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# ANION-EXCHANGE CHROMATOGRAPHY OF NORMAL AND MONOCLONAL SERUM IMMUNOGLOBULINS

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## SUMMARY

Anion-exchange chromatography was used for separations of the three main immunoglobulin classes in human sera. The material included normal sera as well as sera with monoclonal immunoglobulin components. The Mono Q anion-exchange column was eluted with a stepwise gradient formed by 0.05 and 0.3 *M* phosphate buffers. No indications of negative effects on the sample components were observed and the protein recovery was high. A fast, easy and reproducible separation of immunoglobulins G, A and M was obtained.

## INTRODUCTION

Two major categories of procedures are used to separate different protein species: electromigration techniques and liquid chromatography. These methods enable the separation of the sample molecules on the basis of size, hydrophobicity, charge or binding to a biospecific ligand. Ion exchangers suitable for the separation of biological materials were developed three decades ago [1], and ion-exchange chromatography has become an important method for protein fractionation. These media can also be used for chromatofocusing [2] where the sample molecules are eluted on the basis of their isoelectric points (p*I*).

One important advantage of liquid chromatography is that it is well suited for preparative separations. Modern anion-exchange chromatography has given

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TABLE I

BASIC DATA FOR THE THIRTY SERA

The frequencies are given as the number of samples ($n$).

| Sample | Frequency ($n$) | M-Component class and light chain type ($n$) | | | | | Concentration of the immunoglobulin class exhibiting an M-component (g/l) |
|---|---|---|---|---|---|---|---|
| | | IgG | IgA | IgM | $\kappa$ | $\lambda$ | |
| Normal sera | 8 | | | | | | |
| Sera with M-components | | | | | | | |
| Benign monoclonal gammopathy | 12 | 8 | | | 4 | 4 | 14.5–22.0 |
| | | | 2 | | 1 | 1 | 12.0 and 19.3 |
| | | | | 2 | 1 | 1 | 13.6 and 20.5 |
| Myeloma | 8 | 4 | | | 3 | 1 | 25.0–63.5 |
| | | | 2 | | 2 | | 25.7 and 37.0 |
| | | | | 2 | 2 | | 42.0 and 49.5 |
| Macroglobulinaemia | 2 | | | 2 | 2 | | 75.0 and 95.7 |

promising results in the separation of immunoglobulin (Ig) classes in human sera [3,4]. The present study reports data from anion-exchange chromatography performed with the fast protein liquid chromatography (FPLC) system on human sera with normal IgG, IgA and IgM, as well as sera exhibiting monoclonal (M) components of these immunoglobulin classes.

## EXPERIMENTAL

### *Samples*

Thirty human serum samples with normal immunoglobulins or M-components of IgG, IgA or IgM identity were examined. Basic data are listed in Table I.

### *Anion-exchange chromatography*

The FPLC system (Pharmacia, Uppsala, Sweden) had the following components: LCC-500 liquid chromatography controller, two P-500 pumps, 0.6-ml mixer, MV-7 motor value, UV-1 single-path monitor with an HR flow-cell and a 280-nm filter, REC-482 two-channel recorder, FRAC-100 fraction collector with a PSV-100 valve, two prefilters, and 0.5- and 1-ml sample loops. The separations were carried out using an HR 5/5 Mono Q column (Pharmacia) and 0.05 *M* phosphate buffer, pH 6.7 (buffer A), and 0.3 *M* phosphate buffer, pH 6.5 (buffer B). Deionized water was used for the buffers, which were prepared from a stock solution made by mixing 640 ml of 0.3 *M* sodium dihydrogen phosphate (Merck, Darmstadt, F.R.G.) and 500 ml of 0.3 *M* dipotassium hydrogen phosphate (Merck). The buffers were filtered through a sterile 0.22-$\mu$m filter (Acrodisc; Gelman Sciences, MI, U.S.A.) followed by a thorough degassing and final adjustment of the pH with 1.0 *M* hydrochloric acid. The material loaded onto the column was standardized as follows: 1 mg of IgG for normal sera and those with IgG M-components, 1 mg of IgA/M for sera with IgA/M M-components. The sera were diluted with phosphate-buffered saline (PBS) to a level of the respective

immunoglobulin class of 1 mg per 0.1 ml. After this, the samples were diluted 1:5 with buffer A, filtered through a 0.22-$\mu$m filter (Acrodisc) and checked with regard to any unwanted turbidity. A volume of 0.5 ml of these dilutions was loaded onto the column. The elution was performed at a flow-rate of 2 ml/min by use of a stepwise gradient: 12 ml of 100% buffer A, 50% buffer A plus 50% buffer B in the following 12 ml, 100% buffer B during the next 2 ml and finally 4 ml of 100% buffer A. The absorbance of the eluate was measured at 280 nm and the range selector was set in the range 0.1–0.2 a.u.f.s. Fraction collection was volume-based. After each separation, the column was reverse-washed with three injections (1 ml each) of 70% (v/v) acetic acid followed by three injections (1 ml each) of 2 *M* sodium chloride, and finally equilibrated with buffer A.

*Protein analyses*

Enzyme-linked immunosorbent assay, immunoprecipitation nephelometry or radioimmunoassay was used for quantitations of the individual proteins. Isoelectric focusing and protein detection were based on previously described principles [5,6]. The focusing was performed for 70 min at 15 W [initial voltage 300 V (50 mA), final voltage 800 V (18 mA)] in 2-mm thick 0.8% (w/v) agarose gels (Isogel; FMC, Marine Colloids Division, Rockland, ME, U.S.A.) with 5.4% (v/v) Ampholine carrier ampholytes pH 3.5–10 (LKB, Stockholm, Sweden). IgA and IgM were analysed in a native state as well as after a mild reduction. This latter procedure was performed at room temperature by mixing 10 $\mu$l of a sample containing 5 $\mu$g of IgA/M with 10 $\mu$l of 0.8 *M* $\beta$-mercaptoethanol (Sigma, St. Louis, MO, U.S.A.) and 80 $\mu$l of PBS. After incubation for 10–15 min, 10 $\mu$l (i.e. 0.5 $\mu$g of IgA/M) of this solution was applied on the gel. Specific detection of IgG, IgA and IgM after isoelectric focusing was done by blotting to nitrocellulose membranes (BA 85, No. 401198; Schleicher & Schüll, Dassel, F.R.G.) and immunoperoxidase staining. After blotting and blocking of the empty protein-binding spaces, the nitrocellulose membranes were washed for 10 s in PBS followed by incubation for 2 h with a 1:500 dilution in PBS of the primary antisera: rabbit antihuman sera specific for IgG Fc-fragments, $\alpha$-chains or $\mu$-chains (Dakopatts, Glostrup, Denmark). The membranes were then washed four times (15 min each) in PBS and incubated for 2 h with the secondary antiserum: peroxidase-conjugated swine antiserum to rabbit IgG (Dakopatts) diluted 1:500 in PBS. This was followed by four washes (15 min each) in PBS and peroxidase staining. Two-dimensional gel electrophoresis and silver staining of the proteins in the second-dimension gels were performed according to previously described methodologies [7–9].

## RESULTS

Different buffer compositions, gradient designs and flow-rates were tested during the initial part of the study. The results obtained during that stage will not, with one exception, be referred to further. Certain proteins that were eluted in the volumes between IgG and IgA exhibited a significant influence on their chromatographic behaviour by relatively moderate changes of the buffer A molarity.

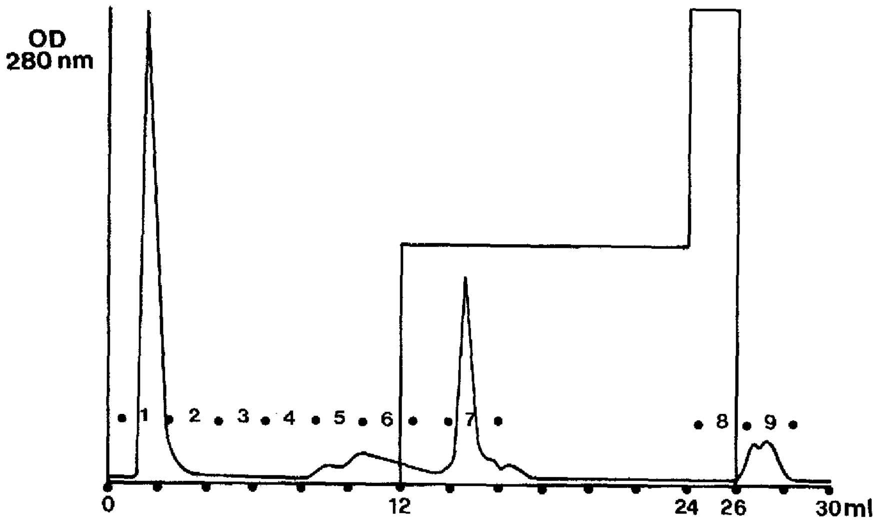


Fig. 1. Chromatography of serum with an IgG M-component. The $x$-axis gives the buffer volumes and the $y$-axis shows the UV absorbance of the eluate. The buffer B gradient is also indicated. Collection of 2-ml fractions was performed as shown by the numbered intervals across the graph: it was started with a 0.5-ml delay and then run consecutively up to 12.5 ml, the next fraction covered the 14–16 ml interval and the final two fractions included the 24.5–28.5 ml interval.

A decrease of this buffer concentration from 0.05 to 0.025 *M* impaired the separation of IgG from these proteins. The chromatographic conditions described in Experimental were found to be well suited for the present application. Duplicate separations of the samples exhibited a high run-to-run reproducibility, and the protein recovery was high. Altered antigenicities or isoelectric focusing patterns of the proteins were not observed. The chromatographic profiles of the sera exhibited the following general characteristics. There were three groups of peaks (Fig. 1), appearing in the initial, intermediate and terminal volumes, respectively. The first and last groups were relatively narrow but the intermediate group exhibited one distinct peak and adjoining lower components. Those of the latter components that preceded the distinct peak were the ones that were found to be eluted closer to IgG if the molarity of buffer A was lowered. IgG, IgA and IgM were eluted from the column with the initial, the most distinct intermediate and the terminal peaks, respectively.

It was found that more than 95% of the IgG eluted was contained in the first 5 ml. A few percent of the IgG appeared later, predominantly the next 2 ml. All sera with IgG M-components exhibited a minor IgG contamination (less than 1% of the IgG) in the IgA fractions, whereas most normal sera gave a complete separation of these two immunoglobulins. No IgG was detected in the IgM fractions. Fig. 1 is a chromatogram of a serum sample with an IgG M-component and Fig. 2 shows the results of isoelectric focusing of the collected material. In this case, ca. 90% of the IgG eluted was obtained in the 0.5–2.5 ml interval and ca. 10% in the 2.5–4.5 ml interval. As can be seen from Fig. 2, these two fractions were the only ones in which IgG was detected with isoelectric focusing followed by nitro-

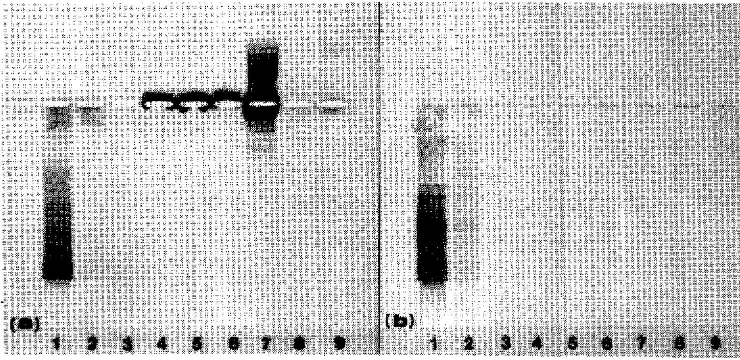


Fig. 2. Isoelectric focusing of the fractions collected during the chromatography shown in Fig. 1. (a) Coomassie staining and (b) immunoperoxidase detection of IgG after nitrocellulose blotting. The anodal end of the gel is at the top and the fraction numbers are shown at the bottom.

cellulose blotting and immunoperoxidase staining. However, radioimmunoassay detected minor amounts of IgG in the volumes up to and including those with IgA. The results from two-dimensional gel electrophoresis of the fractions with the major part of IgG from a normal serum sample are shown in Fig. 3. The second-dimension gel exhibited components of hemopexin and transferrin but otherwise only $\gamma$-, $\kappa$- and $\lambda$-chains as well as some incompletely degraded IgG were clearly visualized.

IgA was eluted within a 4-ml interval beginning after a volume of ca. 14 ml, i.e. 2 ml after the first change of the gradient. By analogy with the results for sera with IgG M-components, samples with monoclonal IgA gave a minor contamination (less than 1% of the IgA) of the immunoglobulin appearing after IgA (i.e.

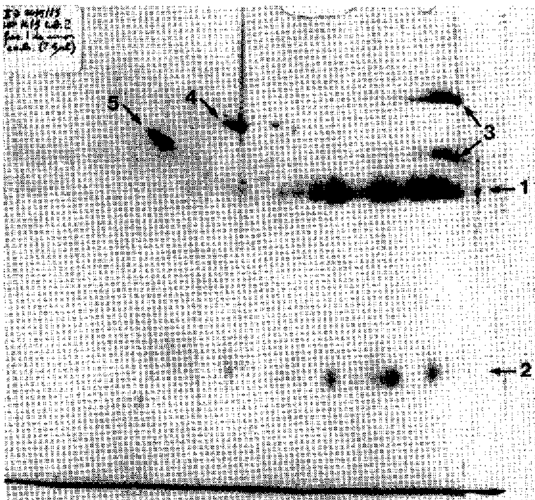


Fig. 3. Two-dimensional gel electrophoresis of the IgG fractions from a normal serum. The cathodic end of the first-dimension isoelectric focusing was to the right and the polyacrylamide gel electrophoresis was run from top to bottom (silver staining). The gel exhibits IgG $\gamma$ (1) and light chains (2), some incompletely degraded IgG (3), transferrin (4) and hemopexin (5).

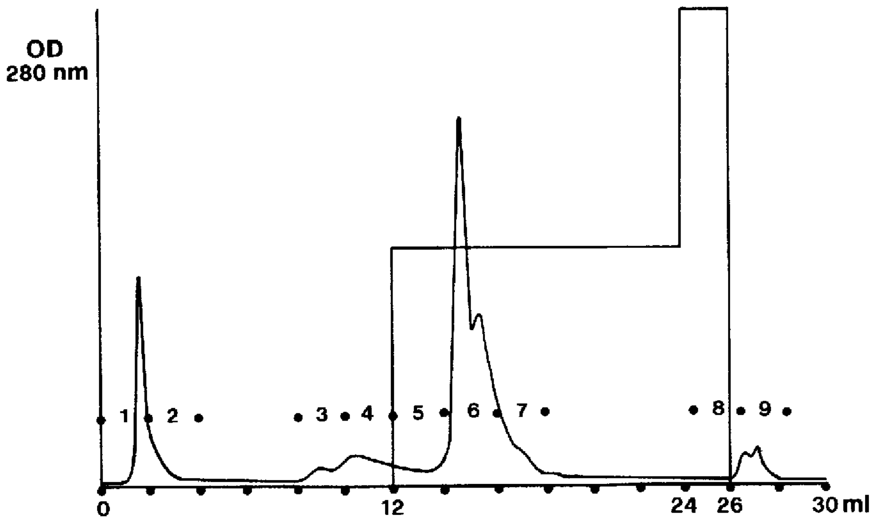


Fig. 4. Chromatography of serum with an IgA M-component. The symbols and experimental conditions are as in Fig. 1. However, the first two fractions were collected between 0 and 4 ml and the ensuing five fractions in the 8–18 ml interval; the final two fractions were collected as in Fig. 1.

IgM), while no IgA was found in the IgG fractions. Fig. 4 exemplifies the results from a separation of serum with an IgA M-component. Approximately 90% of the IgA eluted was in this case obtained in the 14–16 ml interval and ca. 10% in the ensuing 2 ml. Fig. 5 shows that these two 2-ml fractions were the only ones where IgA could be detected by nitrocellulose blotting and immunoperoxidase staining after isoelectric focusing. However, radioimmunoassay showed a minor amount of IgA also in the IgM-containing volume. Fig. 5 also illustrates the deficient migration of native IgA into the agarose gel. Conclusive restricted IgA heterogeneities were observed on isoelectric focusing only after reduction to monomeric IgA by use of $\beta$-mercaptoethanol. This was the case for all IgA M-

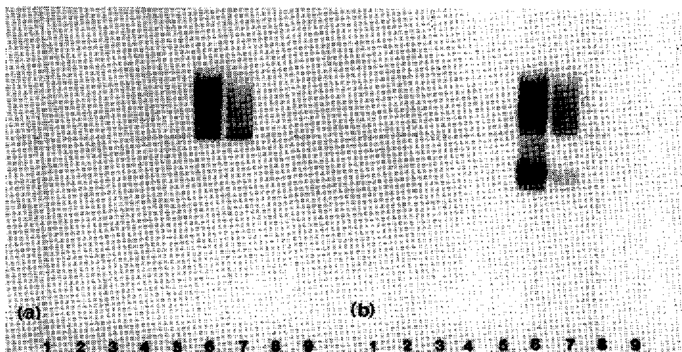


Fig. 5. Isoelectric focusing of the fractions collected during the chromatography shown in Fig. 4 before (a) and after (b) reduction with $\beta$-mercaptoethanol; immunoperoxidase detection of IgA after nitrocellulose blotting. The anodal end of the gel is at the top and the fraction numbers are shown at the bottom.

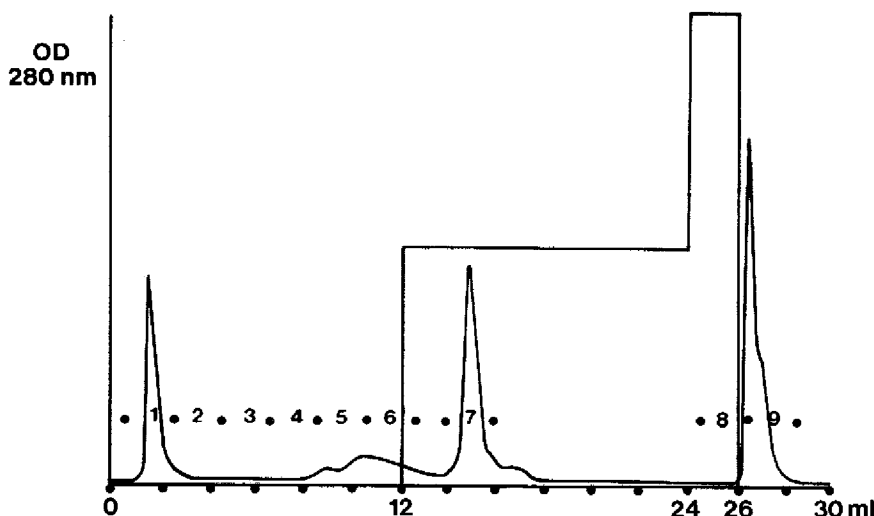


Fig. 6. Chromatography of serum with an IgM M-component. The symbols, experimental conditions and fraction collection mode are as in Fig. 1.

components. After reduction, the M-components gave at least four bands in the p*I* interval of ca. pH 6–7.

The IgM was eluted during the final part of the chromatography. The peak appeared after the change from 100% buffer B to 100% buffer A, i.e. after the 26th ml of the separation, and was obtained in the following 2–3 ml. As opposed to samples with monoclonal IgG or IgA, sera with IgM M-components exhibited no contamination of the other two immunoglobulin classes by IgM. Fig. 6 gives the results from chromatography of serum with an IgM M-component. Small amounts of IgM were obtained in the 24.5–26.5 ml interval, which included the initial part of the IgM peak, and the major portion of IgM (more than 95% of the amount eluted) appeared in the 26.5–28.5 ml interval, which included the predominant part of the peak. Fig. 7 shows the results from isoelectric focusing of the collected fractions. Immunoperoxidase staining after nitrocellulose blotting detected IgM only in the two previously mentioned fractions. The deficient migration into the gel of the native pentameric IgM is also clearly demonstrated. After reduction with $\beta$-mercaptoethanol, the monomeric molecules moved into the gel and showed distinct restricted heterogeneities. The same was found for all IgM M-components which, after reduction, gave at least four bands in the p*I* interval of ca. pH 6–7. Usually, the IgM M-components exhibited a higher heterogeneity than such components of IgA identity.

All samples applied onto the column had a total protein amount of less than ca. 10 mg, and their IgG (normal sera and sera with IgG M-components), IgA (sera with IgA M-components) and IgM (sera with IgM M-components) contents were 1 mg. The mode of standardization that was used, i.e. amount of a certain immunoglobulin class, gave wide differences between the samples with regard to their contents of other immunoglobulins as well as non-immunoglobulin proteins. Regarding these latter proteins, it can be generally stated that their

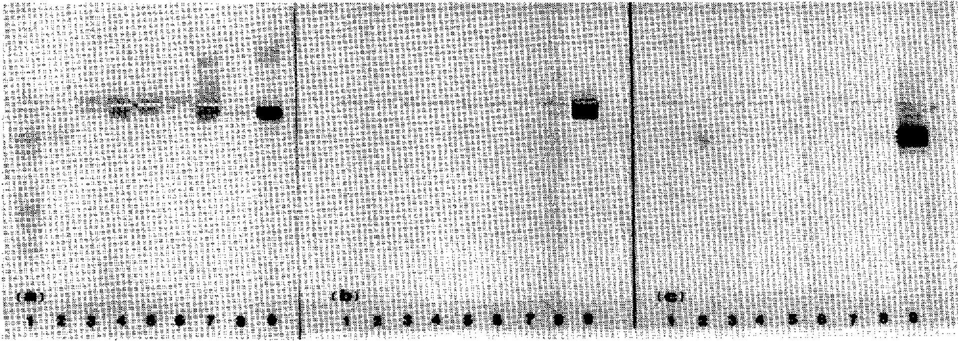

Fig. 7. Isoelectric focusing of the fractions collected during the chromatography shown in Fig. 6. (a) Coomassie staining and (b, c) nitrocellulose blotting with immunoperoxidase staining of IgM before (b) and after (c) reduction with $\beta$-mercaptoethanol. The anodal end of the gel is at the top and the fraction numbers are shown at the bottom.

amounts in the IgG and IgM fractions were low (less than ca. 10%), while the IgA fractions had a higher admixture (more than ca. 50%) of such proteins, predominantly albumin and transferrin. Data on the partition between the three main immunoglobulin classes have already been given. The recoveries of these immunoglobulins were in the range 80–90%. Their dilutions during the chromatography (expressed as the ratio between the volume of the samples and the collected fractions, respectively) were at least 1:4.

## DISCUSSION

This study was aimed at the design and evaluation of a chromatographic system for preparative separations of immunoglobulins in biological fluids. According to the manufacturer, the Mono Q column has a protein capacity in the region of 25 mg per column or 5 mg per single peak. In the present investigation, submaximal protein loads were used firstly to keep an adequate margin against column overloading and secondly to find a program suitable for applications on cerebrospinal fluid. Sera with M-components were included in the material because of our interest in these immunoglobulin aberrations as well as their suitability for evaluation of the chromatographic method. By using such samples, it is possible to apply material having high relative concentrations of either immunoglobulin class onto the column. This will simplify analyses of the separation capacity of the system and reduce the risk of irrelevant artefacts from column overloading by other proteins. Another advantage of these sera is the occurrence of distinct M-component band patterns after isoelectric focusing. Such microheterogeneous components are easy to detect in collected fractions, and an unchanged p*I* pattern after the chromatographic separation will argue against negative effects on the proteins of interest. Finally, it may be relevant to comment briefly on the results from isoelectric focusing of IgA and IgM M-components. Such components have been successfully resolved in agarose gel or composite acrylamide–agarose gels [10,11]. We did, however, observe a deficient migration

of these M-components at agarose gel isoelectric focusing when they were run in a native state.

Column care is an important part of liquid chromatographic methodologies. For the present system, this includes care with regard to lipids in the samples. However, we wanted to collect immunoglobulin fractions in as native a state as possible since they were to be further investigated in another project. Because of this, no procedure for delipidation of the sera was used. A certain risk of a reduced column lifetime will then be introduced, even when the samples are carefully filtered and no turbid material is applied to the column. It was found advantageous to wash the column after each run with an acid as well as a salt solution. If a delipidation is necessary, this can be achieved by a simple chloroform extraction or the use of lipophilic dextran [12]. The compatibilities of such procedures with the proteins of interest should then be evaluated. A chloroform extraction does not, according to our experience, have a negative effect on IgG, such as changed antigenicities or altered isoelectric focusing patterns [13].

There are several examples of situations in which immunological diagnostic tests are optimized by a separation of the immunoglobulin classes [4]. Furthermore, a first-step preparative fractionation prior to immunoglobulin investigations by isoelectric focusing or two-dimensional gel electrophoresis may be necessary in order to improve the standardization and/or reduce the risk of gel overloading. A very efficient separation between IgG and IgM was obtained with the technique used in this study. The amounts of non-immunoglobulin proteins in these fractions were quite low and the admixture of such proteins in the IgA fractions was higher. Sera with IgG or IgA M-components gave some contamination from these immunoglobulins in the IgA and IgM fractions, respectively. The separation of IgG from IgA and IgA from IgM was nevertheless quite satisfactory. The present method for anion-exchange chromatography is easy to perform, fast (15 min analysis time) and utilizes mild chemical conditions. Furthermore, the chromatographed material can be used directly, or after a simple concentration/dilution procedure, for further investigations by quantitative methods, isoelectric focusing or two-dimensional gel electrophoresis.

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