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CHROMATOGRAPHIC ASSAY OF STEROIDS ON IMMUNO-AFFINITY PAPER STRIPS; A RAPID METHOD FOR THE QUANTITATION OF DIGOXIN AND OESTRIOL-16α-GLUCURONIDE CONCENTRATIONS*

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SUMMARY

The combination of affinity chromatography and immunoassay provides a novel method for the quantitation of steroids in biological fluids.

Anti-digoxin and anti-oestriol-16α-glucuronide antibodies were immobilised to cellulose strips, and used for ascending paper chromatography. Labelled digoxin and oestriol-16α-glucuronide were selectively retarded on strips containing the appropriate immobilised antibodies. The degree of retardation was quantitatively affected by the addition of non-labelled steroid to the sample. Calibration curves could, therefore, be constructed. The sensitivity of the assay system can be altered by changing the concentration of immobilised antibody on the cellulose strip. Calibration curves over the range 50–800 pg for digoxin and 50–250 pg for oestriol-16α-glucuronide are presented.

INTRODUCTION

Recent advances in immunoassay techniques have focused on labelling methods including enzyme-linked immunosorbent assay[1], fluoroimmunoassay[2] and luciferase-dependent enzyme-multiplied immunoassay technique systems[3]. Whilst many of these methods have improved sensitivity and have reduced biohazards by avoiding radioisotopes, nevertheless, expensive detectors and carcinogenic substrates are still commonplace. Mass screening by traditional methods of immunoassay is time-consuming and expensive. It is desirable to be able to screen a wide cross-section of newborn for a number of conditions, *e.g.*, phenylketonuria, thyroid and steroid deficiences.

We have sought to develop an assay system which is (i) cheap, (ii) capable of widespread application, (iii) requires little skill by the user, (iv) is rapid and (v) which involves low cost apparatus. The commonplace use of batch techniques in immunoassay limits the ligand–protein interaction to high K_A systems. The disadvantages of

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batch methods have been previously outlined[4]. However, the application of sequential exposures of ligand to immobilised macromolecules does not seem to have been exploited in immunoassay, although recently Glad and Grub[5–7] have described the determination of plasma proteins using a similar technique.

In this paper we describe the use of cellulose-immobilised anti-steroid antisera for the assay of oestriol-16α-glucuronide and digoxin. We chose these two small hapten systems because of the known difficulties of operating some affinity chromatographic separations when the ligands are bound to cellulose matrices.

MATERIALS

[G-^{3}H]Digoxin (specific activity 10.6 Ci/mmol) and [6,9(n)-^{3}H]oestriol-16α-glucuronide (specific activity 30 Ci/mmol) were obtained from the Radiochemical Centre (Amersham, Great Britain). Oestriol-16α-glucuronide was purchased from Steraloids (Wilton, NH, U.S.A.).

Anti-digoxin, code R2BI (titre 1:2500), and anti-oestriol-16α-glucuronide, code R2BI (titre 1:500), were prepared by standard procedures[8]. Digoxin, bovine serum albumin (Cohn Fraction V) and cyanogen bromide were obtained from Sigma (London, Great Britain). Rabbit serum was a gift from Dr. C. D. Green (Department of Biochemistry, University of Liverpool). NE260 micellar scintillant was purchased from Nuclear Enterprises (Edinburgh, Great Britain). Chromatography paper (Grade No. 1 or 3 MM) was obtained from Whatman (Maidstone, Great Britain). All other reagents (Analar grade) were purchased from BDH (Poole, Great Britain).

METHODS

Immobilisation of antisera

Method 1. Whatman 3 MM filter-paper (50 strips, 1 × 8 cm) were suspended in distilled water (200 ml) at 18°C and the pH of the solution raised to 10.8 by the addition of 4 *M* NaOH. Cyanogen bromide (2 g) was added and the solution titrated to pH 10.8 with 4 *M* NaOH. The temperature was maintained at 18–20°C by the addition of crushed ice. After 15 min the papers were transferred to a cooled sinter and washed rapidly with 5% (v/v) acetone–water (1 l) followed by ice-cold 0.1 *M* sodium carbonate–bicarbonate buffer, pH 9.5 (1 l). The paper strips were then immersed in antiserum (1 ml antiserum diluted with 19 ml 0.1 *M* carbonate–bicarbonate buffer, pH 9.5) and incubated on a Coulter rotary mixer for 18 h at 4°C. When dilutions of antisera were required, antisera were diluted to 1 ml with normal rabbit serum followed by dilution to 20 ml with 0.1 *M* bicarbonate buffer. After immobilisation, the paper strips were washed extensively in 100 m*M* phosphate-buffered saline pH 7.0 (5 l), dried and stored at room temperature.

Method 2. The filter-paper (Whatman No. 1, 10 strips, 8 × 0.5 cm) was immersed in 2 *M* potassium carbonate–bicarbonate buffer, pH 11.0, and kept at 4°C. Cyanogen bromide (0.5 g) was dissolved in the minimum volume of N-methylpyrrolidone, and added dropwise to the paper strips without allowing the temperature to rise. The reactants were left for 15 min with occasional mixing before decanting the liquid and washing the activated strips with ice-cold 0.1 *M* sodium bicarbonate, and 5% (v/v) acetone solution. The washing cycle was repeated three times, finishing with

a further bicarbonate wash. The papers were then placed in the antiserum (10 ml) to which 0.1 *M* sodium bicarbonate solution (5 ml) had been added, and rotated on a Coulter mixer for 4 days at 4°C. The papers were washed with 6 × 20 ml 0.1 *M* phosphate-buffered saline pH 7.4, incorporating thiomersal (1 g/l) and bovine serum albumin (1 g/l), and allowed to dry at room temperature, prior to storage at 4°C in a screw-capped bottle.

Chromatography

Each paper strip (8 × 1 cm) was cut in half (8 × 0.5 cm) and marked (pencil) at 0.5-cm intervals down its length. The sample and the labelled steroid (in ethanol) were spotted onto the paper strips 1 cm from the bottom. In the case of [G-^{3}H]digoxin, 32 pg of labelled material were applied; for labelled oestriol-16α-glucuronide, 15 pg were applied. The strips were then developed by ascending chromatography in (i) 0.1 *M* phosphate-buffered saline, pH 7.0 containing 10% (v/v) glycerol until the solvent front was 1 cm from the top of the strip; or (ii) 0.1 *M* phosphate-buffered saline, pH 7.4 for 1 h. Preliminary experiments indicated that the presence of glycerol improved binding to the immobilised antibody. The strips were dried, cut into 0.5-cm segments and radioactivity determined in NE260 scintillant (4 ml) on a Nuclear Chicago Isocap 300 scintillation counter.

RESULTS AND DISCUSSION

Figs. 1 and 2 show the distribution of labelled steroids (digoxin and oestriol-16α-glucuronide, respectively) on paper strips containing the appropriate immobilised antiserum in the presence of increasing amounts of unlabelled steroid. In the absence of unlabelled steroid, the maximum amount of radioactivity was found in segment 3, *i.e.*, the point of application of the steroid. As a control, immobilised normal rabbit serum was used instead of anti-steroid antiserum (Figs. 1 and 2). The maximum radioactivity was observed in segment 12, *i.e.*, immediately prior to the solvent front (segment 14). This indicates that retardation was due to the steroid binding specifically to the immobilised antibody, irrespective of the method of immobilisation used. In both systems, increasing the amount of unlabelled steroid applied to the strips displaced the peak of radioactivity further up the strip (Fig. 1). Thus the application of unlabelled digoxin (10 ng) to anti-digoxin paper strips displaced the peak of radioactivity from segment 3 to segment 11. Preliminary experiments indicated that the presence of human serum in the applied sample did not interfere with the displacement of tritiated steroid by unlabelled steroid.

Calibration curves were constructed relating the displacement of label to the amount of unlabelled steroid applied by measuring either (i) the position of maximum radioactivity (Fig. 3) or (ii) percentage of the total radioactivity found in a particular segment (Fig. 4). In both these plots the major portion of the curve is linear, deviating only at the upper limits of the range. Under the conditions described the sensitivity of this chromatographic method for detecting unlabelled steroid lies within the range 1–5 ng (for digoxin, Fig. 3) and 50–250 pg (for oestriol-16α-glucuronide, Fig. 4).

Because this chromatographic assay system depends on the competition of labelled and unlabelled steroid for immobilised antibody, it is suggested that the repeated exposure of antigens (and cross-reacting substances) in the sample to im-

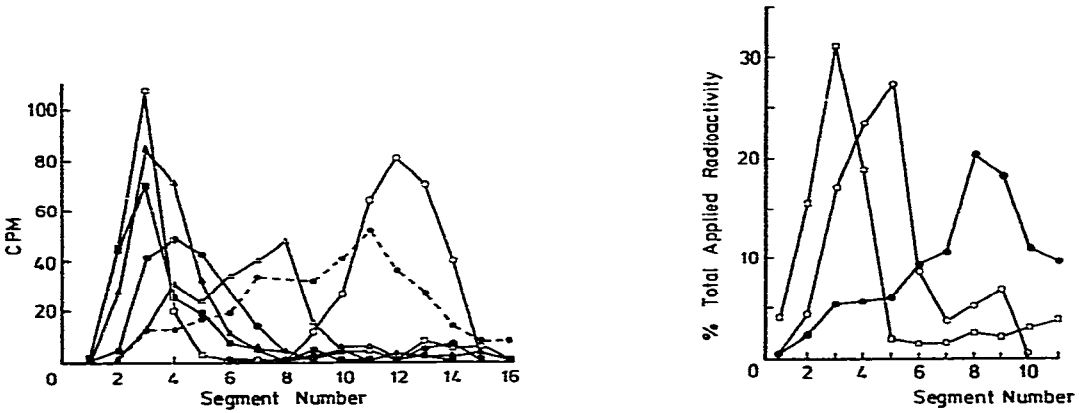


Fig. 1. The movement of [G-^{3}H]digoxin on cellulose strips containing immobilised rabbit anti-digoxin antisera in the presence of increasing amounts of unlabelled digoxin. Unlabelled digoxin (0–10 ng in 5–25 μl ethanol) was applied 1 cm from the end of cellulose strip (0.5 × 8 cm), containing immobilised anti-digoxin antisera or immobilised normal rabbit serum prepared by *Method 1* (see Methods). Tritiated digoxin (36 pg in 5 μl ethanol) was then applied. The cellulose strips were developed by ascending chromatography in 0.1 *M* phosphate-buffered saline, pH 7.0 containing 10% (v/v) glycerol (500 μl) until 1 cm from the top of the strips. The strips were dried, cut in 0.5-cm segments and counted in vials containing scintillant (4 ml). □ – □, 0 ng; ■ – ■, 0.2 ng; ▲ – ▲, 0.6 ng; ● – ●, 1 ng; △ – △, 5 ng; ●--- ●, 10 ng unlabelled digoxin applied; ○—○, immobilised rabbit serum.

Fig. 2. The migration of [6,9(n)-^{3}H]oestriol-16α-glucuronide on paper strips containing immobilised rabbit anti-oestriol-16α-glucuronide antisera in the presence of increasing amounts of unlabelled steroid. Radioactive oestriol-16α-glucuronide (15 pg) was applied as described in Fig. 1 to immobilised antibody strips prepared by *Method 2*. Unlabelled steroids (100 and 400 pg) were applied and the strip developed with 0.1 *M* phosphate-buffered saline, pH 7.4 for 1 h. The strips were dried and assayed for labelled steroid as described in Fig. 1. Radioactivity per segment is expressed as a percentage of that applied to the strip. □ – □, 0 ng; ○ – ○, 100 pg; ● – ●, 400 pg unlabelled oestriol-16α-glucuronide applied.

mobilised antibodies is likely to lead to improved selectivity. Furthermore, it is suggested that the assay sensitivity can be manipulated by the judicious choice of the concentration of the immobilised antibody. Fig. 5 shows the effect of chromatography of 36 pg of tritiated digoxin on paper strips containing decreasing concentrations

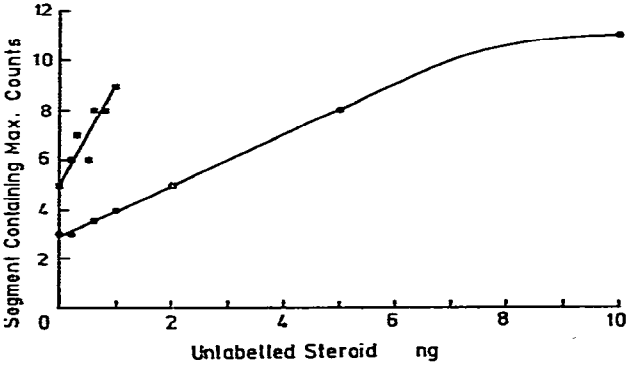
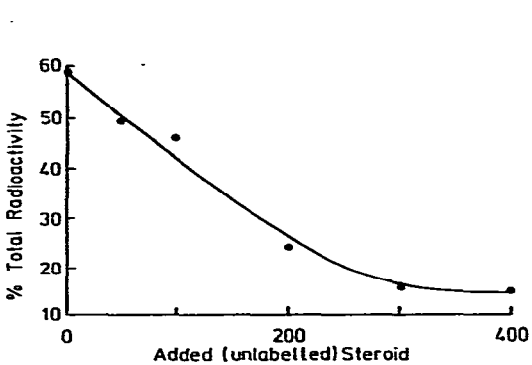


Fig. 3. Calibration curve relating the displacement of [G-^{3}H]digoxin on immobilised anti-digoxin cellulose strips with the amount of unlabelled digoxin applied. Methods see Fig. 1. ● – ●, Undiluted antiserum-paper; ■ – ■, sixty-fold diluted antiserum-paper.

of the immobilised antiserum: as the concentration of immobilised antibody decreased so radioactivity migrated further up the strip. Thus 36 pg of digoxin gave zero migration on R2BI antibody paper strips but gave a peak of maximum radioactivity at segment 7 for a fifty-fold dilution of the original immobilised antibody concentration. Indeed, this displacement is also reflected in the increased sensitivity to cold digoxin. In the "diluted" strip the working range was 50–800 pg (Fig. 3), compared with 1–5 ng in the "undiluted" strip. These data suggest that with a range of immobilized antibody concentrations, a range of assay sensitivities can be produced.



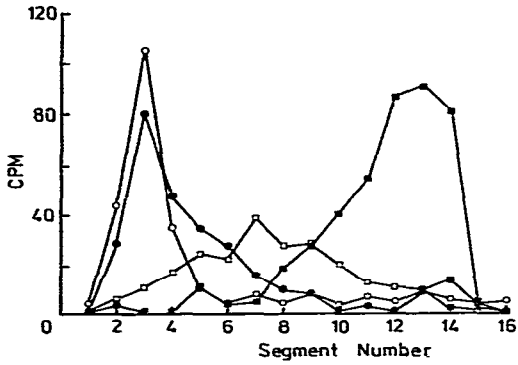


Fig. 4. Calibration curve relating the radioactivity found in segment 3 to the amount of unlabelled oestriol-16α-glucuronide applied to paper strips containing immobilised anti-oestriol-16α-glucuronide. Methods see Fig. 2.

Fig. 5. The effect of antibody concentration on the migration of tritiated digoxin on paper strips containing immobilised anti-digoxin antisera. 36 pg of [G-^{3}H]digoxin were applied to strips containing different dilutions of antisera and the strips treated as Fig. 1. ○—○, Undiluted sera; ●—●, sera diluted by ten-fold; □—□, sera diluted by fifty-fold; ■—■, sera diluted by a hundred-fold prior to immobilisation.

A further interesting possibility exists: the concept of combining chromatography with the separation of free and bound species in immunoassay suggests that the assay of samples need involve measuring only the distance migrated by the label. This idea could be easily applied to a device which discriminates between "high" and "low" samples by whether the label triggers the detector positioned at a fixed location in relation to the point of application. The label could be fluorescent, luminescent or a radioisotope.

CONCLUSION

This paper describes a method which combines the advantages of the chromatographic process and immunoassay: the sequential exposure to antibody as the sample moves through the strip increases sensitivity, specificity and allows rapid quantitation which is suitable for automation.

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REFERENCES

1 E. Engvall and P. Perlmann, *Immunochemistry*, 8 (1971) 871.
2 K. E. Rubenstein, R. S. Schneider and E. F. Ullman, *Biochem. Biophys. Res. Commun.*, 47 (1972) 846.
3 H. Arakawa, M. Maeda and A. Tsuji, *Anal. Biochem.*, 97 (1979) 248.
4 C. R. Lowe, M. J. Harvey and P. D. G. Dean, *Eur. J. Biochem.*, 42 (1974) 1.
5 C. Glad and A. O. Grub, *Biochem. Soc. Trans.*, 5 (1977) 712.
6 C. Glad and A. O. Grub, *Anal. Biochem.*, 85 (1978) 180.
7 C. Glad and A. O. Grub, *Acta Chem. Scand., Ser. B*, 34 (1980) 449.
8 P. D. G. Dean, M. J. Johnson and D. Exley, *Steroids*, 18 (1971) 593.