Specific Micro-Radioimmunoassay for Prednisolone in Serum

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Use of an 125I radioimmunoassay involving antisemur coupled to magnetizable cellulose, after prednisolone-21-phosphate interference is removed by dichloromethane extraction at pH 7.4 and endogenous glucocorticoid interference is removed by selective chemical derivatization with Girard's Reagent T [(carboxymethyl)trimethylammonium chloride hydrazide], allows determination of prednisolone in 10 μL of serum. Results correlate well with those of an established liquid-chromatographic method for separating prednisolone from its metabolites.

Additional Keyphrases: magnetizable cellulose · drug assay · steroids · gastrointestinal disease · pediatric chemistry

Prednisolone is one of the principal drugs used in treating inflammatory bowel diseases. Whether some poor responses to therapy are the result of its malabsorption has not been previously determined.

To facilitate individual therapeutic monitoring in children suffering from such diseases, we decided to assess the extent of prednisolone absorption by comparing, in the same subject, concentrations in plasma after an oral dose with those after intravenous administration of the same dose under similar conditions. The assay to be used for that purpose needed to satisfy two requirements. These were sensitivity, because the volume of each of the 24 samples collected from each child had to be as small as possible, and specificity, because any two main interferences were to be avoided: endogenous cortisol, possibly normal or increased in such patients, and injected water-soluble "pro-drug," prednisolone-21-hemisuccinate or -phosphate, which is converted into prednisolone in vivo (prednisolone itself cannot conveniently be administered by injection because alcohol is required to dissolve it). Existing assays for prednisolone do not meet both of these requirements.

Radioimmunoassay with [3H]prednisolone (1–6) can detect as little as 10 pg of the drug (1), but cross reactivity with cortisol either gives readings that are 10–40% higher if a chromatographic separation step is omitted before the assay (2) or produces blank values up to 80 μg/L (3, 5). Dexamethasone premedication has been used to suppress endogenous cortisol secretion before prednisolone studies (7, 8). In addition, when prednisolone ester is injected, an extraction step must be included in the assay to separate it from prednisolone, to avoid cross reactivity (6).

Exogenous and endogenous glucocorticoids can be determined simultaneously by using thin-layer chromatography with densitometry (9), "high-performance" liquid chromatography with ultraviolet detection (10–16), or gas chromatography with chemical ionization–mass spectrometry (17, 18). These methods demonstrate the possibility of normal or subnormal concentrations of cortisol being present after prednisolone administration, with the same range for both steroids, especially during the absorption phase (12, 15), and also for several hours after the administration, even in patients already so treated for several months (13, 19).

These results confirm earlier assays with fluorometric methods specific for endogenous glucocorticoids (20, 21); however, the sensitivity is 1000 pg for densitometry, 2000 pg or less for measurement of ultraviolet absorbance (13).

We therefore developed a radioimmunoassay for use after prednisolone-21-phosphate is removed by dichloromethane extraction at pH 7.4, and the endogenous steroids are selectively derivatized with Girard's Reagent T (17, 22, 23). This hydrazide reacts preferentially with the 3-keto group of steroids when they have a 1,2-double bond, thus destroying their immunoreactivity and hence their cross reactivity with the immunnoassay. Each determination requires duplicate 10-μL samples of serum.

Materials and Methods

Materials

Prednisolone, succinic anhydride, prednisolone-21-hemisuccinate, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl, N-hydroxyxysuccinimide, histamine, tyramine, and (carboxymethyl)trimethylammonium chloride hydrazide (Girard's Reagent T) were obtained from Sigma, Poole, Dorset, BH17 7NH, U.K.; 20β-hydroxyprednisolone and 20β-hydroxydiprednisone from The Steroid Reference Collection, Department of Chemistry, Westfield College, London, NW3, U.K.; pyridine, dimethylformamide, chloroform, methanol, Chloramine T, sodium metabisulfite, benzene, ethanol, acetic acid, dichloromethane, and Triton X-100 surfactant from BDH Chemicals, Poole, Dorset, BH12 4NN, U.K.; [6,7-3H]prednisolone, [1,2,6,7-3H]cortisone, and Na[125I] from Amershams International, Amersham, Bucks., HP7 9LL, U.K.; 6β-hydroxyprednisolone from Steraloids, Wilton, NH; keyhole limpet hemocyanin in 50% glycerol (product 374817) from Calbiochem-Behring, CP Laboratories, Bishop's Stortford, Herts., CM22 7RQ, U.K.; scintillation mixture (Pico Fluor 30) from Packard Instruments Ltd., Caversham, Berks., RG4 7AA, U.K.; scintillation vials (product no. 505) from Sterilin, Teddington, Middlesex, U.K.; silica gel thin-layer chromatography sheets (DC-Alufolien Kiesegel 60F254 art. 5554) from E. Merck, Darmstadt, P.R.G.; 2-mL polypyrrole conical vials (72/708) from Sarstedt, Beaumont Leys, Leicester, LE4 1AW, U.K.; 1-mL graduated polyethylene Pasteur pipettes (Pastettes, LW 4040) from Alpha Labortories, Eastleigh, Hampshire, S05 4NU, U.K.; Pyrex borosilicate 10 × 75 mm tubes (cat. no. 99445) from Corning, Stone, Staffordshire, ST15 0BQ, U.K.; the vibrator for assay incubation (IKa Vibraf 1) from Sartorius Instruments, Belmont, Surrey, U.K.; the gamma counter (NE 1600) from Nuclear Enterprises, Beenham, Reading, RG7 5PR, U.K.; the beta counter (Pras) from Packard Instruments Ltd.; and the multipolar ferrite block magnets from Magnet Applications, London, EC1, U.K.

Procedures

Preparation of immunogen. Prepare [3H]prednisolone-21-hemisuccinate by mixing 20 μCi of [3H]prednisolone (previously dried under nitrogen) with 2 mg of prednisolone, 2 mg

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of succinic anhydride, and 200 μL of dry pyridine, and refluxing for 5 h in an oil bath at 130 °C. After evaporation under nitrogen add 37 mg of nonradioabeled prednisolone-21-hemisuccinate.

Conjugate prednisolone-21-hemisuccinate to the hemocyanin by activating the preceding final mixture with 20 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide· HCl and 11.5 mg of N-hydroxysuccinimide in 3.5 mL of dimethylformamide, stirring for 1 h, then adding the mixture dropwise to 100 mg of the hemocyanin in 4 mL of water. After overnight stirring, dilute to 20 mL with distilled water. Isolate the conjugate by dialysis against running water for 48 h and lyophilization of the dialysate. Store frozen (-20 °C). Counting tritium radioactivity before and after dialysis showed that we had incorporated 100 nmol of prednisolone per milligram of immunogen.

Immunization. Six New Zealand White rabbits were injected subcutaneously each with 1 mg of immunogen in 0.5 mL of NaCl solution (9 g/L) plus 1 mL of complete Freund’s adjuvant, divided between four sites. Subsequent booster injections with half the initial amount of immunogen were given every month, blood was sampled 12 days later, and the sera were frozen. We used antisera from the fifth sampling of one rabbit for the assay.

Coupling of antisera to magnetizable cellulose. We followed the method of Pourfarzaneh et al. (24), using a ratio of 200 μL of antisera to 1 g of solid phase.

Preparation of 125I tracer. To prepare prednisolone-21-hemisuccinate-histamine, incubate 5 mg of prednisolone-21-hemisuccinate for 2.5 h with 1.5 mg of N-hydroxysuccinimide and 4.5 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide· HCl in 500 μL of dimethylformamide. After adding 2 mg of histamine, let the mixture stand for 2.5 h, then chromatograph it on a silica gel plate in chloroform/methanol (1:1 by vol). Locate the band with Rf = 0.50 by its quenching of fluorescence, elute it into ethanol, dry under nitrogen, re-dissolve to 1 mg/mL in ethanol, and store the solution in a freezer.

To radiiodiniate, dilute the thawed histamine conjugate 20-fold with 250 mmol/L sodium phosphate buffer, pH 7.5. To 20 μL of this add 10 μL (1 mCi) of Na125I and 10 μL of a 5 mg/mL solution of Chloramine T, freshly diluted in the same buffer. After 2 min, add 10 μL of a 30 mg/mL solution of sodium metabisulphite in the same buffer. After chromatography on silica gel in benzene/ethanol/acetic acid (75/24/1 by vol), locate the radioactivity by exposing the gel on roentgenogram film for 2 min. Then elute the major fraction into ethanol, and store the solution at 4 °C until used in the assay. This entire iodination procedure can be completed within 2 h.

Extraction of serum samples. Dispense 10 μL of serum sample or prednisolone standard in human serum into conical vials containing 30 μL of sodium phosphate buffer (200 mmol/L, pH 7.4). Add 100 μL of the same buffer and 1200 μL of dichloromethane. Cap the vials, shake them for 5 min, then centrifuge (5 min, 2000 rpm). Using a Pasteur pipette, transfer 1000 μL of the lower phase into a Pyrex tube and evaporate it under a stream of nitrogen in a water bath at 35–40 °C.

Selective derivatization with Girard’s Reagent T. Add 100 μL of Girard’s Reagent T (100 g/L, in acetic acid/methanol, 1/1000 by vol) to each tube, incubate for 6 h, then evaporate under nitrogen at room temperature.

Radioimmunoassay. Before use, dilute the tracer in sodium phosphate buffer (100 mmol/L, pH 6.5) containing 1 mL of Triton X-100 per liter to give 25 000 counts in 90 s when prepared with fresh 125I. Add to each sample or standard 100 μL of diluted tracer and 100 μL of antisera in the same buffer, keeping the suspension homogeneous while dispensing it (1.56 mg of solid phase per tube). Incubate for 1 h on a vibrator, sediment the suspension on a magnet, wash with 1000 μL of buffer, and re-sediment. Aspirate the supernate, and count the radioactivity of the precipitate.

Results
Stability of 125I tracer. Nearly superimposable standard curves were obtained for as long as four months after preparation of tracer that was kept stored at 4 °C in ethanol, but the dilution in buffer must be done just before assay to avoid loss of immunoreactivity.

Serum effect. A standard curve prepared from standards in a matrix of charcoal-stripped pooled human serum with normal concentrations of protein and <10 μg of cortisol (by RIA) per liter was shifted to the left of a simultaneously prepared curve in which the matrix was pH 6.5 buffer without any serum. Both curves were superimposable if standards were processed by extraction.

Specificity. Table 1 shows the cross reactivities calculated by comparing standard curves for prednisolone or related steroids in pH 6.5 buffer (25), without processing.

Figures 1 and 2, left, compare standard curves for unprocessed (unextracted and underivatized) prednisolone in pH 6.5 buffer, without and with a constant amount of cortisol or prednisolone-21-phosphate exceeding the highest concentration to be expected. Similar standard curves, with 10 μL of stripped serum and processed, are identical (Figures 1 and 2, right). If saline (NaCl, 9 g/L) was used instead of pH 7.4 buffer as the aqueous phase for extraction, the ester interference was not removed.

Several concentrations of Girard’s Reagent T and acetic acid in methanol were tested with 10 μL of serum containing two extreme concentrations of prednisolone or cortisol and traces of [3H]prednisolone or cortisol. Derivatization was assessed by subsequent loss of extractability into 1.2 mL of dichloromethane, the hydrazine being more polar than the parent steroid. Figure 3 shows the most selective combination: 100 g/L of Girard’s Reagent T, in acetic acid/methanol, 1/1000 by volume. Lower concentrations of Girard’s Reagent T had an incomplete effect on cortisol; higher acid concentrations produced an increasingly rapid derivatization of prednisolone. The absence of interference of endogenous steroids was further confirmed by the identity of standard curves prepared with prednisolone in stripped serum and normal human serum containing 162 μg of cortisol per liter, after processing, and by the zero readings with serum from normal subjects collected in the morning, and from patients with above-normal serum cortisol (see below).

Precision and sensitivity. The within-batch coefficient of variation, for four duplicates scattered through a run of samples, was optimum between 500 and 3500 pg per tube (50 and 350 μg/L): 2.1 to 12.9%, mean 8.8% (15 specimens from normal subjects and children with inflammatory bowel disease). Below and above this prednisolone range (from 25

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to 50 and 350 to 750 μg/L), imprecision was higher: 7.6 to 25.7% (mean 17.8%) for five specimens and 3.6 to 30.2% (mean 12.1%) for 14 specimens, respectively.

The between-batch CV, with each specimen being run as duplicates in four or five batches, was 8.9 to 17.1% (mean 14.1%) for eight specimens between 66.5 and 201 μg/L.

Most samples fall within the optimum range, but some have to be diluted (see below).

The lowest detectable amount in stripped serum was 100 pg per tube: a paired t-test with standard 0 on the same batch (12 batches) gave 0.05 < p < 0.10. Twenty-three specimens containing increased cortisol (219 to 2385 μg/L) from patients with adrenal hyperplasia or who were under the stress of thoracic surgery gave blank readings for prednisolone.

Accuracy. Accuracy was studied by use of the same specimens used to assess precision. Five specimens with prednisolone concentrations from 333 to 684 μg/L, diluted threefold with sodium phosphate buffer (200 mmol/L, pH 7.4) gave an analytical recovery of 94 to 112% (mean 103%). Further studies with specimens either undiluted or diluted threefold, showed no significant differences in recovery.

Two specimens with initial concentrations of 213 and 234 μg/L, after addition of 100 μg of prednisolone per liter, showed 88 and 107% recovery of the added steroid, respectively; after addition of 250 μg/L, analytical recovery was 90 and 84%, respectively, where

\[
\text{recovery} = \frac{\text{concentration found}}{\text{concentration expected}} \times 100
\]

Results were the same for specimens collected in plain or heparin lithium tubes, from healthy subjects and patients.

In vitro stability of prednisolone-21-phosphate. After infusion of prednisolone-21-phosphate into a normal subject, specimens collected at 2 h and 4 h 20 min and extracted with dichloromethane 1.5, 6, and 27 h later gave readings unaffected by the time until extraction.
Correlation with liquid chromatography (13). For 19 specimens, four of which were collected 5 to 7 h after prednisolone ingestion from patients with renal insufficiency (creatinine clearance 4 to 52 ml/min), chromatographic values (x) for prednisolone ranged from 0 to 4356 μg/L, with a prednisolone/prednisolone ratio of 0.06 to 0.57. The equation of the regression line of y (the RIA value) on x was: y = 1.04x + 10; the correlation coefficient (r) was 0.96.

Excluding three prednisolone blanks, found to be zero by both methods, the values were: y = 1.02x + 17 (r = 0.95).

Discussion
To attain the sensitivity required, we chose an RIA rather than liquid chromatography with ultraviolet absorbance detection. The antiserum dilution we used gives sub-maximum binding of the tracer; we made no attempt to further increase sensitivity. The use of 125I tracer and magnetizable solid-phase separation results in a quicker and cheaper RIA.

Although 125I has a shorter radioactive half-life than 3H, we found that the 125I tracer could be used for at least four months after preparation. In contrast to 3H, 125I can be counted in a solid phase, thus avoiding the additional steps involved in liquid scintillation counting. The use of a 16-head gamma counter (as opposed to a single-head beta counter) greatly decreases the overall counting time per batch. The cost of scintillation cocktail far exceeds that of any other assay component, and even a 16-head gamma counter is less expensive than the simplest single-head beta counter.

We believe these advantages more than compensate for the need to prepare the 125I tracer in specialized facilities. Samples must be treated as described before RIA to ensure specificity. Sixty specimens are processed in a typical assay batch. The above-cited liquid-chromatographic methods require 10 to 20 min of column-development time per specimen, and complex equipment; for Lipidex chromatography, this time exceeds 5 h; for thin-layer chromatography, the whole batch is eluted simultaneously over 24 h (2) or 15 min (9), but applying samples on plates is tedious unless this step is automated.

The specificity problems are similar to those encountered in previous prednisolone RIAs in which prednisolone-21-hemisuccinate-bovine serum albumin is used as the immunogen and [6,7-3H]prednisolone as the tracer (1–6). The antiserum of lowest reported cross reactivity with cortisol (4%) was raised in sheep (26) and not in rabbits, as in other cases; its use in an RIA after extraction with ethyl acetate gives a satisfactory correlation with results from a liquid-chromatography method for volunteers given a high oral dose of prednisolone (15).

Cross-reactivity problems related to the double bond are also known for cortisol antiserum with respect to prednisolone (27), and for testosterone antiserum with respect to 5α-dihydrotestosterone (28, 29), whatever the site of conjugation of hapten to protein. Fractionation of antibodies to testosterone improves affinity but not specificity (29); this prompted us not to attempt this.

Cross reactivity with prednisone is clinically less important, because the concentration of this metabolite in the circulating blood is usually less than a third of that of prednisolone (2, 5, 10, 12–15). Cross reactivity with other prednisolone metabolites is even less important. After injection of [3H]prednisolone into subjects with normal renal function, more than 90% of the counts extracted into dichloromethane were from unchanged prednisolone up to the fifth hour in one study (30), but in another (31), 30% of the counts corresponded to neither prednisolone nor prednisone at 6 h. Concentrations of prednisolone metabolites in serum are probably higher in cases of renal insufficiency, but no data are available. Among prednisolone metabolites, after 14C]prednisolone administration, 20β-hydroxyprednisolone has been isolated from the unconjugated fraction in urine in only 0.15 of the amount of the parent steroid (32). The correlation we obtained with the chromatographic method demonstrates that interference from prednisolone metabolites in our assay is of little importance.

An alternative procedure, to obtain better sensitivity and specificity, could be dansylhydrazine derivatization of prednisolone at the 3 position, followed by liquid chromatography with fluorescence detection (33, 34). As little as 200 pg can be detected (33).

Our assay has now been used for prednisolone-absorption studies in several children with inflammatory bowel disease (soon to be reported), where its specificity combined with lower serum sample volume requirements are of particular value.

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References


