Separation of Serum Prednisolone and Prednisolone-21-Hemisuccinate by Extraction and Their Concurrent Determination by Radioimmunoassay

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We describe a simple, sensitive, and reliable radioimmunoassay for prednisolone and prednisolone-21-hemisuccinate in serum. The antiserum produced in rabbits to prednisolone-21-hemisuccinate/bovine serum albumin was specific for prednisolone and prednisolone-21-hemisuccinate. A simple dichloromethane extraction permitted the separation of prednisolone from prednisolone-21-hemisuccinate in the serum samples. Interference by cortisol, although not insignificant, is minimized in this assay. We used the method to measure prednisolone and prednisolone-21-hemisuccinate concentrations after a bolus injection of prednisolone-21-hemisuccinate into human beings and mice.

Additional Keyphrases: steroids • biological half-life studies with mice

Since the advent of steroid radioimmunoassay technology (1), this method has been used to measure endogenous steroids as well as exogenously administered steroids.

Radioimmunoassays for synthetic steroid drugs such as prednisolone (2), dexamethasone (3), and betamethasone (4) have been developed recently. Intravenous administration of prednisolone-21-hemisuccinate is often used clinically because it is soluble in water; it is metabolized to the active form, prednisolone, in body tissues.

Here we report the simultaneous determination of prednisolone and prednisolone-21-hemisuccinate in serum by radioimmunoassay, after simple partition of prednisolone and prednisolone-21-hemisuccinate between dichloromethane and water.

Materials and Methods

Reagents and Standards

[6,7-³H]Prednisolone with a specific activity of 58 kCi/mol was purchased from New England Nuclear Co., Boston, MA 02118. Prednisolone and prednisolone-21-hemisuccinate were gifts from Shionogi Pharmaceutical Co., Osaka, Japan. Other steroids were purchased from Sigma Chemical Co., St. Louis, MO 63178, charcoal from Merck Co., Darmstadt, F.R.G., Dextran T-70 from Pharmacia Fine Chemicals Co., Uppsala, Sweden, and bovine gamma globulin and bovine serum albumin from Miles Laboratories, Inc., Kankakee, IL 60901.

Immunization of Animals

Prednisolone-21-hemisuccinate was conjugated to bovine serum albumin according to the method of Erlanger et al. (5). Four male New Zealand albino rabbits were immunized by multiple intradermal injections of the steroid/albumin conjugate suspended in complete Freund's adjuvant. Booster injections were given at four- to five-week intervals. Antiserum obtained three months after the initial immunization was used at a final dilution of 20 000 to 40 000.

Serum Preparation

Twenty or fifty microliters of serum was diluted to 5 mL with isotonic saline (NaCl, 9 g/L), 500 mL of which was extracted with 5 mL of dichloromethane. After centrifugation, 50 mL of the aqueous (upper) phase was used directly for prednisolone-21-hemisuccinate radioimmunoassay, and 500 mL of the lower phase was taken to dryness under air jet and was radioimmunoassayed for prednisolone.

Radioimmunoassay

Standard solutions containing 0.1–20 ng of prednisolone per milliliter of methanol or 0.1–20 ng of prednisolone-21-hemisuccinate per milliliter of saline were used. One hundred microliters of prednisolone standard solution was taken to dryness before radioimmunoassay. A 1 g/L solution of bovine gamma-globulin in saline was used to dilute antiserum and [³H]prednisolone. To samples and prednisolone or prednisolone-21-hemisuccinate standards, 500 mL containing 10 000 dpm of [³H]prednisolone was added, followed by 500 mL of antiserum diluted 10 000 to 20 000-fold. The assay tubes were incubated for 16 to 20 h at 4 °C. We used 200 mL of charcoal and dextran T-70 (5 g of each per liter) in saline to separate antibody bound from free steroid, and the bound fraction was decanted into the counting vial with 10 mL of Bray's solution (6) as fluorophor. The radioactivity was counted by use of a liquid-scintillation system. Logit transformation was used in calculating the results (7). The percent cross reactivities of various steroids with prednisolone antiserum were determined at 50% displacement of [³H]prednisolone.

Experimental Studies

Thirty-six male mice (about 20 g body weight) were administered 1 mg of prednisolone-21-hemisuccinate per kilogram body weight, intraperitoneally. Thereafter, mice were decapitated at appropriate times and serum obtained from them.

Human Studies

Three patients with cerebrovascular diseases each received a single muscular dose of prednisolone-21-hemisuccinate (20 mg), and serum samples were obtained from 5 min through
Table 1. Cross Reactions of Various Steroids with Prednisolone Antiserum

<table>
<thead>
<tr>
<th>Steroids</th>
<th>Cross reactivity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prednisolone</td>
<td>100.0</td>
</tr>
<tr>
<td>Prednisolone-21-hemisuccinate</td>
<td>58.7</td>
</tr>
<tr>
<td>Cortisol</td>
<td>15.0</td>
</tr>
<tr>
<td>Cortisone</td>
<td>8.4</td>
</tr>
<tr>
<td>17-Hydroxyprogesterone</td>
<td>7.5</td>
</tr>
<tr>
<td>Progesterone</td>
<td>1.2</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>0.6</td>
</tr>
<tr>
<td>Deoxycorticosterone</td>
<td>0.5</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>0.4</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.1</td>
</tr>
<tr>
<td>17-Hydroxy pregnenolone</td>
<td>0.0001</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

8 h thereafter. All sera were stored at -20 °C before radioimmunoassay.

Results

Analytical Variables

Specificity: Table 1 shows the cross reactivity of various steroids with prednisolone antiserum. Prednisolone-21-hemisuccinate was 58.7% as active as prednisolone in this assay, and endogenous cortisol showed 15% cross reactivity with this antiserum.

Standard curve: A typical standard curve is shown in Figure 1. The useful range of the prednisolone standard curve was established to be between 40 and 1000 pg, that of prednisolone-21-hemisuccinate between 100 and 2000 pg.

Recovery: The analytical recoveries of unlabeled prednisolone and prednisolone-21-hemisuccinate added to serum was estimated (n = 10) in the range of 100 to 1000 pg. Analytical recovery of prednisolone and prednisolone-21-hemisuccinate from serum was 100.0 (SD 8.5)% and 100.7 (SD 5.0)%, respectively.

Precision: Precision was assessed by repetitive duplicate assays. For the prednisolone assay, the within-assay CV was 7.3%, the between-assay CV 8.7%. For prednisolone-21-

Fig. 1. Standard curve for prednisolone (○) and prednisolone-21-hemisuccinate (●)

Fig. 2. Serum prednisolone (●) and prednisolone-21-hemisuccinate (●) concentrations after intraperitoneal administration of prednisolone-21-hemisuccinate (1 mg/kg body weight) to mice

hemisuccinate these values were 5.7% and 19.7%, respectively.

Animal Studies

Figure 2 shows serum prednisolone and prednisolone-21-hemisuccinate concentrations during a 4-h period after a single intraperitoneal administration of prednisolone-21-hemisuccinate (1 mg/kg body weight) to mice. The serum prednisolone-21-hemisuccinate concentration was the higher value, 126 (SD 32) µg/L, 5 min after the administration. The serum prednisolone concentration was highest at 30 min, 476 (SD 81) µg/L, and then decreased rapidly.

Human Studies

Figure 3 shows prednisolone and prednisolone-21-hemisuccinate concentrations in serum after intramuscular injection of 20 mg of prednisolone-21-hemisuccinate. Prednisolone-21-hemisuccinate in serum reached peak values (637 µg/L, SD 74 µg/L) 5 to 15 min after dosing, then declined rapidly. Serum prednisolone values were highest (288 µg/L,
SD 26 μg/L) 1 h after the dose, then declined gradually. We estimated the biological half-life for serum prednisolone-21-hemisuccinate and prednisolone to be about 45 and 180 min, respectively.

**Discussion**

In this simple, sensitive, and specific radioimmunoassay for prednisolone and prednisolone-21-hemisuccinate, the antisera raised against prednisolone-21-hemisuccinate/bovine serum albumin was specific for prednisolone and prednisolone-21-hemisuccinate. The difference in the solubility of prednisolone and prednisolone-21-hemisuccinate in either dichloromethane or water enables the two compounds to be easily separated in the same sample of serum.

The antibody cross reacted only 15% with cortisol. The high concentrations of prednisolone in serum after a therapeutic dose of prednisolone-21-hemisuccinate decrease the clinical significance of the cortisol cross reactivity. Moreover, administration of the drug results in a rapid decrease in circulating cortisol concentrations (8), further minimizing its interference.

There were marked differences between human and mice in serum prednisolone and prednisolone-21-hemisuccinate concentrations after administration of prednisolone-21-hemisuccinate. Prednisolone-21-hemisuccinate is converted to its biologically active metabolite, prednisolone, more slowly in the human than the mouse.

**References**