minimal concentration of aldosterone that can be distinguished from the zero standard is 0.03 nmol/L, calculated from the assay of 20 duplicates of the zero standard and equal to 2 SD of the mean RLU. The sensitivity of the RIA kit was reported to be 0.03 nmol/L also.

**Precision data:** The precision of the method (Table 1) is quite comparable with that described for RIA. In the latter assay, aldosterone concentrations >0.28 nmol/L were used for the precision evaluation.

**Analytical recovery:** Known amounts of aldosterone were added to a serum pool containing 0.32 nmol of aldosterone per liter. The results (Table 2) indicate an acceptable recovery (97–105%) of the added steroid.

**Interferences:** Bilirubin up to 513 μmol/L and hemo
globin up to 0.16 mmol/L had no significant effect on the aldosterone assay. Grossly lipemic serum, analyzed before and after ultracentrifugation, also showed no significant change in aldosterone concentration.

**Parallelism:** A patient’s serum with an aldosterone concentration of 2.76 nmol/L was serially diluted with zero standard. Table 3 confirms the validity of using the zero standard as a diluent for measuring patients’ samples with increased concentrations of aldosterone.

**Discussion**

Our work indicates that the sensitivity and precision of the CLI are comparable with that of an RIA for the direct assay of serum aldosterone. Clinically, in our hands the CLI assay has produced the appropriate aldosterone values in patients being studied for hypertension with renal artery stenosis and receiving furosemide. The CLI results closely followed the RIA values. Most of the reagents involved are quite stable and

**Table 1. Precision Data for Assay of Aldosterone at Four Concentrations**

<table>
<thead>
<tr>
<th></th>
<th>Mean, nmol/L</th>
<th>SD, nmol/L</th>
<th>CV, %</th>
<th>Interassay variation</th>
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<tr>
<td></td>
<td>Mean, nmol/L</td>
<td>SD, nmol/L</td>
<td>CV, %</td>
<td>Mean, nmol/L</td>
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<tr>
<td></td>
<td>0.04</td>
<td>0.11</td>
<td>0.03</td>
<td>0.10</td>
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<tr>
<td></td>
<td>0.01</td>
<td>0.01</td>
<td>0.04</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>17.8</td>
<td>12.2</td>
<td>6.0</td>
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Table 2. Analytical Recovery of Added Aldosterone

<table>
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<tr>
<th>Added</th>
<th>Endogenous</th>
<th>Expected</th>
<th>Found</th>
<th>Recovery, %</th>
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<tr>
<td>0.28</td>
<td>0.32</td>
<td>0.59</td>
<td>0.60</td>
<td>103</td>
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<td>0.56</td>
<td>0.32</td>
<td>0.87</td>
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<td>1.11</td>
<td>0.32</td>
<td>1.42</td>
<td>1.39</td>
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<td>2.22</td>
<td>0.32</td>
<td>2.53</td>
<td>2.66</td>
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Table 3. Parallellism Study

<table>
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<th>Dilution</th>
<th>Aldosterone, nmol/L</th>
<th>Observed</th>
<th>Percent of expected</th>
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<tr>
<td>Undiluted</td>
<td>2.76</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>3/4</td>
<td>2.07</td>
<td>1.94</td>
<td>93.4</td>
</tr>
<tr>
<td>2/3</td>
<td>1.84</td>
<td>1.88</td>
<td>102.0</td>
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<tr>
<td>1/2</td>
<td>1.38</td>
<td>1.35</td>
<td>98.0</td>
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<td>1/4</td>
<td>0.89</td>
<td>0.67</td>
<td>96.8</td>
</tr>
<tr>
<td>1/8</td>
<td>0.34</td>
<td>0.38</td>
<td>110.0</td>
</tr>
</tbody>
</table>

require replacement weekly or monthly. The ABEl conjugate can be stored for years and may be purified of breakdown products by a simple thin-layer chromatographic step. All other reagents are readily available. The work output can be greatly improved with the use of any of the versatile automatic pipetting stations now available. For assay readout, any of the luminometers that can sequentially inject reagents can be used.

Among the other approaches one can use to achieve adequate sensitivity for a CLI of serum aldosterone is the use of labels with greater efficiency, e.g., acridinium (6, 7), europium (8), and enzymes coupled with light-yielding substrates (9, 10). Assays of aldosterone based on the use of these materials have not yet been described. Such labels could be costly and may not be as stable as the material described in this work.

Although several instrument manufacturers have placed chemiluminescent assay equipment on the market, the assay menus have been relatively limited. The chief analytes available have mostly been restricted to the thyroid hormones and other high-volume assays. We in the endocrine laboratory would like to have such assays extended to include all of the clinically relevant steroid hormones so that we may sooner eliminate the use of isotopic materials and the hazard and waste problems they present.

References

High-Performance Liquid-Chromatographic Method Compared with a Modified Radioimmunoassay of Cotinine in Plasma

Sherry L. Perkins,¹ John F. Livesey,¹ Eduardo A. Escares,¹ Judy M. Belcher,² and Denis K. Dudley²

Cotinine is a sensitive and specific biochemical marker of exposure to cigarette smoke. We describe a simple solid-phase extraction of cotinine from plasma before quantification by HPLC. Extraction recovery was 97.9% ± 11.0% for plasma concentrations of 5-400 µg/L. Baseline separation of cotinine and caffeine was achieved within 11 min of injection onto a C18 reversed-phase column. The mobile phase was citric acid/dibasic potassium phosphate (30 mmol/L each, pH 6.0) containing 100 mL of acetonitrile per liter. Within-day and day-to-day precision (CV) were 4.7% and 8.4%, respectively. We also describe a modification of the Nicotine Metabolite RIA kit (Diagnostic Products Corp.) for quantifying cotinine in plasma. Recovery of cotinine from supplemented plasma was within 10% of the expected value with this RIA kit. Interassay precision averaged 8.1% for samples in the range 50-400 µg/L; intra-assay precision averaged 3.6% at 230 µg/L and 8.7% at 53 µg/L. Correlation between the two methods was RIA = 1.13 HPLC + 14.8 (n = 128, r = 0.957, P <0.001). Both methods are technically simple to perform.

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