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SMALL-COLUMN CHROMATOFOCUSING OF CEREBROSPINAL FLUID AND SERUM IMMUNOGLOBULIN G

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SUMMARY

Chromatofocusing performed by the Pharmacia fast protein liquid chromatographic system equipped with a specially designed small column was applied for examinations of submilligram quantities of cerebrospinal fluid and serum immunoglobulin G The separations were based on a pH gradient between 9 5 and 6 0 Mono- or oligoclonal immunoglobulin G components having p*I* values within the optimal working range of the gradient were easily identified and the findings differed clearly from those of normal immunoglobulin G The capacity to detect abnormal immunoglobulin G components compared well with previous experiences from the commercially available Mono P column The small column offers advantages by having a shorter separation time, a decreased dilution of sample in the eluate and a lower consumption of start and eluent buffers

INTRODUCTION

Chromatofocusing is based on the elution of an anion exchanger adjusted to a certain pH, with a mixture of amphoteric buffers adjusted to a different pH [1—4] This results in the formation of a pH gradient and the sample molecules are eluted and focused on the basis of a well defined physicochemical parameter, the isoelectric point (p*I*)

The technique is promising for analytical and preparative separations of proteins in biological fluids. It combines a high resolving capacity, giving peak widths in the pH range 0 04—0.05, with a relatively fast and easy performance Experiences from studies of immunoglobulin G (IgG) in the cerebrospinal fluid (CSF) and serum by use of commercially available equipment have been encouraging [5, 6] This paper gives data from chromatofocusing, using a specially designed small column, of CSF and serum with normal IgG, CSF

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with intrathecally synthesized oligoclonal IgG and sera exhibiting IgG monoclonal (M) components

EXPERIMENTAL

Fifteen paired CSF and serum samples, as well as twenty sera, were selected for the study All samples were examined by isoelectric focusing including immunoblotting of IgG [7, 8], the albumin and IgG concentrations were determined [9] and the CSF/serum albumin ratio as well as the CSF IgG index [10] were calculated for the CSF samples The results of these examinations are summarized in Table I The total IgG concentrations of the CSF and serum samples were 16—438 mg/l and 6—32 g/l, respectively There were five paired CSF and serum samples with normal IgG patterns on isoelectric focusing, the CSF IgG index and CSF/serum albumin ratio gave normal results in all but one case, which exhibited a CSF/serum albumin ratio consistent with a slight blood—brain barrier dysfunction The remaining ten paired CSF and serum samples showed a number of oligoclonal CSF IgG bands not referable to serum changes. These CSF samples showed spectra of six to fourteen IgG bands covering a pH

TABLE I

BASIC DATA OF THE MATERIAL EXAMINED BY CHROMATOFOCUSING

There were 15 CSF samples (10 samples with oligoclonal IgG and 5 normal samples) and 35 sera (15 samples with monoclonal IgG, 5 normal sera and 15 sera collected in pairs with the CSF samples) The frequencies of the quantitative data and the findings of isoelectric focusing are given as the number of samples (n) The occurrence of mono- and oligoclonal IgG on isoelectric focusing is labelled as "abnormal bands"

Quantitative data	Frequency (n)	Frequency on isoelectric focusing with immunoblotting of IgG (n)	
		Normal pattern	Abnormal bands
CSF samples	15		
IgG index*			
⩽0 7	6	5	1
0 8—1 3	4		4
1 4—3 6	5		5
CSF/serum albumin ratio**			
⩽5 5	8	4	4
5 6— 7 5	3		3
7 6—11 1	4	1	3
Serum samples	35		
IgG level***			
⩽10 g/l	13	13	
11—15 g/l	13	7	6
16—32 g/l	9		9

*Increased when > 0 7

**Age-dependent, slightly increases in 3 samples 1 with normal IgG and 2 with oligoclonal IgG bands

***Increased when > 15 g/l

range of 1.0—2.5 within the p*I* region of pH 6.5—9 5 There was an elevation of the CSF IgG index in all but one case, the CSF/serum albumin ratio was normal in eight of the samples and slightly increased in two cases Fifteen sera exhibited IgG M-components giving isoelectric focusing patterns with three to eight IgG bands within a pH range of 0.2—1.0 and these band spectra were situated in the p*I* region of pH 6 4– 9.0 The total IgG concentration was normal in six samples and elevated in nine cases Finally, there were five sera with normal findings after isoelectric focusing and determination of the IgG concentration.

Chromatofocusing was performed by using the fast protein liquid chromatography (FPLC) system (Pharmacia Fine Chemicals, Uppsala, Sweden) with a GP-250 gradient programmer, two P-500 pumps, a V-7 valve, a UV-1 single-path monitor, an REC-482 two-channel recorder and an FRAC-100 fraction collector A specially designed small column was used that had been prepared by the manufacturer of the FPLC system according to instructions from the authors (P.G. and Å.S.) 1 ml of MonoBeds with Polybuffer exchanger 94 packed in an HR 5/5 column (Pharmacia Fine Chemicals), back-pressure approx 10 bar (1 MPa) at a flow-rate of 1 0 ml/min. The starting buffer was 0 025 *M* diethanolamine titrated to pH 9 5 with hydrochloric acid. Three different eluent compositions were used, which were prepared from Polybuffer 96 (Pharmacia Fine Chemicals) with or without addition of Pharmalyte 8 0–-10.5 (Pharmacia Fine Chemicals). 10% (v/v) Polybuffer 96 titrated to pH 6 0 with hydrochloric acid, 5.2% (v/v) Polybuffer 96 with 1% (v/v) Pharmalyte 8.0—10 5 and titrated to pH 7 0 or 8 0 with hydrochloric acid. All solutions were filtered through a sterile 0.45-μm filer (Analytical filter unit, type A. Nalgene Labware Department, Rochester, MN, U S A) and degassed The samples were delipidated by chloroform extraction addition of an equal volume of chloroform to the sample in a glass test-tube, thorough agitation and, 30 min later, centrifugation at 1460 *g* for 10 min The sera were diluted with 0 15 *M* sodium chloride to an IgG concentration of 1 mg/ml and the CSF samples were concentrated to the same level by vacuum ultrafiltration (Ultrahülsen, UH 100/10, Schleicher & Schüll, Dassel, F R G). Prior to application on the column, the samples were diluted 1 5 in starting buffer (giving an IgG concentration of 0 2 mg/ml) and filtered through a sterile 0 2-μm filter (Acrodisc, Gelman Sciences, Michigan, MI, U S A) The column was loaded with 0.1 mg of IgG via a 0.5-ml sample loop. Separation was performed at a flow-rate of 0 25 ml/min with 2 ml of starting buffer followed by 15 ml of the eluent and finally 2 ml of starting buffer 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 eluent and the range selector was set at 0 05—0 1, the lower sensitivity (0 1) was needed to prevent peaks from being too high when samples with prominent IgG components were examined. Determination of the slope and linearity of the pH gradient was performed by fraction collections (1-ml fractions) and pH measurements on each individual fraction Duplicate separations and blank runs were performed regularly in order to control the reproducibility and background absorbance After each run, the column was regenerated with three injections (1 ml each) of 70% (v/v) acetic acid and three injections (1 ml each) of 2 *M* sodium chloride, followed by equilibration with starting buffer

RESULTS

The protein capacity of this chromatofocusing system is in the range 5–10 mg per column, but since the present study included examinations of CSF, it had to be based on much lower protein quantities A total amount of 0.1 mg of IgG per sample is a realistic amount in CSF examinations and the separation program was designed on the basis of this quantity It also proved possible to detect abnormal IgG components with sample loads of 0 05 and even 0 025 mg of IgG, in these cases, the range selector was set at 0 05 or 0 02 The chromatofocusing program was based on a pH gradient formed by the use of diethanolamine · HCl (pH 9.5) as starting buffer and Polybuffer 96 · HCl (pH 6 0) as eluent Narrower gradients were also used for some samples (vide infra). Different eluent concentrations were tested in order to find the composition giving an optimal ratio between background absorbance and the volume needed to form the pH gradient High eluent concentrations allow the formation of the pH gradient with low volumes; this reduces the dilution of the sample in the eluate, as well as the separation time However, the background absorbance is increased to a degree that was found unsuitable for the majority of samples, especially those with normal IgG There will also be a steep pH gradient unless the flow-rate is reduced The concentrations recommended for the commercially available Mono P column were found to be convenient also for the present system. Flow-rates of 0 25, 0 5 and 1 0 ml/min were compared The flow-rate of 0 25 ml/min gave the best resolution and was, in spite of the longer separation time, chosen for the chromatofocusing program. An easy identification of abnormal IgG components was, however, possible also when using the higher flow-rates Initial start buffer volumes of 2 ml of 0.025 *M* diethanolamine · HCl (pH 9 5) were found to give an adequate wash-out of substances not bound to the column. The elutions were performed with 15 ml of 10% Polybuffer 96 · HCl (pH 6 0), which brought the pH of the eluate down to 6 0.

The chromatofocusing program described in Experimental was designed on the basis of the experiments mentioned above. It gave reproducible linear pH gradients, regularly performed duplicate separations of the samples exhibited a high run-to-run concordance The ratio between background and sample absorbance was compatible with the present analytical application of the method. Generally, the major peaks of the chromatofocusing profiles appeared in the volumes between approx. 3 and 9 ml after initiation of the elution with Polybuffer. These major components were mostly followed by smaller peaks in the remaining elution volumes Some minor peaks could occur within the range of ca. 1 ml prior to the major peaks Obvious differences were observed when the chromatofocusing profiles of normal IgG were compared with those of samples exhibiting mono- or oligoclonal IgG components. These latter IgG components, having a restricted p*I* heterogeneity, gave distinct individual peaks In contrast to this, samples with normal polyclonal IgG gave smoother chromatofocusing profiles because of the more even heterogeneity within the distribution of IgG

The findings from chromatofocusing of paired CSF and serum samples with normal IgG are exemplified in Fig 1. Such separations gave graphs with a number of relatively smooth peaks and since the CSF IgG normally derives

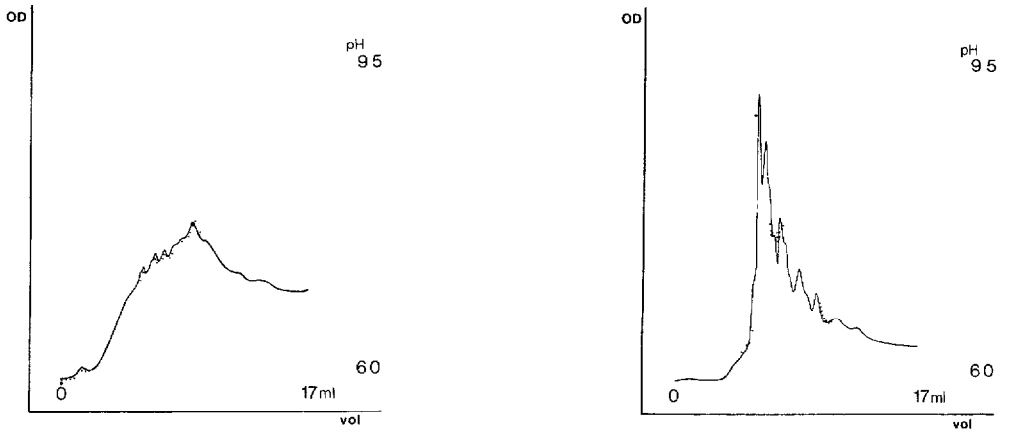

Fig 1 Chromatofocusing of CSF (continuous curve) and serum (dotted curve) from a subject exhibiting normal IgG in both samples The elution was performed with 10% Polybuffer 96 · HCl (pH 6 0) at a flow-rate of 0 25 ml/min The pH values between 9 5 and 6 0 (0 5 units between each dot) are indicated to the right and the dotted line shows the pH gradient The x-axis (vol) gives the volumes (2 ml of starting buffer followed by 15 ml of Polybuffer 96 · HCl, pH 6 0) injected into the column after application of the sample, the y-axis (OD) shows UV absorbance of the eluate at 280 nm There is a very close parallelism between the CSF and serum profiles

Fig 2 Chromatofocusing of CSF (continuous curve) and serum (dotted curve) from a patient with intrathecal synthesis of oligoclonal IgG The symbols and experimental conditions are as in Fig 1 There are distinct peaks in the CSF profile, which correspond to oligoclonal IgG

from serum, the profiles of the paired samples in all cases were found to have a very similar appearance Fig 2 gives the results from chromatofocusing of CSF with intrathecally synthesized oligoclonal IgG and the corresponding paired serum sample. The CSF in all cases gave profiles exhibiting distinct peaks and an obvious difference from the profile of the corresponding serum Fraction collection followed by isoelectric focusing of the material and immunoblotting of IgG verified that these peaks were referable to oligoclonal IgG Furthermore, the components were not found to have changed their isoelectric focusing patterns or antigenicities during the chromatofocusing procedure Fig. 3 shows the results from chromatofocusing of serum with an IgG M-component. The microheterogeneous abnormal IgG in these sera gave prominent peaks in the graph and the findings differed clearly from those of normal IgG, the identity of the components giving these peaks was verified as described for the CSF. There were thirteen sera with M-component band spectra having pI values of pH $>$ 7 0 and all these samples gave chromatofocusing profiles having the general characteristics exemplified in Fig. 3. Two sera had M-components with band spectra restricted to the pI range of pH $\approx$ 7 0—6.5. These two samples exhibited chromatofocusing profiles with prominent, but not well separated, peaks in the terminal part of the pH gradient This is casued by a deterioration of the resolution in the terminal part of the pH gradient, similar experiences have been made with the Mono P column.

Samples with mono- or oligoclonal IgG components having pI values

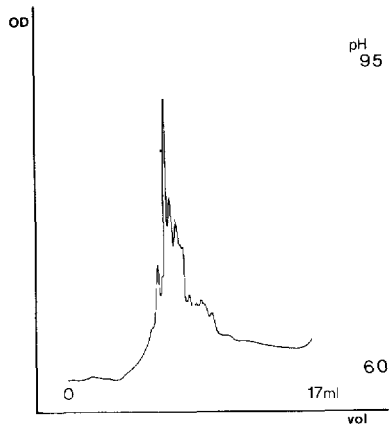

Fig 3 Chromatofocusing of serum with an IgG M-component (continuous curve) The symbols and experimental conditions are as in Fig 1 The profile exhibits prominent peaks referable to the M-component

between pH 9 0 and 8 5 were used in order to compare the resolution capacities of the three different gradients from pH 9 5 down to about pH 6 0, 7 0 and 8 0, respectively The two latter, narrower gradients gave a better peak separation of these components However, the chromatofocusing runs performed using Polybuffer 96 · HCl (pH 6 0) gave quite comparable results This gradient also has a broader optimal working range, which is advantageous in separations of heterogeneous proteins such as IgG

DISCUSSION

Electromigration techniques such as isoelectric focusing performed in agarose or polyacrylamide gels have proved very suitable for examinations of many proteins, including those of the CSF [11, 12]. Another important group of methods for protein separations are the chromatographic techniques where the sample molecules are partitioned on the basis of physicochemical or biological properties Chromatofocusing, a chromatographic analogue of isoelectric focusing, has several points in its favour for examinations of proteins in biological fluids e.g. high resolution, relatively fast and easy performance, comparatively mild separation conditions, capacity to work in analytical as well as preparative scale

The major part of human IgG exhibits neutral or alkaline p*I* values [13] and intrathecally synthesized oligoclonal CSF IgG is generally most prominent in the high alkaline range [12] Most other CSF proteins have more acidic p*I* values [14] and this favours the use of techniques based on isoelectric fractionation in examinations of CSF IgG. The present investigation was based on chromatofocusing of normal and abnormal CSF and serum IgG by using a specially designed small column This method can work with submilligram quantities of proteins, which is a prerequisite for a successful, more general application in CSF protein examinations A pH gradient formed by starting and eluent buffers with pH values of 9 5 and 6 0, respectively, was found to be well suited for examinations of human IgG Narrower gradients improve the peak

separation but they also reduce the working range of the system and this is less advantageous when proteins such as IgG, having a wide range of p*I* values, are examined

Lipids in the samples will cause a deterioration of the column and a chloroform extraction was used to delipidate the material prior to chromatofocusing Such treatment gives some denaturation of certain proteins However, we did not observe any significant problems for IgG, such as changed antigenicities, altered isoelectric focusing patterns or irreproducible chromatofocusing profiles Alternative methods for delipidation could also be applied, e g techniques based on the use of lipophilic dextran [15]

At this stage, we have found major utilities for chromatofocusing in research work, e g. as a first-step preparative procedure, but the method also has potential for clinical applications The capacity of the small chromatofocusing column to detect mono- or oligoclonal IgG components having p*I* values within the optimal working range (pH > 7) of the pH gradient compared well with that of the commercially available Mono P column There is a decrease of the peak separation due to the reduced length of the column However, the resolution capacity is still very satisfying for heterogeneous proteins such as IgG. The small column also offers the advantages of having 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.

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