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SEPARATION OF FREE AND PROTEIN-BOUND LIGANDS IN THE RADIOIMMUNOASSAY BY GEL FILTRATION–CENTRIFUGATION

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SUMMARY

The application of gel filtration–centrifugation to the separation of free and protein-bound ligands in radioimmunoassay is described. The method is simple and rapid. A large number of samples can be handled simultaneously and the bound radioactivity is directly transferred to the scintillation vial. Using a 9 × 36 mm column of Sephadex G-50 (coarse), 400 µl of the reaction mixture can be separated during 2 min centrifugation.

INTRODUCTION

The separation of free low-molecular-weight ligand from high-molecular-weight protein in the radioimmunoassay of steroids, some peptides, nucleotides, drugs, vitamins and other substances is generally performed by adsorption to a suspension of dextran-coated charcoal, adsorption to Florisil or precipitation of the protein–ligand complex with ammonium sulphate, a second antibody or polyethylene glycol. Antibody can also be adsorbed firmly to the test-tube wall or other suitable carrier, the bound activity then being transferred to the solid phase[1]. In some cases, selective adsorption of free ligand can be employed[2]. The majority of these procedures is easy to perform, but dissociation reactions occurring during the time required for adsorption or precipitation may lead to inaccuracies. Florisil, for example, adsorbs at best 80% of free progesterone[3,4]. In another study, 10-min contact of a dextran-coated charcoal suspension with a hapten–antibody complex was reported to be responsible for a 10–42% dissociation of the complex[5]. Barnard *et al.*[6], on comparing the separating potential of various methods, found that the separation of oestradiol–antibody complex by precipitation with ammonium sulphate (with added powdered calcium sulphate) produced a high level of non-specific binding compared with the separation using polyethylene glycol, a second antibody or dextran-coated charcoal suspension. Nevertheless, they recommended this procedure for routine work because the non-specific binding is independent of the amount of oestradiol in the sample and the temperature. Kushinski and Anderson[7] compared the use of ammonium sulphate and dextran-coated charcoal in the radioimmunoassay of oestrogens in plasma. They found that the former method gave concentrations of oestrogens and

oestradiol that were twice as high as those found using dextran-coated charcoal. This is in contrast to the observations of Barnard *et al.*[6] who found a higher oestradiol concentration after separation with dextran-coated charcoal.

From the foregoing observations, it is evident that the routinely employed separation methods do not always give an accurate assessment of the ratio of free to bound radioactivity, which is one of the prerequisites for radioimmunoassay. Separation procedures should also be rapid and practical in terms of costs when serial analyses are envisaged. It is in this respect that precipitation with a second antibody seems rather inconvenient.

In our laboratory, separation of steroid ligands from proteins has been achieved using the method of gel filtration–centrifugation suggested by Ceska *et al.*[8] for the separation of iodinated proteins from $K^{131}I$ in the reaction mixture. The separation of the high-molecular-weight fraction directly into the glass scintillation vial, with a sufficient amount of ligand activity being taken up by the molecular sieve, obviates the necessity of transferring the supernatant and is therefore a distinct advantage. The model substances used to test the separation in our study were ^{131}I-labelled IgG and $K^{131}I$ or [^{3}H]oestradiol. The molecular sizes do not differ essentially from those of routine ligand-binding systems.

MATERIALS AND METHODS

Porcine IgG was labelled with carrier-free $K^{131}I$ according to McConahey and Dixon[9]. The removal of free iodide from the iodination mixture was made by ion-exchange chromatography–centrifugation as described by Fránek and Hruška[10]. Checks on the separation of iodide from the labelled protein were made by thin-layer gel filtration as described by Hruška and Fránek[11].

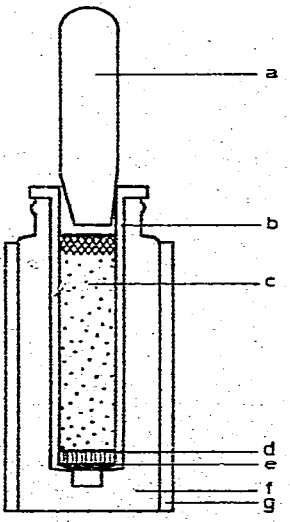

Fig. 1. Gel filtration–centrifugation in the Sephadex column. (a) test tube; (b) column jacket prepared from the barrel of a plastic syringe; (c) Sephadex; (d) cut-off plunger with small holes; (e) Silon tissue; (f) scintillation vial; (g) vial-fixing pad.

[^{3}H]Oestradiol (TRK 322) was supplied by the Radiochemical Centre (Amersham, Great Britain).

Sephadex G-50 (coarse) was swollen in 0.05 *M* borate buffer, pH 7.8, with added sodium azide and gelatine (1 g of each per 1000 ml buffer) before being applied to the columns. These were prepared from the barrels of 2-ml disposable plastic syringes with Silon tissue fixed at the bottom with the cut-off plunger bearing seven small holes (Fig. 1). After the columns were filled with Sephadex, they were placed in the scintillation vial and the interstitial fluid was removed by centrifugation in a chilled Janetzki K-26 centrifuge for 2 min. The final bed volume was *ca.* 2.5 ml (9 × 36 mm). Solutions of ^{131}I-labelled IgG and K^{131}I or [^{3}H]oestradiol were then applied on to Sephadex columns and subjected to centrifugation under the same conditions as used for the removal of the interstitial fluid. The radioactivity of the centrifugates was measured and corrected for changes in volume after the second centrifugation as previously described[10].

The calibration curve for the radioimmunoassay of 17β-oestradiol was obtained as follows. 200 μl of antibody-bound [^{3}H]steroid and 200 μl of unlabelled standard in borate buffer with added sodium azide were pipetted into small test-tubes designed to fit the top opening of the syringe barrel. The mixture was incubated overnight at 4° and applied to the column without pipetting by pouring on to the column as shown in Fig. 1. The tubes were retained in this position throughout the centrifugation period in order to ensure that the whole volume of the mixture was transferred quantitatively to the Sephadex columns. 10 ml of scintillator (SLD 41, Spolana, Neratovice, Czechoslovakia) were added to the centrifugate and radioactivity was measured for 10 min or up to 10,000 impulses, in a Mark 1 liquid scintillation counter (Nuclear-Chicago).

RESULTS AND DISCUSSION

To determine the effect of the speed of centrifugation on the recovery of ^{131}I-labelled IgG and uptake of K^{131}I by Sephadex, 500-μl volumes of these substances were applied to the columns and the activity in the centrifugates was determined as a percentage of the initial activity (Fig. 2). After centrifugation at 800 rpm (180 *g*) for 2 min the proportion of ^{131}I-labelled IgG in the centrifugate was 99.1% of the activity applied, while the uptake of K^{131}I by the column was 98.5% of the initial value. As can be seen from Fig. 2, the recovery of iodinated protein at 1000 and 1500 rpm (260 and 600 *g*) for 2 min was 99.7 and 97.2%, respectively, with a small rise in K^{131}I activity being found in the centrifugate.

When volumes of 200–700 μl were applied to the column and centrifuged at 260 *g*, the centrifugates contained 97.3–100.6% of the activity applied; the uptake of K^{131}I or [^{3}H]oestradiol by the column was 99.5% of the initial value if the volume employed did not exceed 400 μl. The application to Sephadex of 500-μl volumes of K^{131}I and [^{3}H]oestradiol resulted in a small increase of activity in the centrifugates. When 700-μl volumes were applied, the activity in the centrifugates was 3.8 and 2.7% of the initial values, respectively. From the results presented in Fig. 3 it is evident that, after centrifugation of volumes up to 400 μl at 260 *g* for 2 min, approximately 100% of the protein activity was recovered and the uptake of K^{131}I or [^{3}H]oestradiol activity was high. The results are in good agreement with the data reported by Ceska

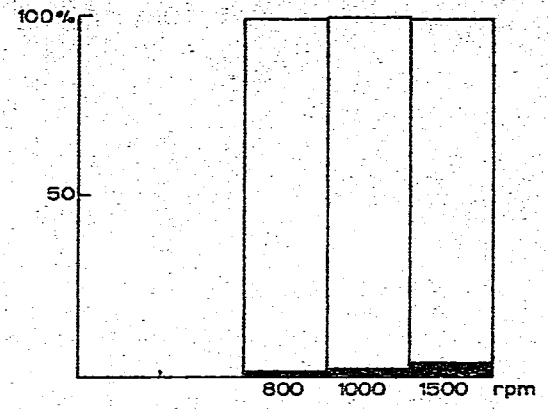

Fig. 2. Recovery of ^{131}I-labelled IgG (open bars) and K^{131}I (filled bars) in the centrifugate after gel filtration–centrifugation at various speeds (500 μl of the mixture).

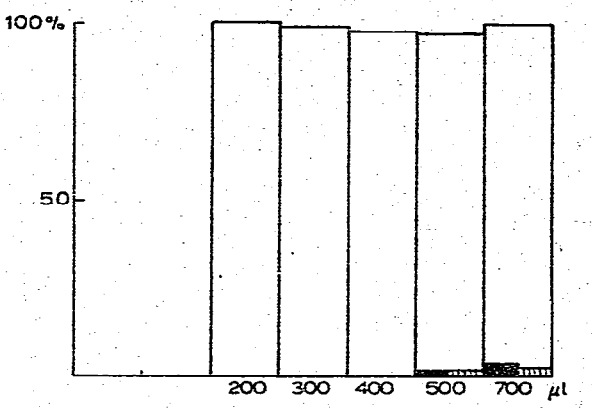

Fig. 3. Recovery of ^{131}I-labelled IgG (open bars), K^{131}I (filled bars) and [^{3}H]oestradiol (hatched bars) in the centrifugate after gel filtration–centrifugation of various volumes of the mixture (260 *g* for 2 min).

et al.[8] who separated radioiodinated proteins from free iodide on Sephadex G-25 (coarse) columns of dimensions similar to those used in our study. However, they employed a more powerful centrifugation (1000 *g* for 5 min).

From the results of 12 gel filtration–centrifugations, it can be seen that the mean recovery of immunoglobulin activity in the centrifugate was 96.97 $\pm$ 2.3% and that the change in centrifugate volume with regard to the sample volume applied was small (102.68 $\pm$ 1.82%). The results provide evidence for low dilution of the sample during the gel filtration–centrifugation process and for good reproducibility of the recovery of ^{131}I-labelled IgG.

Based on these findings, experiments were designed to separate free and bound radioactivity in the systems routinely used in radioimmunoassay or competitive protein-binding assays. The calibration curve constructed from the radioactivity values in the centrifugates obtained by separation of 400-μl volumes of the reaction

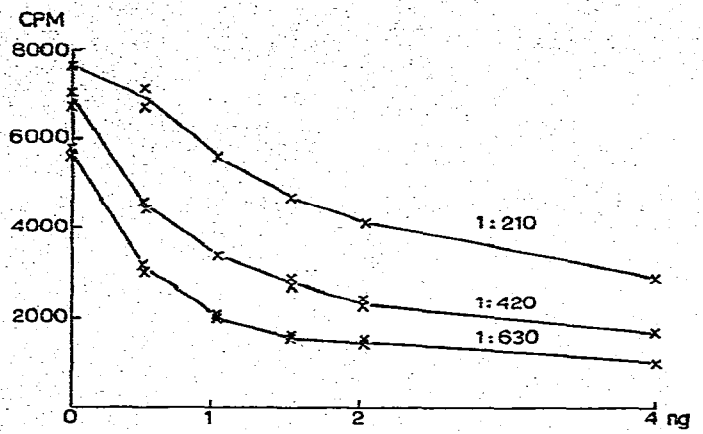

Fig. 4. Calibration curves for the radioimmunoassay of oestradiol at various dilutions of the antibody.

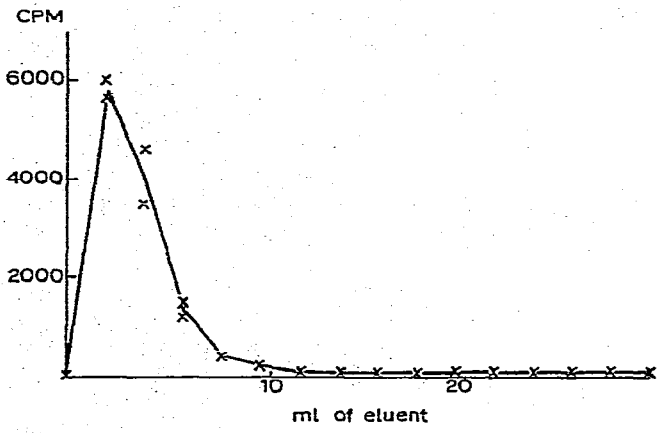

Fig. 5. Elution of [^{3}H]oestradiol from the Sephadex column after gel filtration–centrifugation.

mixture for the determination of oestradiol shows very good reproducibility (Fig. 4), in keeping with the results given above.

The low-molecular-weight radioligand taken up in the column can easily be removed by washing with a small volume of buffer. As little as 10 ml of elution buffer proved sufficient to remove the radioactivity, a distinct advantage, permitting repeated use of the columns (Fig. 5). For this step, a mass washing chamber is convenient to use.

The method of gel filtration–centrifugation is a convenient tool for the separation of free and protein-bound activity in the radioimmunoassay of steroids. Its main advantages over present methods are the following: (1) it is rapid and simple; (2) the composition of the reaction mixture is the same throughout the separation procedure, thus reducing the danger of dissociation; (3) the separation can be carried out directly into the scintillation vials; (4) measurement is made of the whole bound

activity of the reaction mixture; (5) a large number of samples can be handled at the same time.

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