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RADIOIMMUNOASSAY OF PROSTAGLANDIN $F_{2\alpha}$ USING SEPHADEX G-75 GEL CHROMATOGRAPHY

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SUMMARY

The development of a "bound—free" separation technique and its application to the radioimmunoassay of prostaglandin $F_{2\alpha}$ is described. The method is simple, rapid, free of non-specific binding and could be performed either at 4°C or at room temperature. A total of 100 tubes could be subjected to "bound—free" separation in 30 min at 4°C. The bound fraction is collected directly into scintillation vials. The total column length was 9.5 cm, of which the bed volume was 2.5 ml. The $PGF_{2\alpha}$ radioimmunoassay incubation volume of 0.3 ml when bedded in required 1.4 ml of elution buffer to elute the antibody-bound fraction. The free fraction was washed out with 4.0 ml of buffer and the columns were ready for further use. A standard curve of high sensitivity (5 pg) and good reproducibility (CV %: intra-assay = 6.54; inter-assay = 9.68) was obtained.

INTRODUCTION

In the radioimmunoassay systems for prostaglandins, prostaglandin metabolites, steroids, peptides and other substances, the antigen—antibody complex and the free antigen occur in soluble form. It is therefore necessary to separate the antibody-bound fraction from the free antigens. A variety of methods have been utilized for the bound—free separation in the radioimmunoassay of prostaglandin $F_{2\alpha}$. These include dextran-coated charcoal suspension [1], double-antibody [2, 3], ammonium sulphate [4], ammonium sulphate in conjunction with calcium sulphate suspension [3], polyethylene glycol [5], and nitrocellulose membranes [6, 7]. The use of the solid-phase method originally described for oestradiol-17β radioimmunoassay [8] has not been utilized in the radioimmunoassay of prostaglandins. The use of gel chromatography for the separation of protein-bound insulin from free insulin was described for study-

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ing insulin antibodies [9]. The use of gel chromatography with Sephadex G-75 was reported for insulin radioimmunoassay [10]. A modified gel chromatographic technique using Sephadex G-50 in conjunction with centrifugation has been reported for the bound–free separation in the radioimmunoassay of oestradiol [11].

Although the majority of the techniques described are easy to perform, the unstable equilibrium of the antigen–antibody complex is easily disrupted during the process of bound–free separation. The dextran-coated charcoal method, despite being quick and cheap, causes "stripping" of the antigen–antibody complex, which can be minimized by performing the assay in the cold [1]. Chemical precipitation techniques [3–5] have the disadvantage of precipitating free (unbound) antigen. The double-antibody technique is an expensive method if the second antibody is obtained commercially. Furthermore, it requires rigorous titration from batch to batch. It is also time-consuming, requiring in most instances 24 h to complete the second incubation [2].

The present report describes the development and the application of a bound–free separation technique, using Sephadex G-75 and gravitational flow, in the radioimmunoassay of prostaglandin $F_{2\alpha}$.

MATERIALS AND METHODS

[5,6,8,11,12,14,15(n)-^{3}H]$PGF_{2\alpha}$ ($^3HPGF_{2\alpha}$) of specific activity 150 Ci/mmol and radioactive concentration 0.1 mCi/ml was obtained from the Radiochemical Centre (Amersham, Great Britain). $PGF_{2\alpha}$-1–BSA antiserum was raised in rabbits [2]. Pure prostaglandin $F_{2\alpha}$ was donated by the Upjohn Company, Kalamazoo, MI, U.S.A.

Radioimmunoassay diluent (assay buffer) was a 0.01 *M* phosphate-buffered saline (pH 7.40) with 0.1% human gamma globulin (IgG; supplied by the Commonwealth Serum Laboratories, Parkville, Australia) and 0.1% sodium azide (NaN_3) as the bacteriostatic agent.

Sephadex G-75 (Pharmacia Fine Chemicals, Uppsala, Sweden) was soaked in the assay buffer for a period of 12–24 h in a conical flask with a side-arm. The Sephadex columns consisted essentially of 2-ml disposable plastic syringes supplied by Pharma-Plast Australia (New South Wales, Australia). The base of each column was covered with a vyon porous disc of diameter equal to the internal diameter of the syringe barrel (Fig. 1).

The swollen Sephadex gel was deaerated by connecting the side-arm of the capped conical flask to an aspirator. The development of excessive negative pressure inside the flask is avoided, to prevent any rupture of swollen Sephadex beads. The removal of trapped air bubbles is necessary for the preparation of uniform Sephadex columns free of air bubbles. The Sephadex was layered in the column to a height of 4.0 cm. The top of the bed was covered with a vyon disc. To carry out the elution of bound and free fractions by gravitational flow, it was found necessary to increase the effective volume of the space above the Sephadex bed. This was achieved by attaching a plastic tube 5.5 cm long to the top of the 2-ml syringe. One hundred columns were prepared and mounted on a stand. The stand was constructed

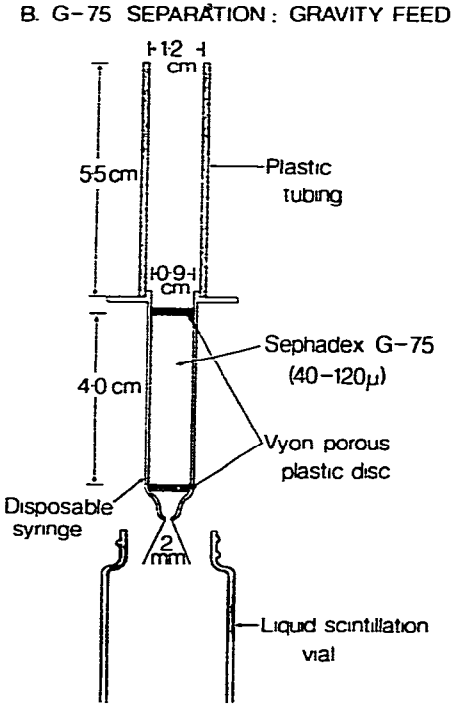


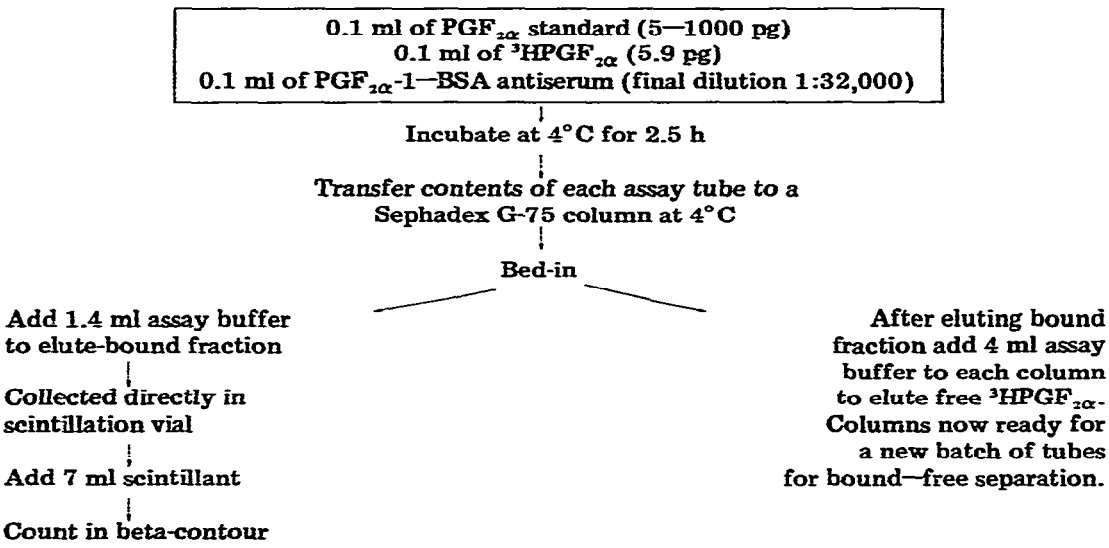
Fig. 1. Schematic diagram of a Sephadex G-75 minicolumn used in the "bound–free" separation of prostaglandin radioimmunoassay.

to the dimensions of a box of 100 scintillation vials purchased from the Radiochemical Centre (Australia) Ltd. This design allows the tip of the columns to point directly into the mouth of scintillation vials. When the columns were not in use for more than one week, they were individually placed in vials containing phosphate-buffered saline at 4°C. This prevents drying up the gel and the development of cracks in the bed. $^3HPGF_{2\alpha}$ solution in assay buffer was prepared by diluting 5 μl of the stock $^3HPGF_{2\alpha}$ in 25 ml of assay buffer. This solution, when used at 100 μl volume per assay tube, yielded 5.5 pg of $^3HPGF_{2\alpha}$. $PGF_{2\alpha}$-1—BSA antiserum was diluted in assay buffer and used in 100 μl volume per assay tube to yield a final dilution of 1:32,000 in a $PGF_{2\alpha}$ incubation volume of 300 μl.

Equal volumes of $^3HPGF_{2\alpha}$, diluted $PGF_{2\alpha}$-1—BSA antiserum and assay buffer were mixed in a glass vial. The reaction mixture was incubated at 4°C for a minimum period of 2.5 h. The time for $PGF_{2\alpha}$ radioimmunoassay to attain equilibrium had been previously established by a time–course study. A 300-μl volume of the reaction mixture was applied to the Sephadex G-75 column and allowed to bed-in. The void volume was collected into a scintillation vial. This was followed by the addition of 1.2 ml of assay buffer to the column and the void volumes were collected in fractions of four drops per vial. This procedure was repeated with increasing volume of eluting buffer. The elution pattern was studied in duplicate for each volume of eluting buffer studied. To the vials containing the eluted fractions, 7 ml of a toluene—

Triton X 100 (1.9:1) scintillant containing PPO and POPOP were added. The vials were counted in a beta-spectrometer (Searle Isocap/300, 6868 liquid scintillation system) after 1 h of dark equilibration.

Having established the appropriate volume of assay buffer which eluted only the antibody-bound fraction, standard curves for prostaglandin $F_{2\alpha}$ were established. $PGF_{2\alpha}$ standards (5, 10, 25, 50, 100, 300, 500, and 1000 pg per 100 μl per assay tube) were prepared in assay buffer. The preparation of standard curve tubes and the incubation procedure were carried out as illustrated in the flow diagram in Scheme 1.



Scheme 1.

RESULTS

The volume of buffer which eluted only the bound fraction completely, but left the free $^3HPGF_{2\alpha}$ held by Sephadex beads, was determined by studying the effect of sequential increase in elution volume on the elution pattern. Each fraction eluted from the column in this study was four drops per vial. Addition of 1.4 ml of buffer to the column resulted in the elution of only the bound fraction (Fig. 2). It is evident that any increase in elution volume above 1.4 ml resulted in the elution of "free" as well. This was demonstrated by the occurrence of a second wave of counts when 1.5, 1.6, 1.7, and 1.8 ml eluting volume was used (Fig. 2).

The fraction eluted in the first 1.4 ml was, in fact, antibody-bound and was confirmed by carrying out an elution profile in the presence and in the absence of $PGF_{2\alpha}$ antiserum (Fig. 3). The resolution of the elution profile was enhanced by collecting two drops per fraction per vial. It could be seen that in the absence of $PGF_{2\alpha}$ antiserum (open circles in Fig. 3) no $^3HPGF_{2\alpha}$ was eluted in the first 1.4 ml elution volume. However, the addition of 4 ml of buffer resulted in the elution of $^3HPGF_{2\alpha}$. The elution of this free fraction

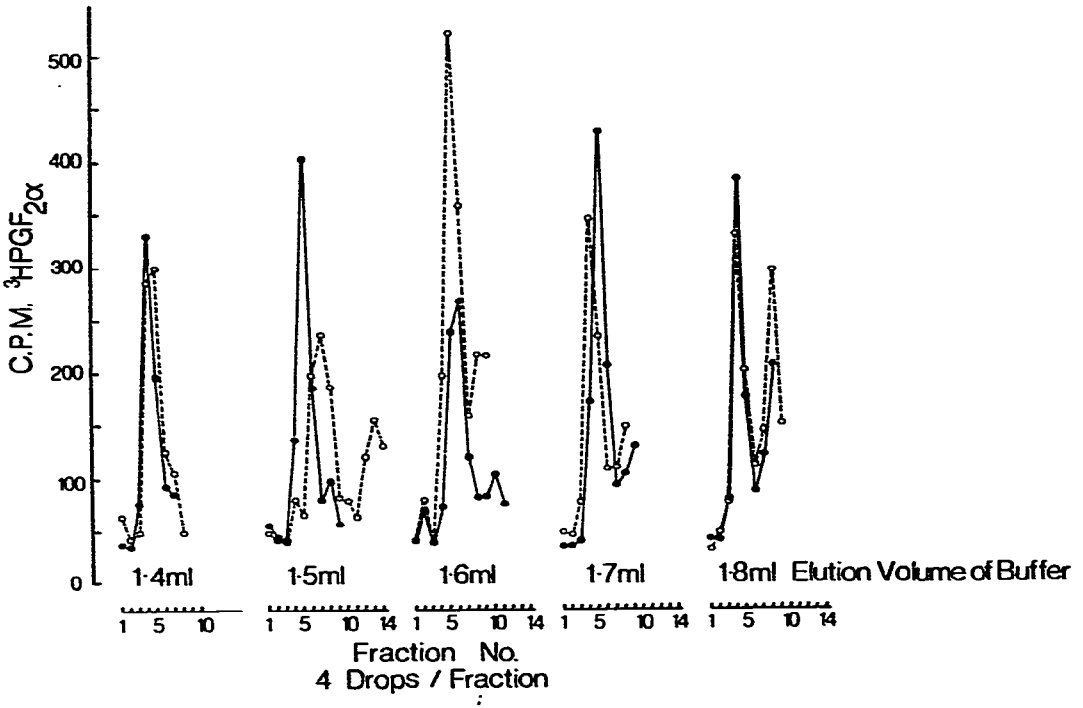


Fig. 2. Determination of the optimum volume of buffer for the elution of antibody-bound fraction in the radioimmunoassay of prostaglandin $F_{2\alpha}$. Each fraction collected represents four drops. Elution profiles at each elution volume were studied in duplicate. Closed circles represent column 1; open circles represent column 2. Note that the elution of bound fraction takes place within first 7 or 8 fractions.

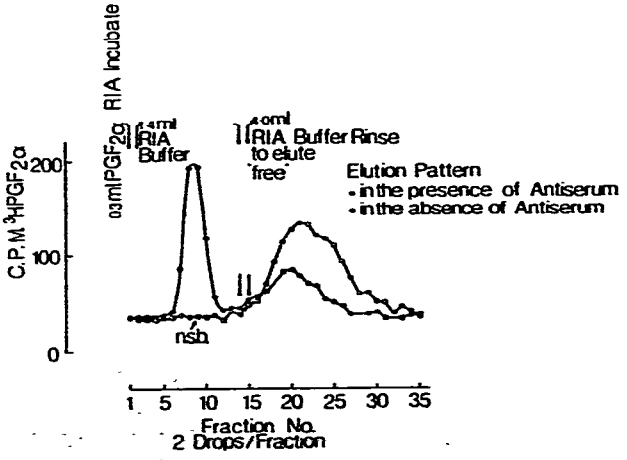
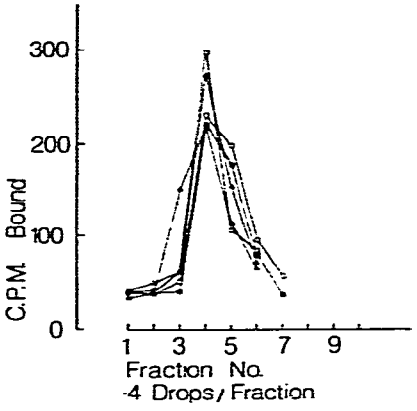


Fig. 3. Elution profile of antibody bound and free $^3HPGF_{2\alpha}$. Elutions were carried out both in the presence of $PGF_{2\alpha}$—BSA antibodies (closed circles) and in the absence of antibodies (open circles). Fractions were collected at the rate of two drops per vial.

was complete, indicating that the columns were ready for further use.

The reproducibility of the elution of antibody-bound fraction was studied from column to column. It can be seen from Fig. 4 that the elution of bound $^3HPGF_{2\alpha}$ was similar provided the column dimensions and Sephadex bed volume were identical.

Based on these observations, standard curves of $PGF_{2\alpha}$ radioimmunoassay were established. The content of each standard curve tube was transferred to a column using a pasteur pipette. One pasteur pipette per set of tubes was adequate to obtain reasonable within-standard variability. The bound fraction was eluted with 1.4 ml of assay buffer using an Oxford pipetter. Fig. 5 shows the standard curve of $PGF_{2\alpha}$ radioimmunoassay. Each point of the standard curve represents the mean of fifteen individual standard curves. The standard curve was of a high degree of reproducibility (intra-assay CV % = 6.45 ± 0.54, I.S.D.; inter-assay CV % = 9.68 ± 0.96, I.S.D.) and sensitivity (5.0 pg per tube per 0.3 ml reaction mixture was different from 0 tube at the 95% confidence limit) with a working range of 5—500 pg.



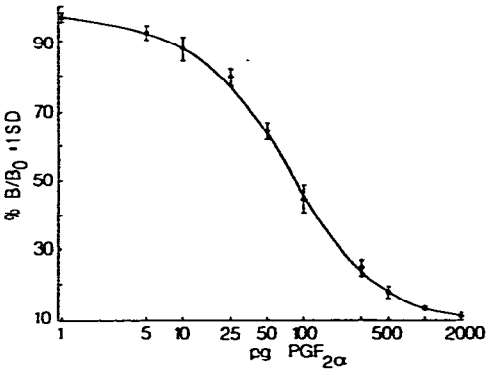


Fig. 4. Reproducibility of "bound—free" separation on Sephadex G-75 columns using $PGF_{2\alpha}$ radioimmunoassay system. Note that the elution of bound fraction using 0.3 ml incubation volume and 1.4 ml of eluting buffer is complete within 8 fractions.

Fig. 5. Standard curve for prostaglandin $F_{2\alpha}$ radioimmunoassay. Each point is the mean of fifteen individual standard curves. "Bound—free" separation was achieved by means of Sephadex G-75 gel chromatography at 4°C.

DISCUSSION

The method of gel chromatography by gravitational flow is a convenient technique for the separation of antibody-bound fraction from the free in the radioimmunoassay of prostaglandins. The use of gel chromatography in conjunction with centrifugation in the radioimmunoassay of oestradiol reported earlier [11] may not be a suitable technique because of the development of cracks in the Sephadex bed during centrifugation. In the method described in the present report no such problem was encountered and the columns were ready for use for another 100 tubes at the end of the wash with

4 ml of buffer to remove free radioactivity. Unlike the earlier report of Fránek and Hruška [11], who used 10 ml of elution buffer to remove free ligands at the end of the separation of antibody-bound fraction, in the technique reported in this paper a volume of buffer as low as 4 ml was found to be sufficient. This is a considerable saving on the quantity of buffer used, besides allowing repeated use of the columns.

The use of gel chromatography has the advantage of allowing the separation on the basis of molecular size alone [12]. The low molecular weight $PGF_{2\alpha}$ is taken up by the swollen Sephadex beads through the pores on its surface. The large molecular weight immunoglobulins with the attached (ionic bond) ligand are larger than the largest pores of the swollen Sephadex. Because the immunoglobulins are above the exclusion limit, they cannot penetrate the gel particles and therefore pass through the bed in the liquid phase between the Sephadex beads. This is the reason for the smaller volume of buffer which elutes the bound fraction and for the bound fraction to be eluted first. Smaller molecular weight $PGF_{2\alpha}$ retained within the gel particles requires a much larger volume to be eluted out of the column.

Bound—free separation techniques by chemical precipitation have yielded adequate standard curves [3—5]. However, these techniques are beset with the problem of non-specific precipitation of unbound $^{3}HPGF_{2\alpha}$ [3]. The double-antibody technique [2] requires rigorous titration of the second antibody for each assay and for each dilution of first antibody. This may prove costly unless the second antibody is raised in the same laboratory. Furthermore, this technique is time-consuming, requiring at least 24 h for the separation. The dextran-coated charcoal technique [1], although a cheap and commonly used method, has the distinct disadvantage of "stripping" the bound fraction.

The main advantages of the gel chromatographic technique presented in this paper over the existing techniques are that it is rapid and simple to operate. The columns can be used repeatedly over a considerable period of time. The bed volume can be maintained constant by adding freshly soaked Sephadex G-75 for any lost by leaching during the washing procedure.

Unlike previous methods, the gel chromatographic method offers the advantage of maintaining the composition of the reaction mixture throughout the separation procedure, which minimises dissociation of the antigen—antibody complex. The design of the stand on which columns were placed facilitates the collection of the bound fraction directly into the scintillation vials, which enables the measurement of the antibody-bound radioactivity. The stand was constructed to the measurements of a standard scintillation vial box supplied by Radiochemical Centre (Australia) Ltd. A total of 100 tubes could be subjected to bound-free separation in 30 min.

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REFERENCES

1 B.V. Caldwell, S. Burstein, W.A. Brock and L. Speroff, J. Clin. Endocrinol. Metab., 33 (1971) 171.
2 K.T. Kirton, J.C. Cornette and K.C. Barr, Biochem. Biophys. Res. Commun., 47 (1972) 903.
3 J.F. Hennam, D.A. Johnson, J.R. Newton and W.P. Collins, Prostaglandins, 5 (1974) 531.
4 B.M. Jaffe, J.W. Smith, W.T. Newton and C.W. Parker, Science, 171 (1971) 494.
5 D.E. Van Orden and D.B. Farley, Prostaglandins, 4 (1973) 215.
6 H. Gershman, E. Powers, L. Levine and H. Van Vunakis, Prostaglandins, 1 (1972) 407.
7 S. Bauminger, U. Zor and H.R. Lindner, Prostaglandins, 4 (1973) 313.
8 G.E. Abraham, J. Clin. Endocrinol. Metab., 29 (1969) 866.
9 V. Manipol and H. Spitzy, Int. J. Appl. Radiat., 13 (1962) 647.
10 S. Genuth, L.A. Frohman and H.E. Lebovitz, J. Clin. Endocrinol. Metab., 25 (1965) 1043.
11 M. Fránek and K.J. Hruška, J. Chromatogr., 119 (1976) 167.
12 P.G. Squire, Arch. Biochem. Biophys., 107 (1964) 471.