Journal of Chromatography, 327 (1985) 293–299
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 577

METHODS FOR CHROMATOFOCUSING OF CEREBROSPINAL FLUID AND SERUM IMMUNOGLOBULIN G

PAOLO GALLO, OVE OLSSON and ÅKE SIDÉN*
Department of Neurology, Huddinge University Hospital, S-141 86 Huddinge (Sweden)

SUMMARY

Chromatofocusing programs were designed for separations of submilligram amounts of normal and abnormal human IgG. The Pharmacia FPLC system, equipped with a Mono P column or a specially designed, small column was used for the separations. Normal IgG in paired cerebrospinal fluid and serum samples, paired samples from patients with intrathecal immunoglobulin G synthesis, as well as sera with IgG M components were examined. Abnormal immunoglobulin G components, especially those with p*I* values greater than *ca.* 7.0 pH units, were easily identified.

INTRODUCTION

Isoelectric focusing, which separates the sample molecules on the basis of a well-defined physico-chemical parameter, the isoelectric point (p*I*), has proved very useful for examinations of cerebrospinal fluid (CSF) and serum proteins[1]. New developments in ion-exchange chromatography such as chromatofocusing[2–6], offer interesting alternatives for high resolution of proteins. Chromatofocusing is based on the elution of an anion exchanger, adjusted to a certain pH, with a mixture of amphoteric buffers, adjusted to another pH. This results in the formation of a pH gradient, and the proteins are eluted and focused in the order of their p*I* values. The method has been used for studies of proteins in biological fluids, such as CSF and serum[7,8]. So far, however, there are few data available from examinations of CSF proteins in health and disease.

This paper summarizes some methodological data from the application of chromatofocusing to studies of relatively low concentrations of normal and abnormal immunoglobulin G (IgG). The capacity to detect oligoclonal CSF IgG or serum monoclonal components (M components) is evaluated, and some experiences with a specially designed small column are presented.

MATERIALS AND METHODS

Thirty paired CSF and serum samples (ten paired samples with normal IgG and twenty paired samples from patients with intrathecal IgG synthesis) as well as 20 sera with IgG M components were selected for the study. All samples were exam-

0021-9673/85/$03.30

ined by isoelectric focusing, including immunoblotting of IgG[9,10], and the levels of albumin and IgG were determined[11]. The total IgG concentrations of the sera were 6–32 g/l and those of the CSF samples were 16–438 mg/l. All CSF samples from the 20 patients with intrathecal IgG synthesis exhibited oligoclonal bands of IgG on isoelectric focusing, and the CSF IgG index[12] was elevated in the majority of cases. The sera with monoclonal IgG were collected from 20 subjects with benign M components, and all these samples showed distinct bands of microheterogeneous IgG on isoelectric focusing.

Chromatofocusing was performed with the fast protein liquid chromatography (FPLC) system (Pharmacia, Uppsala, Sweden), equipped with a Mono P column or a specially designed small column, based on the same medium. The starting buffer was 0.025 *M* diethanolamine–HCl (pH 9.5); the eluent was prepared from Polybuffer 96 (Pharmacia) and a 10% solution titrated to pH 6.0 with hydrochloric acid was used. All solutions were filtered through a sterile 0.2-μm filter and degassed. The samples were delipidated by chloroform extraction and the IgG concentration was adjusted to 1 mg/ml by dilution with 0.15 *M* sodium chloride or by concentration with vacuum ultrafiltration. Prior to application, the samples were diluted 1:5 in starting buffer, and filtered through a sterile 0.2-μm filter. The column was loaded with 0.1 mg of IgG via a 0.5-ml sample loop. Chromatofocusing was performed at a flow-rate of 0.25 ml/min with 5 ml of starting buffer, followed by 35 ml of the Polybuffer eluent and finally 5 ml of starting buffer; the corresponding volumes used for the small column were 2, 15 and 2 ml. A chart speed of 0.25 cm/min was used for the recorder, and the optical density of the eluate was measured at 280 nm. The reference cell of the UV monitor was filled with the Polybuffer eluent and the range selector was set at 0.02 (Mono P column) or 0.05–0.1 (small column). Determination of the slope and linearity of the pH gradient was performed by measurements on the eluate. Duplicate separations and blank runs were regularly performed in order to control the reproducibility and background absorbance. After each run the columns were regenerated with three injections (1 ml each) of 70% acetic acid and three injections (1 ml each) of 2 *M* sodium chloride, followed by equilibration with starting buffer.

RESULTS

A pH gradient from 9.5 to 6.0 was used since the study was aimed at separations of IgG. The compositions of the starting and eluent buffers were based on instructions from the manufacturer of the equipment. Polybuffer concentrations of 10, 15 and 20% were tested. The higher concentrations make it possible to form the pH gradient with a lower volume, which reduces the dilution of the sample in the eluate. Some samples with distinct IgG bands on isoelectric focusing gave well-separated and very prominent peaks, corresponding to these components when chromatofocused with 15 or 20% Polybuffer. However, these latter eluent concentrations gave a high background absorbance and were unsuitable for the major part of the samples, especially those with normal IgG.

The total protein capacity of the Mono P column is *ca.* 25 mg and that of the small column is about one-third of this amount. Since the present investigation included separations of CSF, it had to be based on much lower amounts of protein.

A total of 0.1 mg of IgG per sample was considered as a realistic amount if chromatofocusing should be more generally applicable for studies of IgG in CSF. It also proved possible to work with this amount protein when the separation program had been designed. Abnormal IgG components could, in fact, be detected with even lower sample loads. All samples were delipidated and carefully filtered in order to avoid deterioration of the column. Flow-rates of 0.25, 0.5, and 1.0 ml/min were compared. The rate of 0.25 ml/min was found to give the best results and was, in spite of the longer separation time, chosen for the present study. However, abnormal IgG fractions were also easily identified when higher flow-rates were used.

Suitable initial volumes of starting buffer for the Mono P column and the small column were found to be 5 and 2 ml, respectively. A total volume of *ca.* 40 ml of 10% Polybuffer 96, (pH 6.0), was needed to bring down the acidic end of the pH gradient to pH 6.0 when the Mono P column was used; the corresponding volume for the small column was *ca.* 15 ml. Most of the separations on the Mono P column were performed on paired CSF and serum samples. The predominant goal of these tests was to evaluate the utility of the method for investigations of oligoclonal CSF IgG. Intrathecally synthesized IgG generally has an alkaline p*I*, which makes the terminal (acidic) part of the pH gradient less important for this application, and the Polybuffer volume was restricted to 35 ml. The choice of the UV-monitor sensitivity has to be based on a compromise between the protein concentration in the eluate and the background absorbance when small amounts of sample are examined. The optimal value of the range selector was found to be 0.02 for the Mono P column and 0.05–0.1 for the small column. Finally, it was observed that a more thorough regeneration and washing of the column after each separation than that recommended by the manufacturer improved the results.

The chromatofocusing programs were found to give well reproducible pH gradients with good linearity, and regularly performed duplicate separations of the sam-

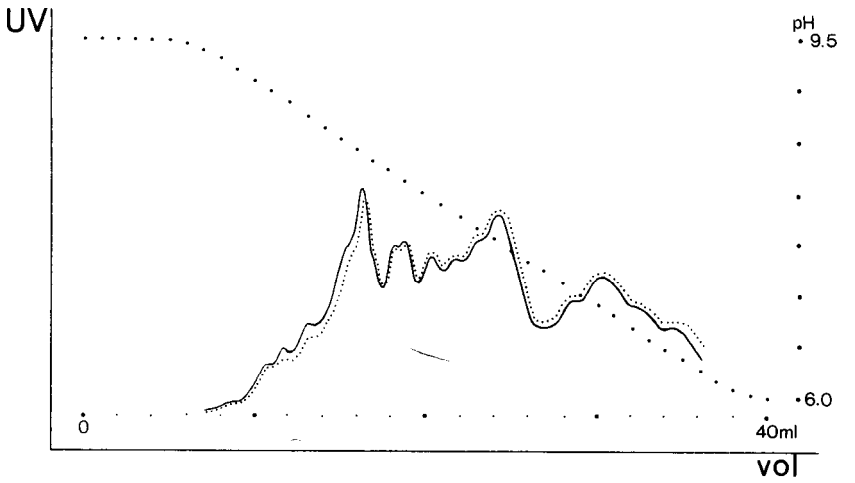

Fig. 1. Chromatofocusing on the Mono P column of CSF (continuous line) and serum (dotted line) from a subject with normal IgG in both samples. The *x* axis (vol) gives the volumes injected onto the column from onset of the elution with Polybuffer 96 and the *y* axis (UV) shows the UV absorbance of the eluate at 280 nm. The pH gradient is indicated by large dots. The CSF and serum profiles are very similar.

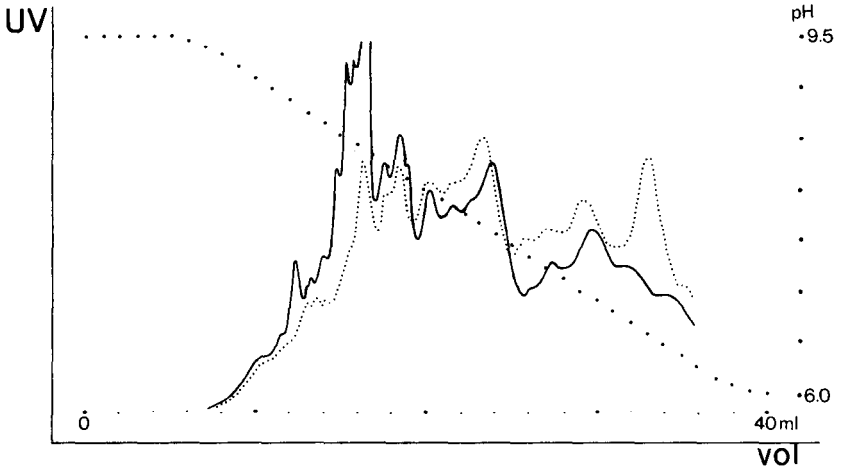

Fig. 2. Chromatofocusing of CSF (continuous line) and serum (dotted line) from a patient with intrathecal IgG synthesis; experimental conditions as in Fig. 1. The CSF profile exhibits distinct peaks, corresponding to oligoclonal IgG.

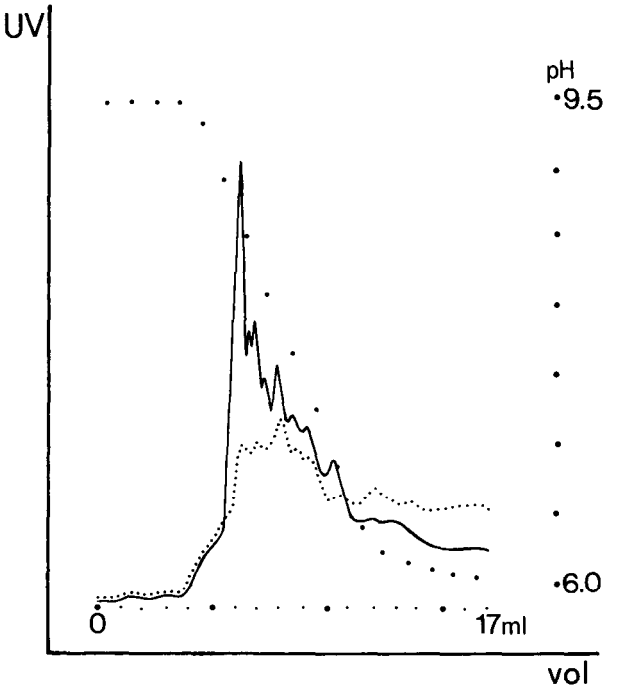

Fig. 3. Chromatofocusing on the small column of CSF (continuous line) and serum (dotted line) from a patient with intrathecal IgG synthesis. The buffer volumes of the separation (2 ml of starting buffer, followed by 15 ml of Polybuffer 96) are indicated along the x axis (vol) and the UV absorbance of the eluate at 280 nm is shown along the y axis (UV). The pH gradient is indicated by large dots. The CSF profile exhibits distinct peaks, corresponding to oligoclonal IgG.

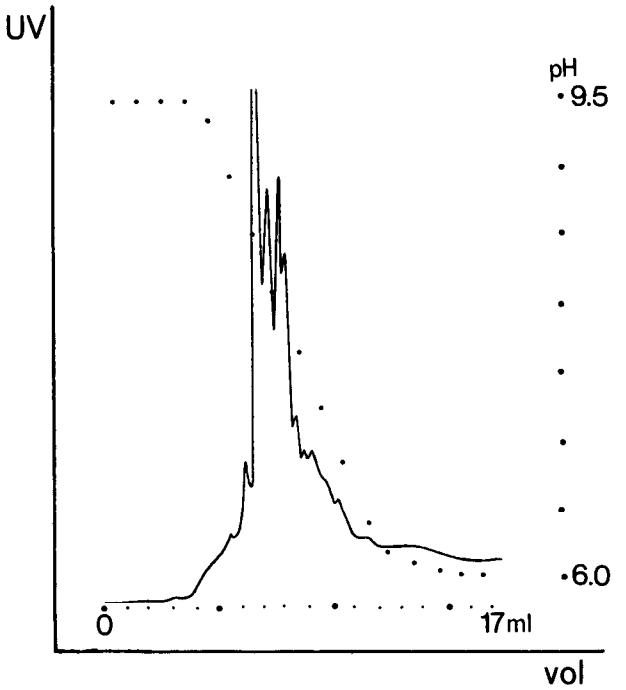

Fig. 4. Chromatofocusing of serum (continuous line) with an IgG M component; experimental conditions as in Fig. 3. The serum profile exhibits distinct peaks, corresponding to the M component.

ples exhibited no significant run-to-run variation. Figs. 1 and 2 show examples from chromatofocusing of paired CSF and serum samples on the Mono P column. The profile with a number of relatively smooth peaks was typical for samples with normal IgG. In these cases, the graphs have a very similar appearance, since the CSF IgG under normal conditions represents a fraction of the serum IgG transported to the CSF. Separations of samples from patients with intrathecal IgG synthesis gave CSF profiles with a number of distinct peaks, and the graphs were clearly different from those of the corresponding sera. Small-column chromatofocusing also showed the same type of general parallel for paired normal samples, as well as distinct CSF/serum differences for subjects with intrathecal synthesis of IgG (Fig. 3). Separations of sera with IgG M components on the Mono P column or the small column (Fig. 4) in all but two cases gave profiles with a number of distinct abnormal peaks. These 18 samples exhibited IgG-band spectra with p*I* of greater than values *ca.* 7.0 pH units on isoelectric focusing. Two sera had IgG bands with p*I* values in the range *ca.* 7.0–6.5 pH units. These two sera exhibited chromatofocusing profiles with prominent but not well-separated peaks in the terminal of the pH gradient.

Biological fluids, such as CSF and serum, contain a mixture of different proteins. Chromatofocusing is based on an isoelectric fractionation of the samples, and the neutral or alkaline p*I* values of the major part of IgG reduce the admixture of other proteins. However, the chromatofocusing profiles should, especially during the period of method development and initial application, be carefully compared with results from examinations of the eluate. The separations on the Mono P column and

the small column were combined with fraction collection. Besides pH measurements, these fractions were used for isoelectric focusing, followed by non-specific protein staining and immunoblotting of IgG. Careful comparisons between these results and the chromatofocusing profiles definitely showed that the peaks were referable to IgG and that mono- or oligoclonal IgG components gave the prominent peaks exemplified in Figs. 2–4. Furthermore, these examinations of the eluate did not indicate that the chromatofocusing procedure introduced false heterogeneities or antigenic changes.

DISCUSSION

Chromatofocusing, based on this broad and predominantly alkaline pH gradient, shows promise for studies of IgG in CSF. The analyses could be performed on submilligram amounts, and all CSF samples with oligoclonal IgG were identified. Intrathecally synthesized IgG generally has the most prominent components in the alkaline p*I* range[13], whereas the majority of other CSF proteins show more acidic p*I* values[14]. This fact favours separation techniques based on isoelectric fractionation.

The method also appears to be a versatile tool for examinations of IgG M components in sera. Samples with monoclonal IgG having p*I* values greater than *ca.* 7.0 pH units gave distinct abnormal peaks. However, a minority of the sera had more acidic M components, and these samples gave equivocal results. This reflects inferior resolution in the terminal part of the pH gradient. The detection of acidic IgG components is also complicated by the admixture of other proteins with similar p*I* values.

Lipids in the samples will decrease the lifetime of the column. Standard procedures for delipidation based on chloroform or ether are simple to perform; chloroform extraction was used in the present study. This will produce some denaturation of certain proteins. However, we did not observe any significant problems for IgG, such as irreproducible chromatofocusing profiles, alterations of the isoelectric focusing patterns, or changed antigenicities. Lipophilic matrices could also be used to delipidate the samples. There are reports of simple methods for the removal of hydrophobic substances by the use of lipophilic dextran[15].

Our results from the specially designed small column are promising; its ability to detect oligo- or monoclonal IgG fractions seems to be comparable with that of the Mono P column. There is a decrease of the peak-to-peak distance, which is caused by the reduced length of the column. However, the small column offers the advantages of a shorter separation time, a decreased dilution of the sample in the eluate (including a more favourable ratio between the sample and background UV absorbances) and a lower consumption of starting and eluent buffers.

The present investigation indicates that chromatofocusing is a useful method for examination of heterogeneous proteins, such as IgG in biological fluids. This technique will probably prove most useful for research work, *e.g.* as a first-step preparative method, aimed at the isolation of different components for complementary investigations. However, in certain situations it may also be a valuable tool for a relatively fast and easy detection of abnormal IgG components.

ACKNOWLEDGEMENTS

This work was supported by grants from the Swedish Society of Medical Sciences and the Tore Nilson Foundation for Medical Research.

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