

QUANTITATIVE DETERMINATION OF RICININE IN VARIOUS SAMPLES OF MEAL BY AN IMMUNOMETRIC ENZYMATIC METHOD USING AFFINITY COLUMNS.

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A method has been developed for the quantitative estimation of ricinine in extracts and meal from two castor-oil plant seed testers.

The high specificity of the antigen-antibody interaction and the sensitivity of this reaction to slight structural changes in its participants are responsible for the broad possibilities of the immunochemical approach to this problem

Heterogeneous enzyme-linked immunoassay (EIA) with a definite enzyme label in solution is, as a rule, assigned to the methods of immunometric analysis in which the role of the component detected quantitatively is played by labeled antibodies.

J. W. Freytag et al. [1] have described a variant of immunometric analysis using affinity columns, which combines the advantages of the noncompetitive principle of sandwich analysis with the demand for the presence in the antigen of only one binding center, as in the one-center variant. In the method under consideration, an excess of enzyme-labeled monovalent antibodies is incubated with a sample containing the antigen to be tested. Even at a very low concentration of antigen, the use of a large excess of antibodies leads to the situation that the antigen is bound rapidly and completely. The excess of labeled antibodies not bound with antigen is eliminated from the mixture by passing it through a column with an affinity sorbent.

The labeled antibodies-antigen complex does not bind with the sorbent. The activity of the enzyme label, which is proportional to the initial concentration of antigen in the sample, is measured in the eluate obtained in this way, which contains no free antibodies. Furthermore, the use of an enzyme label in this method is extremely favorable, since, to obtain a sufficiently strong signal it is possible where necessary to use prolonged times of incubation with the substrate. The sensitivity of this method is limited only by the sensitivity of the system for detecting the label. It can be used in equal measure for testing both small and large antigens [2, 3].

Horseradish peroxidase from the firm Reanal was selected as label. The enzyme was purified by a known method [4].

An immunoglobulins IgG-peroxidase conjugate was obtained by a method based on the oxidation of the carbohydrate groups of the enzyme with sodium metaperiodate followed by the formation of a covalent bond with the NH_2 groups of the immunoglobulins IgG molecules. The conjugate was separated from the nonbound enzyme by gel filtration on a column of Sephadex. The first peak corresponded to molecules of a different composition, and the second to the unchanged peroxidase. The concentration of the conjugate was calculated from its peroxidase content. It was deduced from the results of UV spectrometry that 22-25 molecules of hapten were conjugated with one protein molecule.

The demands placed upon the matrix for the preparation of affinity columns [5] are practically identical with the demands on the material of the immunosorbents in other methods of heterogeneous EIA. It is possible to use any insoluble supports similar to those used in column chromatography: agarose, various dextrans, porous and nonporous silica gels, polyacrylamide, etc. Since the activity of the enzyme in the method using affinity columns is determined in the fraction not binding with the sorbent, the absence of nonspecific sorption is not such an important parameter as in solid-phase methods.

As the matrix for the affinity sorbent we used aminoethyl-Sepharose which we synthesized from BrCN-activated Sepharose 4B.

The affinity sorbent was obtained by immobilizing the hapten bovine serum albumin (BSA)-acid (1) on the matrix.

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Extracts from meal and oil-cake were incubated with peroxidase-labeled immunoglobulins and were deposited on a column of the affinity sorbent. The optical density of the solution was measured on a SF-16 spectrophotometer at 530 nm in cells with a layer thickness of 1 cm. The control cell contained no ricinine.

The Lambert–Bouguer–Beer law was satisfied in the range of 0.1–0 mg, a regular relationship being observed between the amount of ricinine and the optical density of the solution. The relative error of the method was 5–7%.

Thus, conditions have been worked out for obtaining an affinity sorbent and a procedure has been proposed for the quantitative determination of ricinine. It was established that the amount of ricinine in an acetone powder was 0, in oil-cake 126, and in toaster meal 218 mg-%.

EXPERIMENTAL

Preparation of the Immunogen. Ricinine was hydrolyzed in a water bath with a 5% solution of caustic soda for 20 min. This gave 4-hydroxy-1-methyl-2-oxo-1,2-dihydronicotinonitrile, mp 286–287°C, mol. mass M^+ 150.

The hydrolysate (0.5 g) was dissolved in 5 ml of 4% KOH, 50 ml of absolute benzene was added, and the mixture was evaporated to dryness in a rotary evaporator. The dry residue was treated with 25 ml of absolute alcohol and 0.5 g of sodium monochloroacetate. The resulting mixture was boiled under reflux for 3 h, cooled to room temperature, and evaporated to dryness. The dry residue consisted of 3-cyano-1-methyl-2-oxo-1,2-dihydro-4-pyridoxacetic acid (acid (1)). A solution of BSA (0.06 g) in 5 ml of 0.1 M NaHCO_3 (pH 8.3) was treated with an equal volume of a 2% solution of acid (1) in 0.1 M NaHCO_3 containing 5 M NaCl and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride. The reaction mixture was stirred in the cold for a day. The hapten formed was dialyzed against 0.01 M buffered physiological solution with pH 7.2. Immunoglobulins IgG from rabbit blood serum were obtained by a method described previously [7].

Preparation of the Conjugate. Peroxidase purified on CM-cellulose was oxidized with 0.02 M sodium metaperiodate at room temperature for 30 min. After the addition of a 0.32 M aqueous solution of ethylene glycol, the reaction mixture was left for 1 h and was then dialyzed against 0.01 M sodium carbonate buffer with pH 9.5.

To 5 mg of IgG was added an equal amount of activated peroxidase, the pH of the solution was brought to 9.5 with the aid of 0.2 M Na_2CO_3 , the mixture was stirred at room temperature for 2 h, and, after the addition of sodium tetrahydroborate to a final concentration of 0.3 mg/ml, it was incubated at the same temperature for 1 h. Then it was dialyzed against buffered physiological solution (pH 7.2) at 4°C overnight. After gel filtration on a column (1.5 × 100 cm) of Sephadex G-200, fractions with a volume of 3 ml were collected and their protein contents were determined spectrophotometrically at 280 and 403 nm. Fractions for which the E_{403}/E_{280} ratio was between 0.1 and 0.4 were combined.

Peroxidase activity was determined spectrophotometrically from the absorption of the product of the oxidation of *ortho*-dianisidine [6].

Preparation of the Affinity Sorbent. To BrCN-activated Sepharose 4B was added an equal volume of distilled water, cooled to 4°C, containing 0.002 mole of ethylenediamine to each milliliter of Sepharose, and the pH was brought to 10 with the aid of 6 N HCl. After 16 h at 4°C, the gel was washed with a large volume of distilled water. Then 10 ml of a solution of the immunogen BSA-acid (1) in 0.01 M of PBS (pH 7.2) and 100 mg of carbodiimide in the same buffer were added, and the mixture was stirred for 3 h. The gel was transferred to a porous glass filter and was washed with 0.1 M NaHCO_3 containing 0.5 M NaCl (pH 8.3). A suspension of the prepared sorbent was transferred to a column and was equilibrated with 0.01 M PBS, pH 5.8.

Quantitative Determination of Ricinine. A 100-g sample of oil-cake (meal) was heated on the water bath and was extracted first with 70% alcohol and then with distilled water for 3 h with constant stirring. The extracts were centrifuged at 6000 rpm for 20 min. The supernatant liquid from the alcoholic and aqueous extracts were combined and evaporated in a rotary evaporator. The residue was dissolved in 30 ml of PBS (pH 5.8).

An aliquot of the sample (10 ml) was incubated with 0.1 ml of a solution of labeled antibodies at 30°C for 30 min and was then deposited on the column containing the affinity sorbent. Elution was performed with PBS (pH 5.8) at a rate of 12 ml/h. The free volume of the column was 15 ml, and elution was performed with 1.5 free volumes, 2-ml fractions being collected. Optical densities were measured on a spectrophotometer at 530 nm (A_1 value).

The eluates were each treated with 32 ml of substrate mixture and 0.2 ml of 0.03% H_2O_2 . The resulting mixtures were incubated at 30°C for 30 min, and, after the treatment of each with 5 ml of 50% sulfuric acid, their optical densities were

measured at 530 nm (A_2 value). The difference $A_2 - A_1$ corresponds to the optical density on the calibration curve for pure ricinine.

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