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RADIOIMMUNOASSAY OF DIGITONIN

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We describe a radioimmunoassay for digitonin which utilizes the ability of digotonin to compete with ^{125}I-labeled digoxigenin for binding to anti-digoxin antiserum. As performed using the Gammaflo automated radioimmunoassay system and commercially available reagents, the assay can detect as little as 20 μg/ml (15 μM) digitonin. The assay is insensitive to interference by cholesterol or other cell membrane constituents and is useable above and below the critical micelle concentration of digitonin. It should be useful for the monitoring of digitonin concentrations in solubilized biochemical preparations.

Introduction

Digitonin is widely used for the solubilization of membrane proteins, particularly in studies of mitochondria [1], visual pigment [2], and hormone receptors [3,4]. Chemical assays for digitonin, however, are neither simple, direct, nor sensitive, and usually include tedious extraction and chromatographic separation. In order to monitor the removal of digitonin from biological samples, we found that its structural similarity to digoxin (Fig. 1) enabled the development of a radioimmunoassay based upon crossreactivity of the detergent with the binding of a ^{125}I-labeled digoxigenin derivative to digoxin-specific antibodies.

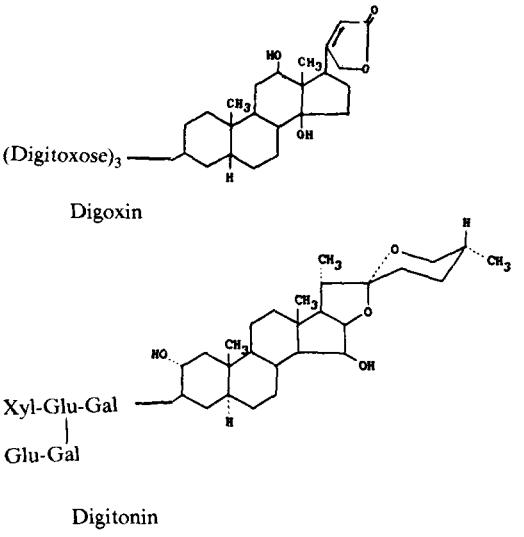

Fig. 1. Structural formulae of digoxin and digitonin. The immunologic determinants of relevance are on the aglycone moiety.

Methods and Materials

All reagents used in the radioimmunoassay procedure, with the exception of the buffer, were part

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of the digoxin assay kits supplied by E.R. Squibb and Sons, Inc., for use with the Gammaflo™ automated RIA system. Digitonin (lot 19C-0328), digoxin, and Brij 35 were obtained from Sigma Chemical Co., and [^{3}H]digitoxin from New England Nuclear Co.. Rat erythrocyte plasma membranes were prepared and extracted according to the method of Fleming and Ross [5]. The critical micelle concentration of digitonin was determined according to Schrock and Gennis [6] using 1,6-diphenyl-1,3,5-hexatriene.

Both digitonin and digitoxin are products of *Digitalis purpurea*. To insure that immunologic crossreactivity was due to the digitonin itself, a sample of dry digitonin was washed three times with twenty volumes of chloroform to remove any contaminating digitoxin which might crossreact with the antidigoxin antibodies. A tracer quantity (10^6 cpm) of [^{3}H]digitoxin was dried onto the digitonin powder prior to extraction to monitor removal of cardiac glycosides. At least 97% of the [^{3}H]digitoxin was removed in this procedure.

The Gammaflo™ automated radioimmunoassay system has been described previously [7]. It automatically mixes sample, iodinated antigen, and antiserum in appropriate proportion, incubates the mixture for a fixed interval, and then separates antigen-antibody complex from unbound antigen on an ion exchange column. Bound radioactivity is then counted in a self-contained gamma counter. The procedure used for radioimmunoassay of digitonin followed the procedure suggested for the radioimmunoassay of cyclic AMP [7] with two exceptions. Serum albumin was omitted from the buffers, and 0.5 M NaCl was routinely added to the buffers. The salt slightly (30%) increases the total amount of ligand bound and suppresses any variability of binding caused by different ionic strengths in the samples. Data are expressed as the fraction of ^{125}I-labeled digoxigenin bound in the presence of sample relative to the maximal amount bound (B/B_0) in the absence of competing ligand.

Results

Using the procedures described above, digoxin typically competes half-maximally with ^{125}I-labeled digoxigenin at a concentration of about 7 nM (Fig. 2). Digitonin competes with much lower but

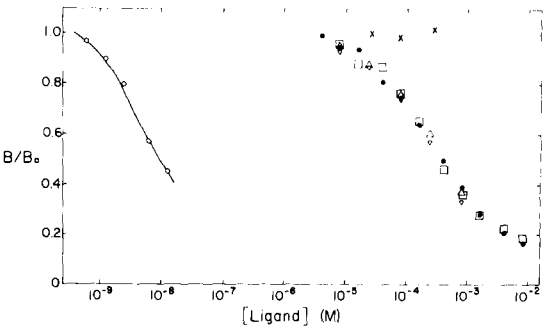

Fig. 2. Competitive radioimmunoassay of digoxin and digitonin. The assay was performed as described in Methods and Materials. The samples which were assayed are: ○, digoxin; □, commercial digitonin; ●, $CHCl_3$-washed digitonin; △, washed digitonin in a sonicated suspension of 1 mM dimyristoylphosphatidylcholine; ▽, washed digitonin in a 1 mg/ml solution of Brij 35; ×, cholesterol in a 1 mg/ml Brij 35 solution. Neither Brij 35 nor phosphatidylcholine altered B_0. 'Non-specific binding', i.e., that which is not competed for either by digoxin or digitonin, is typically 15%. Non-specific binding is not subtracted from the data shown.

still considerable affinity, displacing the iodinated ligand half-maximally at a concentration of 300 μM. It is likely that this displacement reflects true immunologic crossreactivity by the following criteria. Displacement occurs over a roughly 100-fold range of concentrations, as is consistent with non-cooperative binding to a single set of sites. The displacement of radioactive ligand does not reflect the surfactant properties of digitonin, since the displacement curve crosses the critical micelle concentration (0.2 mM) without a discontinuity and the entire Gammaflo system operates in a buffer containing 0.15 mg/ml Brij 35 [7]. Furthermore, the assay of digitonin is not altered by the addition of 0.1% Brij 35 (Fig. 2) or 0.1% Lubrol 12A9 (not shown) to the sample. The assay is thus not sensitive to added detergent or to phospholipid (Fig. 2), nor is it affected by variation of ionic strength of the sample (0 to 0.5 M NaCl). Variation between replicate samples is less than 10%, and variation in total binding of the iodinated ligand is less than 15% over several weeks. Sucrose in the sample increases the variation among replicate determinations, presumably by increasing the viscosity of the sample and thereby interfering with the mixing or the quantitatively metered flow. Therefore, triplicate determination of digitonin in

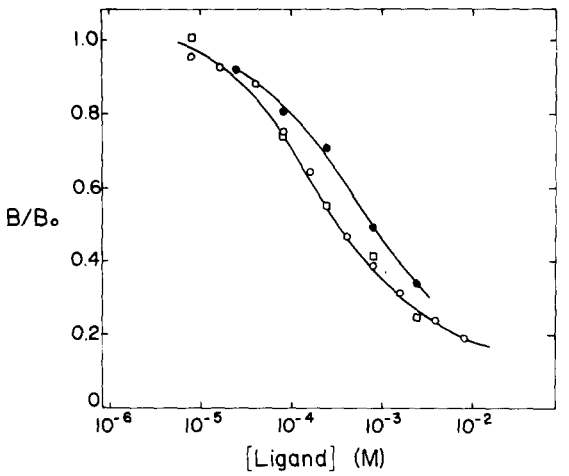

Fig. 3. Radioimmunoassay of digitonin in the presence of biological materials. Digitonin was assayed as described, either in aqueous solution (○) or in the presence of a Brij 35 extract of rat erythrocyte membranes (□). In the latter case, the protein concentration in the samples was 0.8 mg/ml and the Brij concentration was 2 mg/ml. Also shown is a digitonin extract of plasma membranes (●). Membranes (2 mg/ml) were stirred for 60 min in a solution containing 7.5 mM digitonin. After centrifugation (45000 rev./min; 30 min), the supernatant was serially diluted in water and assayed for digitonin. The data are plotted according to the assumption that no digitonin is lost during this procedure.

the presence of more than 10% sucrose is necessary.

Both digitonin and digitoxin are natural products of *Digitalis purpurea*. Since it is known that digitoxin binds anti-digoxin antibodies with considerable affinity [8], it is necessary to show that the crossreactivity that we observe is an intrinsic property of digitonin and not a result of any possible contamination of commercial digitonin with about 0.1% of digitoxin. Fig. 2 compares the binding affinity of commercial digitonin and a sample of digitonin that was extracted with chloroform to remove the less polar cardiac glycosides. Extraction of added [^{3}H]digitoxin indicated that the efficiency of extraction was 97%. If contamination of the digitonin with digitoxin were significant, such extraction would be expected to decrease the apparent binding affinity. As shown, however, the extraction did not alter the affinity of the interaction at all, suggesting that crossreactivity of anti-digoxin immunoglobulin with the digitonin molecule is the cause of the observed competitive binding.

In order to be useful, an assay for digitonin must be insensitive to other materials found in biological membranes. Data in Fig. 2 show that cholesterol does not crossreact with the antibody, nor does it interfere with the assay of digitonin. This is important since digitonin and cholesterol form a tight equimolar complex, and cholesterol or similar sterols are present in most eukaryotic membranes. Fig. 3 shows that a crude detergent extract of erythrocyte plasma membranes does not interfere with the radioimmunoassay for digitonin. A second test of this possible interference is to compare the assay of pure digitonin and digitonin in an extract of plasma membranes. Membranes at a protein concentration of 2 mg/ml were extracted in a mixture containing a final concentration of 7.5 mM digitonin, and insoluble material was removed by centrifugation. The supernatant was then assayed for digitonin by the radioimmunoassay procedure. As shown in Fig. 3, the supernatant titrated in the radioimmunoassay with a slope similar to that of pure digitonin, but behaved as though its concentration was only about 2 mM. This loss of digitonin into the pellet during the

TABLE I

RADIOIMMUNOASSAY OF DIGITONIN IN MIXTURES OF PURE DIGITONIN AND PLASMA MEMBRANE EXTRACTS

A rat erythrocyte membrane extract was prepared as described in the legend to Fig. 3. The digitonin concentration in the extract was determined by radioimmunoassay, and the extract was then diluted to the calculated concentration shown. One volume of extract was mixed with an equal volume of standard digitonin solutions. The concentrations of digitonin in the mixtures were then determined by radioimmunoassay.

Initial concentration (μM)		Concentration in mixture (μM)	
Digitonin solution	Membrane extract	Expected	Assayed
81	8	45	41
81	27	54	49
400	27	210	190
160	81	120	130
81	81	81	66

extraction of membranes is reproducible, varying from 50% to 75%. It probably reflects binding of digitonin to bulk hydrophobic insoluble material in the pellet, but is also consistent with the known instability of digitonin solutions and with the ability of digitonin to form insoluble complexes with sterols. The assay of digitonin which remains in the supernatant is reliable, as shown in Table I. In this experiment, equal volumes of membrane extracts and standard digitonin solutions were mixed, and digitonin was then assayed in the mixtures. As shown, the predicted concentrations and the assayed concentrations were in good agreement. Taken together, the data of Table I and Fig. 3 support the validity of the radioimmunoassay procedure for the determination of digitonin in biological samples. We have used this assay routinely to monitor digitonin concentrations during column chromatography [4] and sucrose density gradient centrifugation of β-adrenergic receptors both in detergent solution and during and after reconstitution into phospholipid vesicles [5]. It has proved reliable in the presence of high concentration of phospholipids and in samples of varying sucrose and salt content. The ability to assay digitonin in these experiments has been crucial.

Discussion

Digitonin is a widely used detergent in membrane research in spite of its several drawbacks. These include low and unstable solubility in water and batch-to-batch variability [9] caused by typical impurity of the commercial product [10]. A third major problem is difficulty in measuring the amount of digitonin in a mixture of biological molecules. Radiolabeled digitonin is not commercially available and its synthesis is difficult. Chemical analysis involves extraction, separation by thin -layer chromatography [10] and either chemical analysis or binding assay using [^{3}H]cholesterol. Assays by dye-extraction [11] or fluorescence enhancement [6] which are typically used for detergents are only applicable above the critical micelle concentration and are interfered with by phospholipids and by any other detergent. The sensitive hemolytic assay suggested by Hubbard [2] and Thron [12] is also not useable in the presence of other detergents or of bulk quantities of lipid.

The experiments reported here demonstrate the feasibility of assaying digitonin according to the commercially available procedure for the assay of the cardiac glycoside digoxin. The assay is sensitive and is not interfered with by other compounds usually encountered when digitonin is used. The present assay has been performed using the Gammaflo automated assay system, which offers the advantage of speed (less than 10 min for initial data) and excellent reproducibility [7]. We see no reason, however, why digitonin should not be easily assayable using one of the several manual methods for digoxin radioimmunoassay as well (see Ref. 8). We have recently begun to use other anti-digoxin sera with results similar to those shown here for commercial Squibb antiserum. We have also shown crossreactivity over the same range of concentrations in a manually performed assay. The only peculiarity of the Gammaflo system which may be of importance in this assay is the presence of Brij 35 in the buffers. Although the intended purpose of the detergent is to improve the flow characteristics of the automated system, it may also serve to disperse what would otherwise be large digitonin micelles. There is no reason not to use a detergent in performance of a manual radioimmunoassay procedure, and we would recommend it.

In conclusion, we would also like to point out the possible general utility of subverting an established radioimmunoassay procedure to the assay of a substantially different but still crossreacting molecule, with the resultant savings in time and effort.

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