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RAPID DUAL-COLUMN CHROMATOGRAPHIC ASSAY FOR RECOMBINANT LEUKOCYTE INTERFERON α-2

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SUMMARY

A rapid dual-column chromatographic assay for determining recombinant leukocyte interferon α-2 in complex mixtures is described. The assay relies on the use of a high-performance monoclonal antibody affinity column connected in tandem with a reversed-phase column. The high specificity and selectivity of these columns permits the quantitation of subcomponent species, such as interferon oligomers that may be present in assay samples. The assay has a limit of sensitivity equal to 1 μg/g over a range of 1–20 μg/g. The precision of the assay was estimated to be about 5%.

INTRODUCTION

The advantage of dual-column chromatography lies in enhancement of resolution. Complex mixtures very often produce clusters of unresolved peaks when chromatographed on a single column. If these unresolved clusters are isolated and loaded onto a second column, which operates with different selectivity, often baseline separation of individual components is achieved. This principle is well documented in a number of papers on dual-column gas chromatography[1–5]. Several authors[6–15] have extended this concept to liquid chromatography of proteins and metabolites. One example is the analysis of ribonucleotides in urine or deproteinized serum by means of a boronic acid column connected with a conventional reversed-phase column[8].

In this paper, we describe the use of a high-performance immunoaffinity column, containing an immobilized monoclonal antibody directed against leukocyte interferon, connected to a reversed-phase column, for a rapid, automated assay of recombinant interferon. The rapid assay not only determines the concentration of interferon but provides information on the relative amounts of its subcomponents present in crude mixtures. The term high-performance in the case of the immunoaffinity column means merely that the packing material is stable to high flow-rates and high pressure.

EXPERIMENTAL

Preparation of immunosorbent

The procedure for immobilization of monoclonal antibody to the silica support

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was essentially as described by Roy *et al.*[16] and Ohlson *et al.*[17]. Polyhydroxylated silica support (4 g) (GF-200, 20 nm pore size, 20–40 μm, Sota Chromatography, Crompond, NY, U.S.A.) was suspended in distilled water and fines were removed by repeated decantation of the turbid supernatant. To the wet silica cake was added 5 ml of distilled water, containing 100 mg of sodium periodate (Sigma, St. Louis, MO, U.S.A.). The mixture was gently agitated for 30 min at room temperature, then quickly collected on a coarse sintered-glass filter, and washed several times with cold, distilled water.

The monoclonal antibody, LIT-1 (Charles River Biotechnical Services, Wilmington, MA, U.S.A.), was produced in mice from a hybridoma cell line, developed according to the technique described by Kohler and Milstein[18]. The LIT-1 monoclonal antibody was purified from ascitic fluid by ammonium sulfate precipitation and anion-exchange chromatography[19]. The activated support was suspended immediately in 11 ml of purified monoclonal antibody solution, containing 40 mg of protein and 3 mg of sodium cyanoborohydride (Aldrich, Milwaukee, WI, U.S.A.), which was previously dialyzed against the coupling buffer (0.1 *M* sodium phosphate–0.1 *M* sodium chloride, pH 7.0). The suspension was agitated gently overnight at 4°C. The packing material was collected on a sintered-glass filter and washed three times with coupling buffer. The protein content of the filtrate indicated nearly quantitative binding of the antibody to the silica. Therefore, the coupling density was about 10 mg/g of dry sorbent material.

In order to block the unreacted active sites, the resulting immunosorbent was suspended in 40 ml of 1 *M* ethanolamine hydrochloride (Fisher, Springfield, NJ, U.S.A.) at pH 8.1, containing 40 mg of sodium cyanoborohydride. The suspension was agitated gently overnight at 4°C, then washed three times with coupling buffer and stored in distilled water, containing 0.1% (w/v) sodium azide at 4°C. A stainless-steel column (3 × 0.47 cm; bed volume 0.5 ml) was packed with the immunosorbent.

Description of the automated system

The automated system for routine analysis integrated the following components: an automatic gradient controller (Model 680), an automated valve station (Model WAVS, Millipore, Waters Chromatography Division, Milford, MA, U.S.A.), and an autoinjector (Model SIL-6A, Shimadzu Scientific Instruments, Columbia, MD, U.S.A.). The immunoaffinity column was connected to a reversed-phase (RP) column (Chromegabond C-4, 10 cm × 0.2, 5 μm, ES Industries, Marlton, NJ, U.S.A.) via a six-way valve (Rheodyne, Cotati, CA, U.S.A.). This arrangement enabled simultaneous loading, washing, and reequilibration of both columns, or simultaneous elution of the affinity column and loading and elution of the RP column (Fig. 1).

The gradient program, all valve switching and the start command for the integrator (Model 3392A, Hewlett-Packard, Palo Alto, CA, U.S.A.) was controlled by the automatic gradient controller, while the autoinjector initiated the programming sequence.

Preparation of solutions, samples and standards

The following solutions and buffers are used in the assay procedure: (A) 0.2

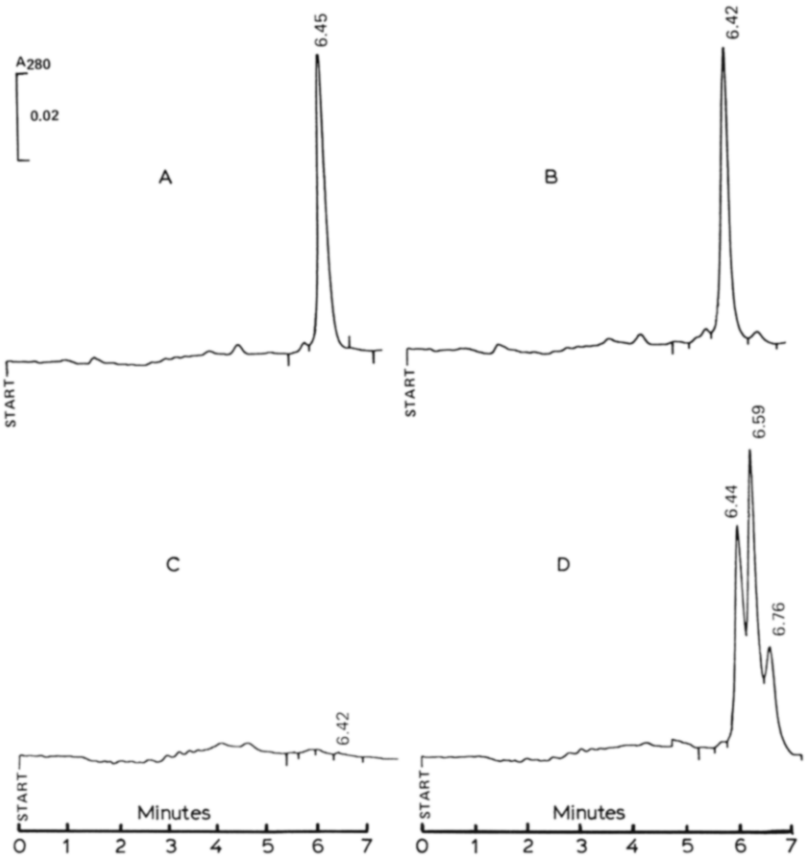

Fig. 3. (A) Loading buffer spiked with interferon α-2 monomer; (B) yeast lysate interferon α-2 gene deleted) spiked with interferon α-2 monomer; (C) yeast lysate (interferon α-2 gene deleted); (D) yeast lysate (interferon α-2 gene inserted).

the corresponding concentrations. Typical chromatograms of standard and crude cell extracts of *S. cerevisiae* (with interferon gene inserted or deleted) are shown in Fig. 3.

RESULTS AND DISCUSSION

Interferon emerges from the reversed-phase column as a series of three peaks of varying magnitude. The relative ratio of the peak areas depends on the source, treatment, and purity of the analyte. The first peak (t_R 6.4 min) was shown to contain mostly interferon monomers with two disulfide bonds, the second peak (t_R 6.6 min) is enriched in monomers with less than two disulfide bonds, while the third one (t_R 6.8 min) is associated with disulfide-linked interferon oligomers. If a longer gradient is applied to the reversed-phase column, further resolution of individual interferon species is achieved (Fig. 4). Thus, the assay gives not only quantitative data on the concentration of interferon in the analyte, but provides information on the amount of the individual interferon components as well.

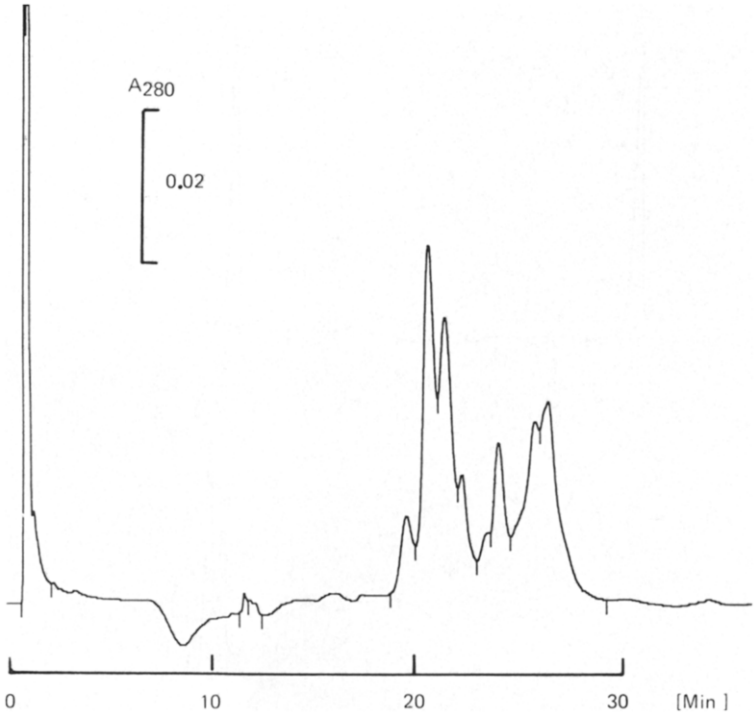

Fig. 4. Chromatogram of interferon α-2 sample from rapid assay. Gradient conditions: flow-rate, 0.3 ml/min; initial 80:20 mixture of solutions E and F, isocratic 1 min, step gradient to 43% F, isocratic 10 min, then 20-min linear gradient to 59% F, followed by 5-min linear gradient to 95% F.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)[22] of elution peaks of crude *S. cerevisiae* extracts and standards are shown in Figs. 5 and 6. The elution peaks (Figs. 5 and 6, lane C, E) show typical patterns of homogeneous interferon bands and the absence of non-interferon (yeast) proteins. Comparison of the load and the corresponding elution peak of the interferon standard gives band patterns that are identical, showing that interferon in the analyte is not affected by the assay. The high specificity of the assay is evident from Fig. 3. The chromatogram (Fig. 3C) of the crude *S. cerevisiae* extract (interferon gene deleted) confirms the absence of any non-interferon bands in SDS-PAGE analysis of peak eluates (see Fig. 5, lane E) by the absence of any peaks in the region where interferon peaks are eluted.

The detection limit of this assay is about 1 μg/g over the range of the assay. In a precision study where the standards were assayed seven times in succession, the relative standard deviation is 3.5% (Table I). The reproducibility of the assay was checked by analyzing the same crude cell extract seven times in succession. The relative standard deviation was 4.7%. In addition, the system was cycled several hundred times without any measurable deterioration in the performance of either column.

Convenient assays for interferon that have been previously described were based on reduction of cytopathic effect[23] and radioimmunoassay, utilizing mono-

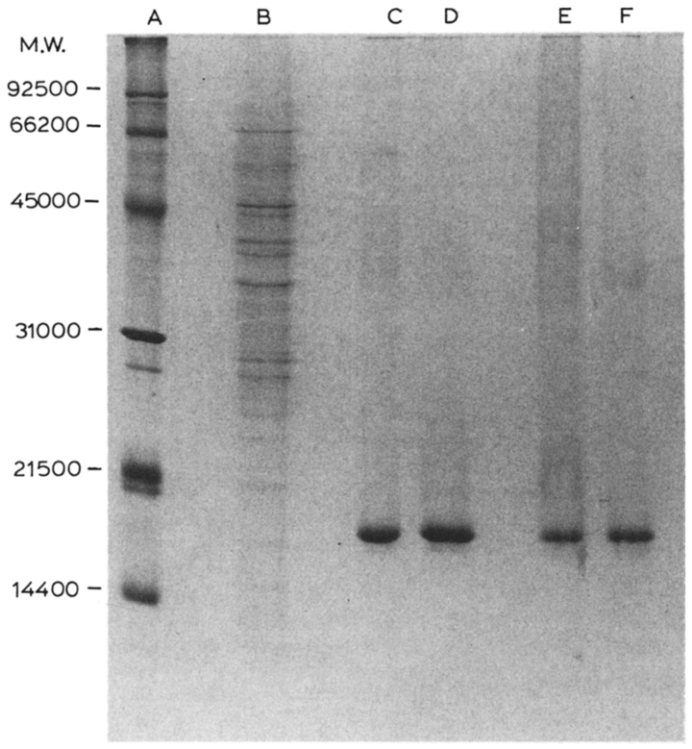

Fig. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, reducing conditions. Peak fractions were dried down in Savant Speed-Vac Concentrator, protein reconstituted in gel loading buffer (Laemmli[22]) containing 20 m*M* of 2-mercaptoethanol, and heated at 90°C for 5 min. (A) Low-molecular-weight markers (Bio-Rad), (B) yeast lysate (interferon α-2 gene deleted), (C) peak of yeast lysate (interferon α-2 gene deleted) spiked with interferon α-2 monomer (8 μg), (D) interferon α-2 monomer (8 μg), (E) peak of yeast lysate (interfgeron α-2 gene deleted) spiked with interferon α-2 standard (5 μg), (F) interferon α-2 standard (5 μg).

clonal antibodies[24]. Both assays had lower limits of sensitivity than the assay described here, but the cytopathic effect assay required 16 h and it was sensitive to biological variations. Neither assay could be used to determine the relative proportions of the interferon subcomponents present in a sample. Pestka *et al.*[25] have described a radioimmunoassay which measured dimers and oligomers in interferon preparations, yet, their assay did not directly measure the monomer concentration. Roy *et al.*[16] described a high-performance monoclonal antibody affinity assay for recombinant leukocyte interferon α, which measured total interferon concentration at a limit of sensitivity of about 30 μg/ml.

The isolation, or formation, of disulfide-linked oligomers of recombinant interferon from *E. coli* has been documented[26]. Although yeast was used as the host organism in this study, it did not provide a simple solution to the formation of disulfide linked oligomers. It has become increasingly important to remove these impurities from preparations of recombinant proteins destined for therapeutic use because, (1) the pharmacological consequences are unknown, and (2) they frequently

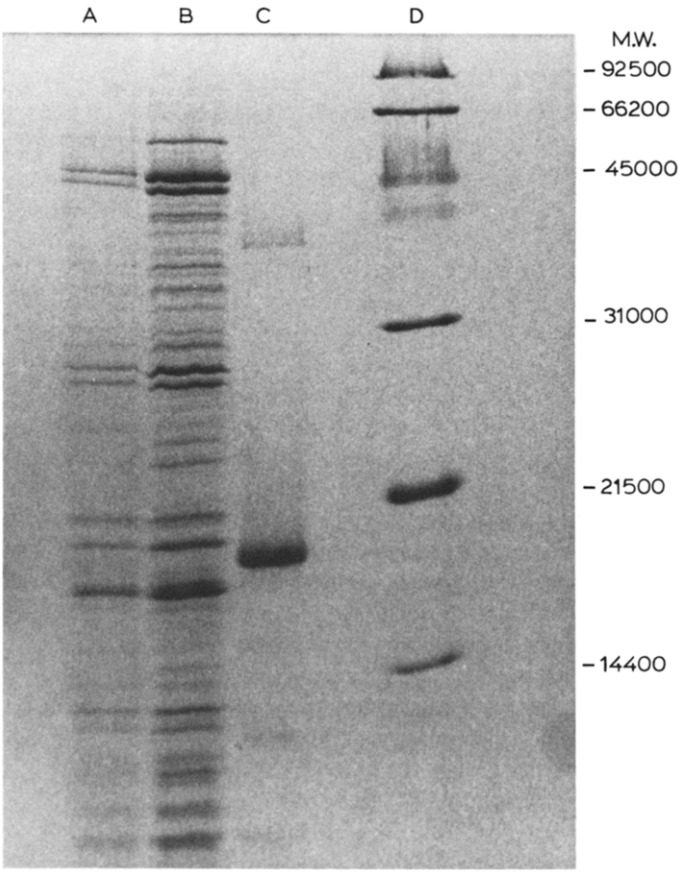

Fig. 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, reducing conditions. Peak fractions were dried down in Savant Speed-Vac Concentrator, protein reconstituted in gel loading buffer (Laemmli[22]) containing 20 m*M* 2-mercaptoethanol and heated at 90°C for 5 min. (A) Yeast lysate (interferon α-2 gene inserted), (B) rapid assay flow-through from yeast lysate (interferon α-2 gene inserted, (C) peak of yeast lysate (interferon α-2 gene inserted, 11 μg), (D) low-molecular-weight markers (Bio-Rad).

TABLE I

ACCURACY STUDY

Concentration added, 15.1 μg/g.

Injection No.	*Concentration (μg/g)*
1	14.8
2	14.4
3	15.0
4	15.6
5	14.7
6	14.3
7	15.6
Mean ± S.D.	14.91 ± 0.52*

* Relative standard deviation, 3.5%.

possess lower *in vitro* biological activity. Also, the appearance of protein oligomers may adversely affect the step yields for a given purification process. Therefore, we recommend the assay described here for process control and process optimization, especially in regard to the origin and formation of disulfide-linked oligomers during purification processes. In addition, the high specificity and selectivity of the assay makes it especially well suited to the evaluation of the effects of excipient ingredients in protein dosage forms.

CONCLUSION

The assay described here has general applicability to any recombinant or natural antigen/antibody system, providing that the antigen is sufficiently hydrophobic to be bound by the reversed-phase column. However, the non-interference of any matrix proteins which may have been non-specifically bound to the affinity column and then eluted together with the antigen into the reversed-phase column must be established in each case.

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