Journal of Chromatography, 470 (1989) 385–389
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1561

PURIFICATION OF LABELLED ANTIBODIES BY HYDROPHOBIC INTERACTION CHROMATOGRAPHY

HENRIK ALFTHAN* and ULF-HÅKAN STENMAN
Departments I & II of Obstetrics and Gynecology, Helsinki University Central Hospital, Haartmaninkatu 2, SF-00290 Helsinki (Finland)

SUMMARY

In order to improve the sensitivity of immunometric assays, a chromatographic technique was developed that virtually eliminates components causing non-specific background. Labelled antibodies were applied to a phenyl-Sepharose column in physiological buffer. When labelled antibodies were purified by this technique, the non-specific background of various time-resolved immunofluorometric assays was reduced 3- to 10-fold and was very close to the instrument background. The assay sensitivity was simultaneously increased by a factor of 2 to 16. This purification method might be used to improve the results of immunometric assays in general.

INTRODUCTION

Time-resolved immunofluorometry is a highly sensitive immunochemical technique[1–3], with which it is possible to develop methods 100 times more sensitive than corresponding radioimmunoassays[4]. The method is based on the sandwich principle, in which two antibodies are used[5,6]. One antibody is immobilized on the walls of microtitre strip wells and the other one is labelled with a europium chelate[2]. The high sensitivity of the technique is related to the use of large amounts of antibodies in combination with an highly fluorescent label[7,8]. In order to achieve maximum sensitivity, the background signal, *e.g.*, the fluorescence of the sample containing no analyte, must be low and the specific signal must be as high as possible. Gel chromatography is used mostly to purify the labelled antibody, but after this purification step, some antibodies still show high non-specific binding, causing an high background.

Hydrophobic interaction between the labelled antibody and the solid phase is a potential cause of non-specific binding. To test this hypothesis we have purified antibodies by hydrophobic interaction chromatography (HIC). On the basis of the results we have developed a rapid and simple technique by which the non-specific background can be reduced 2- to 10-fold and the sensitivity improved by a similar factor.

0021-9673/89/$03.50

EXPERIMENTAL

Materials

hCG and free hCGα and hCGβ subunits were obtained from Boehringer Mannheim (Mannheim, F.R.G.).

T-buffer is 0.05 mol/l Tris–HCl (pH 7.7), containing 9 g/l NaCl and 0.5 g/l NaN_3. C-buffer is 0.1 mol/l Na_2CO_3 (pH 9.1). The washing solution contained 9 g/l NaCl, 0.2 g/l Tween 20 and 0.5 g/l NaN_3. The assay buffer used in the immunofluorometric assays (IFMA) was T-buffer containing 5 g/l bovine serum albumin (BSA), 0.5 g/l bovine globulin and 0.1 g/l Tween 40. The enhancement solution contained 6.8 mmol/l potassium hydrogenphthalate, 100 mmol/l acetic acid, 1 g/l Triton X-100, 50 μmol/l tri-*n*-octylphosphine oxide and 15 μmol/l 2-naphthoyltrifluoroacetone (Wallac Biochemical Labs., Turku, Finland).

Six monoclonal antibodies (MAbs) were used. MAbs 1–5 were kindly provided by Drs. Jim Schröder (Department of Genetics, University of Helsinki, Helsinki, Finland) and Robin Fraser (Monoclonal Antibodies, Mountain View, CA, U.S.A.). MAbs 1 and 2 are specific for the free α subunit of the gonadotropins, MAb 3 is specific for the free β subunit of hCG and MAbs 4 and 5 detect both hCG and the free β subunit of hCG. MAb 6 was obtained from Medix Biochemica (Helsinki, Finland). The antibody is specific for hCG and the free β subunit of hCG.

Methods

MAbs 1–5 were purified from ascites by precipitation with Na_2SO_4. Equal amounts of ascites and 36% Na_2SO_4 were mixed and incubated for 10 min at 20°C. After centrifugation at 5000 *g* for 10 min, the precipitate was washed twice with 18% Na_2SO_4 and finally dissolved in C-buffer. After purification with Na_2SO_4, 1 mg of MAb 5 in T-buffer was applied to an 11-ml chromatography column equipped with a frit (Econo-Column®; Bio-Rad Labs., Richmond, CA, U.S.A.), packed with 1.5 ml phenyl-Sepharose (Pharmacia LKB Biotechnology, Uppsala, Sweden) and eluted with T-buffer. Fractions containing non-adsorbed protein were pooled and used to coat microtitre strip wells. The columns were discarded after a single use.

Coating

Antibodies were coated onto the walls of microtitre strip wells (Eflab, Helsinki, Finland). A 200-μl volume of C-buffer, containing 5 mg/l of antibody, was pipetted into the wells and incubated overnight at 20°C. To block non-specific adsorption on the plastic surface, 250 μl of T-buffer, containing 10 g/l BSA, were added to the wells and incubated overnight. The solution was discarded, and the wells were stored in a moist atmosphere at 4°C.

Labelling

MAbs, purified from ascites by precipitation with Na_2SO_4, were labelled with an isothiocyanatophenyl derivative of diethylenetriaminetetraacetate (isothiocyanatophenyl-DTTA), complexed with europium at 100-fold molar excess in C-buffer[2]. After 20 h at 4°C, unreacted chelate was separated from labelled antibody by gel chromatography on a 50 cm × 1 cm Sephacryl S-300 column (Pharmacia LKB Biotechnology) in T-buffer at a flow-rate of 15 ml/h. Fractions of 0.5 ml were collected in tubes, prefilled with 100 μl assay buffer.

HIC

Labelled MAbs were further purified by HIC. The sample was applied to a chromatography column (Econo-Column) packed with 0.8 ml phenyl-Sepharose (Pharmacia LKB Biotechnology) and equilibrated with T-buffer. After application of the sample, the column was eluted at hydrostatic pressure with 10 ml T-buffer. The non-adsorbed fractions were collected and used as a label in IFMA. The columns were discarded after a single use.

Immunofluorometric assay

A 25-μl volume of sample and 200 μl assay buffer were pipetted into the coated wells. After incubation for 2 h, the wells were washed, and 50 ng europium-labelled antibody in 200 μl assay buffer were added. After a further incubation for 1 h, the wells were washed four times and filled with 200 μl enhancement solution. The fluorescence was measured after 5 min in an Arcus 1230 fluorometer. The sensitivity of the assays was calculated from the mean +2 S.D. of twelve replicates of assay buffer (Table I).

RESULTS

Europium-labelled antibodies were purified by gel chromatography. The MAbs were eluted as one smooth peak, and the peak fractions were used as tracers in immunofluorometric assay (Fig. 1). In the immunofluorometric assay with labelled antibody 5 the background was moderately elevated (2200 $\pm$ 363 cps, mean $\pm$ S.D.). When the tracer was further purified by HIC, 80–90% was recovered in the non-adsorbed fractions. When this fraction was used as tracer the background dropped to 275 $\pm$ 29 cps (Fig. 2). This should be compared with the instrument background (53 $\pm$ 8.4 cps), the background of the microtitre strip wells (147 $\pm$ 11 cps) and the enhancement solution (256 $\pm$ 17 cps). The fraction adsorbed to the column was not studied further. The assay sensitivity before and after HIC purification was 2.9 and 0.18 pmol/l, respectively. Similar results were obtained with other HIC-purified antibodies (Fig. 3, Table II). Purification of the europium-labelled MAbs by HIC did not change the maximum signal obtained by immunofluorometric assay.

Normally, the solid phase antibody was purified only by sodium sulphate precipitation. When this antibody was further purified by HIC, 5% was retained on the column. When the non-adsorbed fraction was used for coating, no further reduction of non-specific binding was obtained. However, a two-fold increase in the maximum signal was observed when HIC-purified solid-phase MAb was used in

TABLE I

SPECIFICITY OF THE IMMUNOFLUOROMETRIC ASSAYS

Solid-phase antibody	*Labelled antibody*	*Specificity*
1	2	hCGα
6	3	hCGβ
4	5	hCG, hCGβ

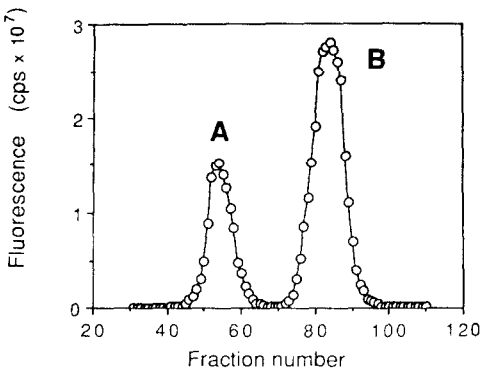
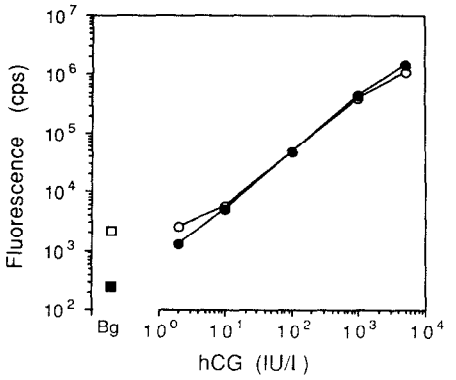


Fig. 1. Gel chromatography of anti-hCG antibody 5, labelled with isothiocyanatophenyl-DTTA-Eu. The labelled antibody corresponds to peak A and the free chelate to peak B.



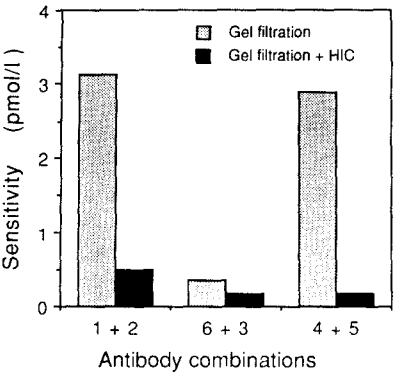


Fig. 2. Standard curve of an hCG immunofluorometric assay. The labelled antibody was purified by gel chromatography (@) and by gel chromatography and HIC (⅛). The background signal (Bg) is denoted by squares.

Fig. 3. Sensitivity of the immunofluorometric assays before and after purification of the labelled antibody by HIC.

TABLE II

SENSITIVITY OF hCG IMMUNOFLUOROMETRIC ASSAYS BEFORE AND AFTER PURIFICATION OF THE LABELLED ANTIBODY BY HIC

Purification method	*Solid-phase antibody*	*Labelled antibody*	*Background fluorescence (mean ± S.D.)*	*Sensitivity (pmol/l)*	*Sensitivity increase (factor)*
Gel chromatography	1	2	2017 ± 250	3.1	
Gel chromatography + HIC	1	2	443 ± 37	0.5	6.2
Gel chromatography	6	3	2156 ± 265	0.36	
Gel chromatography + HIC	6	3	622 ± 91	0.17	2.1
Gel chromatography	4	5	2200 ± 363	2.9	
Gel chromatography + HIC	4	5	275 ± 29	0.18	16.1

combination with a labelled antibody that had been purified by gel filtration and HIC (results not shown).

DISCUSSION

We have developed a novel technique for purification of antibodies used in immunometric assays, based on HIC. This method lowers the background and improves the sensitivity 2- to 16-fold in three different model assays. Earlier, the labelled antibodies were purified by gel chromatography. By this method we have obtained extreme sensitivity for immunofluorometric methods, *i.e.*, an 100-fold improvement over conventional radioimmunoassays. Even these sensitive assays can be further enhanced by a factor of 2 to 3 by using HIC.

The purification technique is simple and rapid. It utilizes small disposable polypropylene chromatography columns, packed with phenyl-Sepharose, and the chromatography is completed within 10 min. After this step, the labelled antibodies show virtually no non-specific binding in the immunofluorometric assay. Our results indicate that the main cause of the background is the presence of hydrophobic europium-labelled proteins. These may be formed as a result of denaturation during the labelling procedure, or hydrophobic proteins may be labelled, contaminating the immunoglobulin G (IgG) preparation. When HIC was used to purify the solid-phase antibody, an increased maximum signal was also obtained. This may have resulted from removal of hydrophobic components from the rather crude IgG preparation which interfered with the binding of the specific MAb to the solid phase.

The sensitivities of the immonofluorometric assays used in this study were all improved remarkably by purifying the labelled antibody by HIC. We use this purification technique routinely for all of our assays.

This technique should also be applicable to other immunometric techniques in which different labels are used and may also improve the specificity of other immunochemical techniques, *e.g.*, immunohistochemistry. Studies are in progress to clarify this possibility.

ACKNOWLEDGEMENTS

This study was supported by grants from the Sigrid Juselius Foundation, the Academy of Finland and the Finnish Cancer Society.

REFERENCES

1 E. Soini and H. Kojola, *Clin. Chem.*, 29 (1983) 65.
2 I. Hemmilä, S. Dakubu, V.-M. Mukkala, H. Siitari and T. Lövgren, *Anal. Biochem.*, 137 (1984) 335.
3 T. Lövgren, I. Hemmilä, K. Pettersson, J. U. Eskola and E. Bertoft, *Talanta*, 31 (1984) 909.
4 U.-H. Stenman, H. Alfthan, T. Ranta, E. Vartiainen, J. Jalkanen and M. Seppälä, *J. Clin. Endocrinol. Metab.*, 64 (1987) 730.
5 L. E. M. Miles and C. N. Hales, *Nature (London)*, 219 (1968) 186.
6 L. Wide, H. Bennich and S. G. O. Johansson, *Lancet*, ii (1967) 1105.
7 T. M. Jackson and R. P. Ekins, *J. Immunol. Methods*, 87 (1986) 13.
8 H. Alfthan, *J. Immunol. Methods*, 88 (1986) 239.