Radioimmunoassay of Colchicine

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Antibodies to colchicine were raised in rabbits after injection of *N*-desacetylthiocolchicine conjugated to bovine serum albumin. Antisera diluted 1:100 bound 50% of 1.6 ng of [³H]colchicine. Cross-reaction of the antisera with colchicine derivatives and other drugs was measured and a radioimmunoassay of colchicine in biological fluids was proposed.

Colchicine has been used for more than 150 years in the treatment of acute gouty arthritis. Owing to the great toxicity of this drug the daily doses are low (1 or 2 mg/day). As a result, measuring the quantity of colchicine in biological fluids has always been tedious and unreliable (Kovatsis & Heyndricks, 1969; Wallace *et al.*, 1970).

In addition to its uses in the measurement of protein hormones, radioimmunoassay is finding increasing use in the assay of smaller organic molecules such as steroids, digitalis glycosides (Oliver *et al.*, 1968; Smith *et al.*, 1969), cyclic AMP (Steiner *et al.*, 1969), morphine (Spector & Parker, 1970) and barbiturates (Flynn & Spector, 1972). These developments prompted us to devise a radioimmunoassay for colchicine that was capable of measuring this drug at very low concentrations.

Fig. 1 shows the formulae of colchicine and its derivatives.

Experimental

Materials

Bovine serum albumin was obtained from Miles Laboratories Inc., Elkhart, Ind., U.S.A. 1-Ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride and colchicine were obtained from Merck, Darmstadt, Germany. *N*-Desacetylthiocolchicine hydrochloride was a gift from Roussel-Uclaf Laboratories, Paris, France. [³H]Colchicine (5 Ci/mmol) was purchased from New England Nuclear Corp., Boston, Mass., U.S.A. Freund's complete adjuvant was obtained from BD-Merieux, Marcy l'Etoile, Charbonnières, France. Sheep anti-(rabbit γ -globulin) serum was obtained from the Institut Pasteur, Paris, France. Instagel was obtained from Packard Instrument Co., Downers Grove, Ill., U.S.A.

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Fig. 1. Colchicine and related derivatives

Compounds	R'	R''	R'''
Colchicine	-NH-CO-CH ₃	-O-CH ₃	-O-CH ₃
<i>N</i> -Desacetylthiocolchicine	-NH ₂	-S-CH ₃	-O-CH ₃
<i>N</i> -Desacetyl- <i>N</i> -methylcolchicine	-NH-CH ₃	-O-CH ₃	-O-CH ₃
Colchicine	-NH-CO-CH ₃	-OH	-O-CH ₃
<i>N</i> -Desacetylcolchicine	-NH ₂	-OH	-O-CH ₃
Colchicoside	-NH-CO-CH ₃	-O-CH ₃	-O-Glc
Thiocolchicoside	-NH-CO-CH ₃	-S-CH ₃	-O-Glc
<i>N</i> -Desacetylthiocolchicine-protein conjugate	-NH-CO-Glu or -NH-CO-Asp	-S-CH ₃	-O-CH ₃

Preparation of *N*-desacetylthiocolchicine-protein conjugate

As colchicine does not possess a suitably reactive chemical group for the preparation of a hapten we have used *N*-desacetylthiocolchicine, a derivative of colchicine prepared by Velluz & Muller (1954). This compound possesses a free amino group, which can react with the carboxyl groups of the dicarboxylic acids of bovine serum albumin, giving an amide bond. *N*-desacetylthiocolchicine was conjugated to bovine serum albumin by the carbodi-imide method described by Goodfriend *et al.* (1964).

Bovine serum albumin (40mg) and the hapten desacetylthiocolchicine hydrochloride (30mg) were suspended in 4ml of 0.15M-NaCl. The reaction was carried out, with constant stirring, by the dropwise addition of 500mg of 1-ethyl-3-(3-dimethylamino-propyl)carbodi-imide hydrochloride dissolved in 2ml of 0.15M-NaCl. The conjugate was purified by dialysis in Visking tubes [Union Carbide (UK) Ltd., London, W.1, U.K.] over 5 days at 4°C, against distilled water (with daily changes) and freeze-dried.

Analysis of the conjugate

A portion (1mg) of the conjugate was applied to a column (30cm×0.9cm internal diam.) of Sephadex G-25 equilibrated with 0.15M-NaCl and eluted by the same solution with a flow rate of 0.4ml/min. The effluent was monitored by its absorption at 280nm. The first peak corresponded to the *N*-desacetylthiocolchicine-bovine serum albumin conjugate, the second to free *N*-desacetylthiocolchicine. Spectrophotometric measurements at 375nm and determination of the protein content by the method of Lowry *et al.* (1951) indicated a ratio of 7mol of *N*-desacetylthiocolchicine/mol of bovine serum albumin (assuming a molecular weight of 70000 for the protein) in the conjugate isolated after gel filtration (peak I). The free *N*-desacetylthiocolchicine in the crude conjugate represented less than 5% of the total *N*-desacetylthiocolchicine present in the antigen.

Immunization procedure

The freeze-dried hapten-protein conjugate (2mg) was dissolved in 0.5ml of 0.15M-NaCl, emulsified in an equal volume of Freund's complete adjuvant and injected subcutaneously into four or five different sites on the back and sides of each of seven New Zealand albino rabbits, each weighing about 2 kg. Booster injections of 2mg of conjugate were administered every 15 days for a total of four series of injections, then subsequently each month. Blood was collected by ear venipuncture or cardiac puncture before the immunization and 12 to 15 days after each injection. After clotting, serum was separated by centrifugation at 600g for 5 min at 20°C and stored at -20°C.

Detection of antibodies and radioimmunoassays

Assays were performed with semi-automatic pipettes in polystyrene disposable tubes. 0.15M-NaCl-0.02M-phosphate buffer, pH 7.0, containing 0.1% NaN₃ was used as diluent in all assays.

The ability of the sera from immunized rabbits to bind [³H]colchicine was demonstrated by using the following system: 100μl of serum diluted 1:10 in

diluent buffer, 20μl of [³H]colchicine (20nCi, equivalent to 4pmol, or 1.6ng) and 1ml of diluent buffer. The mixture was incubated for 18 h at 4°C in the dark. Free colchicine was separated from antibody-bound colchicine by the double-antibody method (Morgan & Lazarow, 1962) by the addition of 10μl of non-immunized rabbit serum and 0.1 ml of a convenient dilution of sheep anti-(rabbit γ-globulin) serum. After 6h at 4°C, the precipitate separated by centrifugation at 2000g for 10min at 4°C was resuspended in 0.7ml of 0.15M-NaCl and poured into counting vials containing 10ml of Instagel. Radioactivity was counted in a liquid-scintillation counter (Intertechnique SL 30).

For radioimmunoassay the same protocol was used except that 0.5ml of diluent buffer and 0.5ml of sample or standard were used in place of 1ml of buffer. The final antiserum dilution was chosen so that 40% of the total radioactivity was bound. This result was generally obtained with a 1:100 final dilution. A standard curve was obtained by using normal serum or normal urine to which were added various known amounts of pure colchicine. The radioactivity precipitated (d.p.m.) is plotted against the weight of added unlabelled colchicine per tube (on a log scale) (Fig. 2).

Determination of specificity

Various amounts of colchicine derivatives and other substances were added to incubation tubes in place of non-radioactive colchicine and the bound radioactive colchicine measured as described above. The cross-reactivity was then expressed as $(x/y) \times 100$ where *x* is the weight of colchicine and *y* the weight of cross-reacting substance required to decrease by 50% the degree of binding of [³H]colchicine to the antiserum.

Results

After 2 months of immunization, three rabbits produced antibodies binding 30–50% of the introduced [³H]colchicine at 1:100 final dilution of serum. At the same dilution, the serum of the rabbits before immunization bound only 3–5%. The standard curve shown in Fig. 2 is a d.p.m.-dose plot and has a linear portion in the range 5–50ng of colchicine. Any value more than two standard deviations from the mean was considered significant.

Only *N*-desacetylthiocolchicine, *N*-desacetyl-*N*-methylcolchicine and colchicine cross-reacted, by 8, 8 and 2.5% respectively. The following compounds showed negligible cross-reactivity: *N*-desacetylcolchicine, colchicoside, thiocolchicoside, tropolone, podophyllotoxine, trimethoprim, papaverine and 3,4-dimethoxyphenethylamine.

Fig. 2. Standard curve for the colchicine radioimmunoassay

Each point is the mean of the two values joined by the bar. For details, see the text.

Discussion

It was surprising that *N*-desacetylthiocolchicine did not cause greater displacement than colchicine. This might be explained by the presence of an amide bond in both colchicine and the *N*-desacetylthiocolchicine-protein complex but not in *N*-desacetylthiocolchicine itself.

As colchicine is apparently not metabolized in the body (Walaszek *et al.*, 1960), radioimmunoassay can be applied to a colchicine metabolism study.

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