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FAST PROTEIN LIQUID CHROMATOGRAPHY SCALE-UP PROCEDURES FOR THE PREPARATION OF LOW-MOLECULAR-WEIGHT PROTEINS FROM URINE

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SUMMARY

A system for the rapid isolation of low molecular weight proteins from urine has been devised, and illustrated by α_1-microglobulin, β_2-microglobulin, retinol binding protein, lysozyme and monoclonal light chains. Urine proteins from patients with tubular dysfunction were concentrated, either by ultrafiltration or ammonium sulphate precipitation. This was followed by gel chromatography on Sephadex G-50.

The appropriate fractions were then separated by chromatography on Pharmacia monobead columns. A Mono Q strong anion exchanger was used for β_2-microglobulin, retinol binding protein, α_1-microglobulin and free monoclonal light chains. Lysozyme was separated on a Mono S cation exchanger. The chromatography was first optimized on HR 5/5 columns and then scaled up to HR 16/10 columns.

INTRODUCTION

Low-molecular-weight plasma proteins (<40 kilodalton) are cleared from the circulation by renal glomerular filtration and subsequently reabsorbed and catabolized by the renal proximal tubular cells[1]. Failure of the kidney to reabsorb the proteins, their over-production and combination of both of these factors can cause increased amounts of low-molecular-weight plasma proteins in the urine. Several of these small trace proteins have become important in a wide variety of pathophysiological disorders, and their isolation is a key procedure in developing assays for their measurement as well as in more fundamental investigations of their function.

Multistep chromatography by classical gel and ion-exchange chromatography have been devised for isolating low-molecular-weight proteins from urine, but they take several days[2–4]. Studies of the separation of urinary LMW proteins by fast protein liquid chromatography (FPLC) have shown a desalting procedure, followed by anion exchange on a Mono Q column could be used to separate β_2-microglobulin (β_2-m), retinol binding protein (RBP) and α_1-microglobulin (α_1-m)[5] and monoclonal

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kappa and lambda light chains[6]. Later, a purification procedure of the main peaks from the Mono Q column by chromatofocusing on Mono P columns was devised[7]. However, this high-performance chromatofocusing is restricted to analytical studies and is unsuitable for any scale-up procedure. We present methods for the rapid isolation of low-molecular-weight proteins from urine by FPLC and scale-up procedures to enhance the yield per chromatographic run by use of the large-capacity high-performance ion-exchange columns.

MATERIALS AND METHODS

Urine

Urine for the isolation of β_2-m, α_1-m, and RBP, was obtained from patients with impaired renal tubular function; the urines were selected to have an initial β_2-m concentration of > 50 mg/l; urine from patients with chronic granulocytic leukaemia or myelomonocytic leukaemia were used as a source of lysozyme. Free kappa or lambda light chains were isolated from the urine of patients with myelomatosis excreting > 10 g free light chain per litre, but without renal impairment. Materials were obtained from the following sources: Sephadex G-25, and G-50, gel chromatography media, Mono Q HR 5/5 and HR 16/10 prepacked strong anion-exchange columns, Mono S HR 5/5 and HR 16/10 prepacked strong cation-exchange columns from Pharmacia (Uppsala, Sweden); antisera against human β_2-m (Dako, Copenhagen); RBP, α_1-m, lysozyme (Behringwerke, Marburg, F.R.G.); Bis-Tris-propane {1,3-bis-[tris(hydroxymethyl)-methylamino]-propane}; succinic acid; N(2-hydroxyethyl)piperazine-N′-3-propanesulphonic acid (EPPS) (Sigma, Poole, U.K.), sodium dihydrogen orthophosphate, disodium hydrogen orthophosphate, sodium azide, ammonium sulphate, ammonium hydrogen carbonate (BDH Chemicals, Poole, U.K.).

Buffer solutions

Sephadex G-50 gel chromatography was performed with a 50 m*M* phosphate buffer (pH 7.0), containing 0.01% sodium azide. The buffers for ion-exchange chromatography are given with the figure legends.

Equipment

Urine and eluate concentration was by ultrafiltration with an Amicon (Upper Mill, U.K.) 50- or 200-ml pressure cell, fitted with YM5 membranes (cut-off 5000 daltons). Freeze-drying was carried out in an SB4 freeze-drier, supplied by Chemlab (Hornchurch, U.K.). All high-performance chromatographic separations were performed with the FPLC system, supplied by Pharmacia. The instrument was equipped with a UV-1 monitor and S-2 flow cell, and the absorbance of the eluate was recorded at 280 nm. The elution of proteins was monitored by radial immunodiffusion, using specific antisera. Rapid identification of β_2-m and α_1-m was also made by using a latex agglutination test provided by Professor T. Kawai, Jichi Medical School, Japan.

Scaling-up procedures

All exploratory chromatograms and optimizations of buffers and flow-rates were made on the analytical HR 5/5 columns. Scale-up to the HR 16/10 columns was made by using the scaling up factors advised by Pharmacia. The scaling-up factors

are as follows: flow-rate: HR 5/5, 1 ml/min; HR 16/10, 10 ml/min; linear gradient: HR 5/5, 0–100% B in 20 ml; HR 16/10, 0–100% B in 400 ml; chart speed: HR 5/5, 1 cm/ml; HR 16/10, 0.05 cm/ml. No adjustment of gradients were made on the HR 16/10 columns. Fractions (30–200 ml) from the gel filtration columns were loaded onto the HR 16/10 columns using a 50-ml superloop. When scaling up from HR 5/5 columns to HR 16/10 columns the standard mixing chamber was replaced with a 6-ml mixing chamber.

Electrophoretic separation

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed on vertical slab gels, as modified from the method described by Hashimoto *et al.*[9] in order to improve the separation of the proteins in the range of 10 to 30 kdalton and to demonstrate any smaller fragments.

RESULTS AND DISCUSSION

Pre-column gel chromatography

The urine samples were first centrifuged at 5000 *g* for 30 min and then concentrated about 40 times by pressure filtration through YM5 membranes. This gave very small losses of low-molecular-weight proteins as tested by lysozyme activity in the ultrafiltrate or retention of ^{125}I-labelled β_2-m. Dialysis by Visking 20/32 membrane was found to be unsatisfactory for desalting β_2-m and lysozyme as these proteins leaked through the pores. Urine concentrate (15 ml) was applied to a Sephadex G-50 (90 cm × 2.6 cm I.D.) column. The gel chromatography performed two functions. Firstly, the removal of a wide spectrum of small peptides and organic molecules with a strong absorption at 280 nm as well as salt exchange; secondly, the separation of the low-molecular-weight proteins into broad groups, as is shown in Fig. 1, where the positions of the "peaks" of α_1-m and β_2-m are being used to illustrate the separation.

Precipitation of the urine proteins by saturation with ammonium sulphate (750 g/l, 100%) was also used as an alternative preparative step prior to gel chromatography on Sephadex G-50. After precipitation at 4°C overnight, the precipitate was collected by centrifugation at 4000 *g* for 1 h, dissolved in phosphate buffer (pH 7.0), recentrifuged, and concentrated by pressure ultrafiltration before loading onto the gel column. Both ultrafiltration and ammonium sulphate precipitation were found to be satisfactory for concentrating the urine proteins.

Previous analytical studies used the proteins from 0.5 ml of urine desalted on Sephadex G-25[5] but this resulted in the protein fraction containing a wide range of molecular weights, in particular albumin, transferrin, and IgG as contaminants of the low-molecular-weight proteins. By using Sephadex G-50, desalting and partial separation of the proteins of interest could be achieved. The choice of fractions from the gel chromatography was designed to reduce contamination rather than to optimize the yield.

β_2-Microglobulin and retinol-binding protein

The separations of β_2-m and RBP on the Mono Q HR 16/10 column with the Bis-Tris-propane buffer system is shown in Fig. 2.

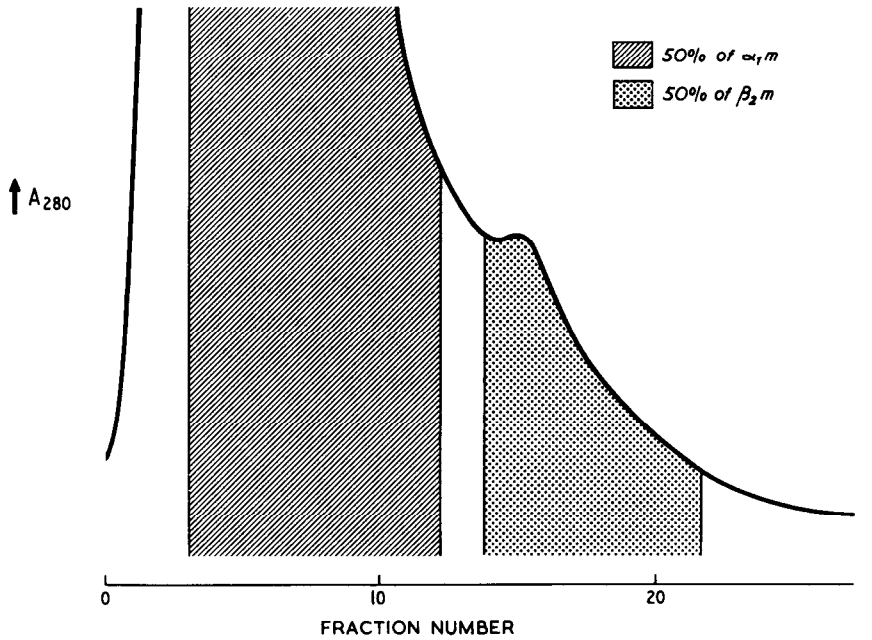

Fig. 1. Separation of urinary proteins on a Sephadex G-50 column. Shaded areas show fractions chosen to contain the highest concentration of α_1-m and β_2-m. Note: α_1-m tends to be eluted together with higher-molecular-weight proteins. Volume applied, 15 ml urine concentrate (× 40).

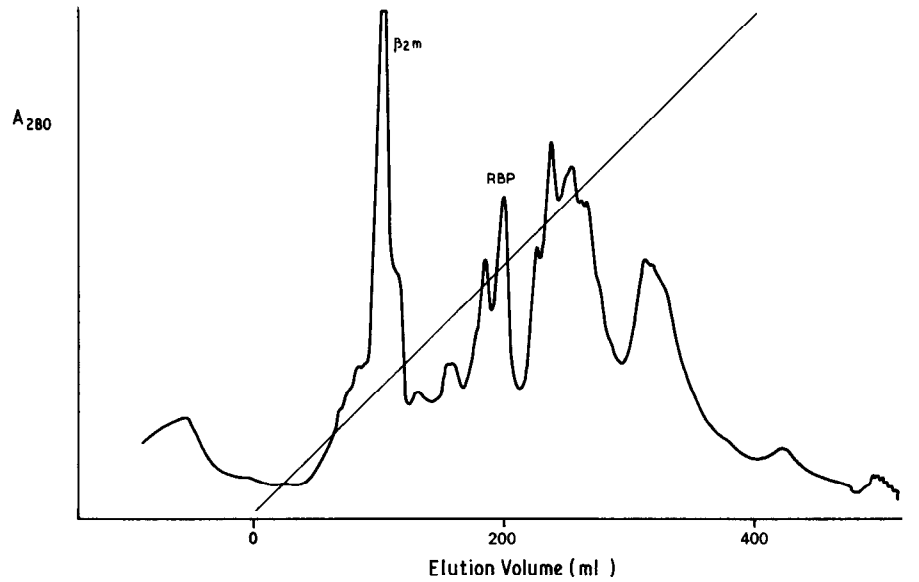

Fig. 2. Isolation of β_2-m and RBP by chromatography of a fraction from the Sephadex G-50 column on a Mono Q HR 16/10 column. Eluent A, 6.25 m*M* Bis-Tris-propane (pH 7.5); B, A + 0.35 *M* sodium chloride (pH 9.5); flow-rate, 10 ml/min, chart speed, 0.05 cm/ml, gradient 0–100% B in 400 ml, detection 280 nm, 0.2 a.u.f.s. flow cell S-2.

The scale-up of the preparation of β_2-m and RBP was based on the previously described system using Mono Q anion-exchange and Bis-Tris-propane buffer. Preliminary gel chromatography on a Sephadex G-50 was used to partially resolve these proteins. The scale-up to HR 16/10 produced a chromatogram almost identical to that on the HR 5/5 analytical column (Fig. 2). The SDS-PAGE patterns of the β_2-m and RBP produced after a re-chromatography on the Mono Q column are shown in Fig. 3.

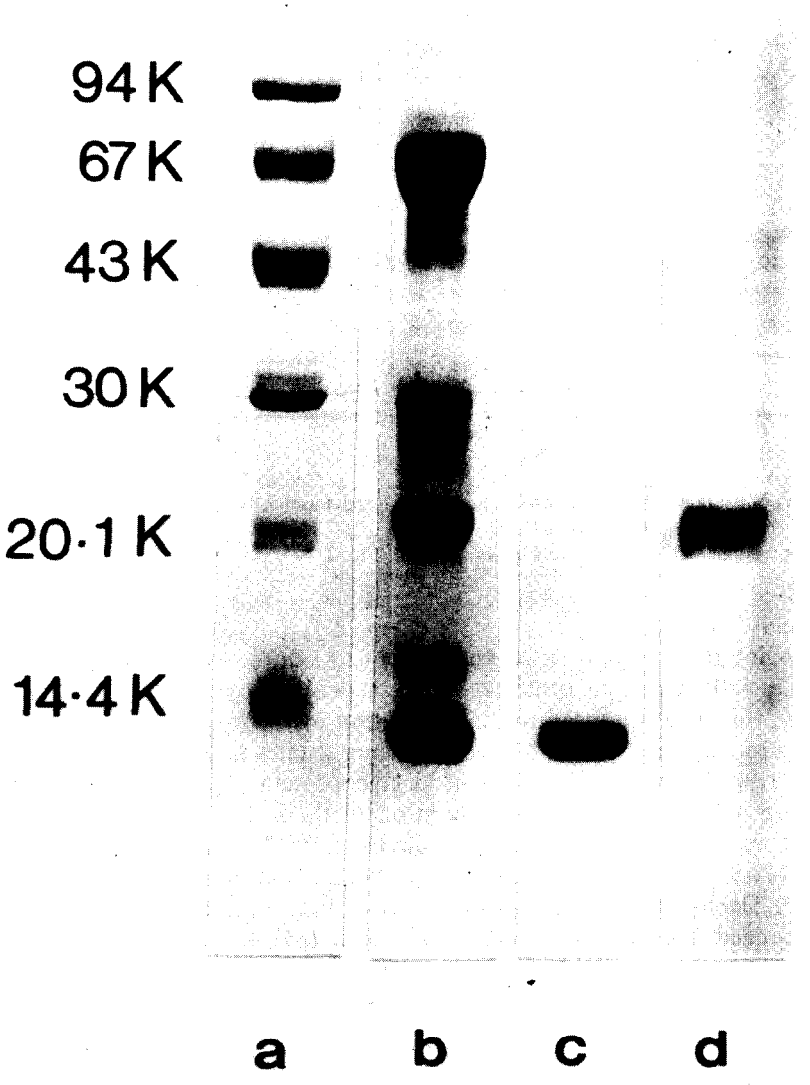

Fig. 3. SDS–PAGE of β_2-microglobulin and retinol-binding protein. (a) Low-molecular-weight standard; (b) G-50 eluate β_2-m and RBP fraction; (c) β_2-m final product; (d) RBP final product. K = Kilodalton.

Lysozyme

Lysozyme occurring in the urine of patients with chronic granulocytic or myelomonocytic leukaemia, as a result of overload, is partially purified by Sephadex G-50 chromatography. Final chromatography on Mono S cation-exchange provides a pure product with the preservation of its enzyme activity (Fig. 4).

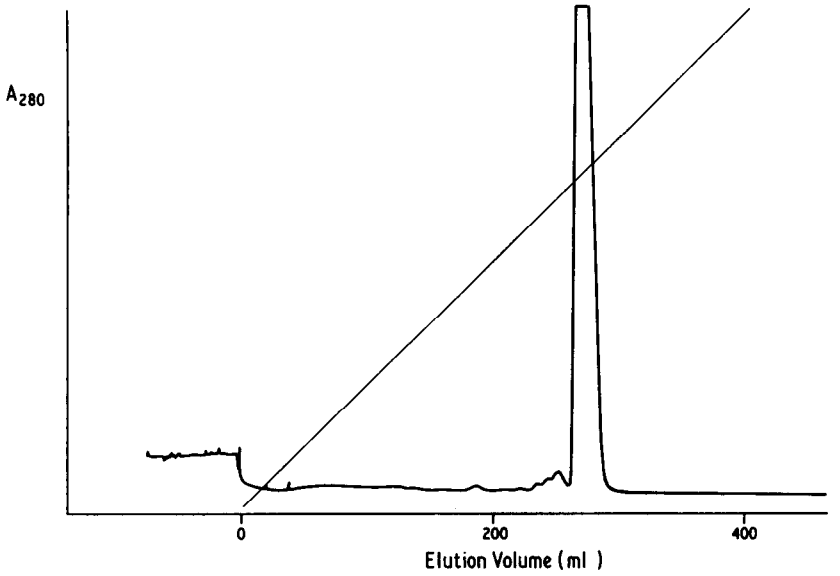

Fig. 4. Sephadex G-50 isolation of urinary lysozyme from a lysozyme-rich fraction by chromatography on a Mono S HR 16/10 column. Eluent A, 0.01 *M* phosphate (pH 7.1); B, A + 0.35 *M* sodium chloride (pH 7.1); flow-rate, chart speed, gradient, and detection as in Fig. 2.

α_1-Microglobulin

α_1-Microglobulin is glycosylated and known to exhibit a wide heterogeneity of charge (p*I* 4.3–4.8)[2]. Urine concentrates were first desalted by chromatography on Sephadex G-50, this also produced partial resolution of the α_1-m. The subsequent ion-exchange chromatography was devised following studies of a model mixture of purified α_1-m, α_1-antitrypsin (AT) and α_1-acid glycoprotein (AGP). The following 4-step procedure became the definitve separation method. The α_1-m-rich fraction from the G-50 was diluted with an equal volume of Bis-Tris-propane buffer and loaded onto a Mono Q column, and chromatographed using the same system as for β_2-m and RBP. The α_1-m-rich fraction was isolated (Fig. 5), desalted on Sephadex G-25 and exchanged into 20 mM succinic acid buffer (pH 4.5) and loaded onto a Mono Q column equilibrated with this buffer. The protein was then eluted with a salt gradient (Fig. 6) and purified by rechromatography.

Isolation of monoclonal light chains

Urine was selected from patients with myelomatosis excreting more than 10 g/l free light chain without impaired renal function as the total proteins contain only a few percent of albumin and low-molecular-weight proteins[8].

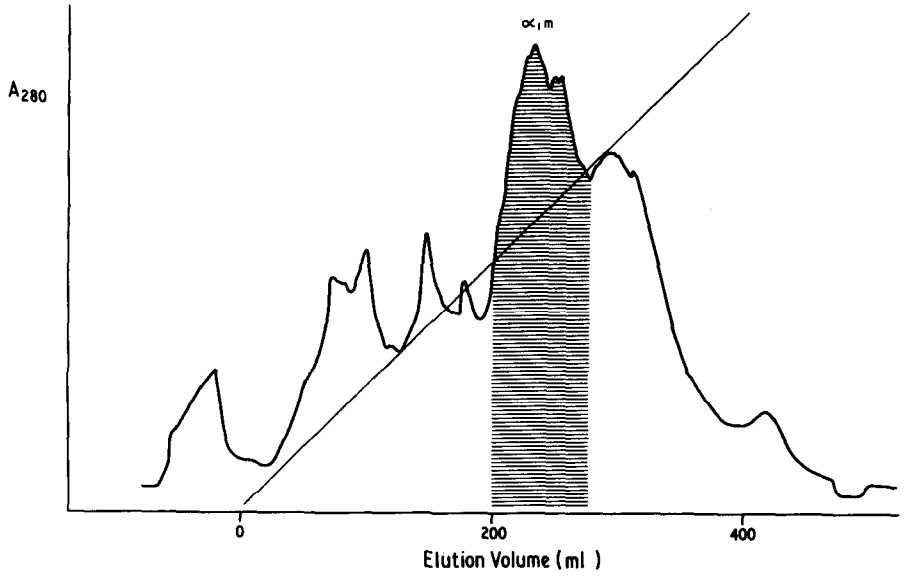

Fig. 5. First step in isolation of α_1-m by chromatography on Mono Q HR 16/10 column from an α_1-m-rich fraction from Sephadex G-50. Eluent A, 6.25 m*M* Bis-Tris-propane (pH 7.5), B, A + 0.35 *M* sodium chloride, (pH 9.5); flow-rate, 10 ml/min; chart speed, gradient, and detection as in Fig. 2.

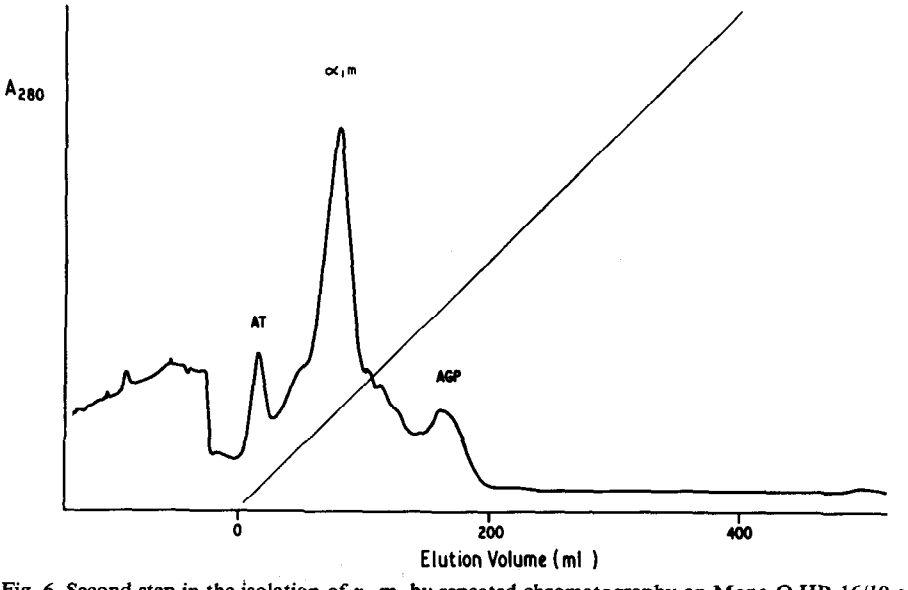

Fig. 6. Second step in the isolation of α_1-m, by repeated chromatography on Mono Q HR 16/10 column following buffer change. Eluent A, 0.02 *M* succinate (pH 4.5); B, A + 0.35 *M* sodium chloride (pH 4.5); flow-rate, chart speed, gradient, and detection as in Fig. 2.

The isolation of monoclonal kappa and lambda light chains could generally be achieved by a desalting step on Sephadex G-25 followed by examination of the eluate on an analytical Mono Q column with the Bis-Tris-propane buffer system. This analysis divided the light chains into two types: light chains with a single well-defined peak and light chains that produced two or more peaks, resulting from a marked post-translational modification of the original protein. The types producing a single peak were purified by scaling-up to the Mono Q 16/10 column, isolating the main peak, desalting, and rechromatographing on the Mono Q 16/10 column. The light chains, eluted as complex patterns, required careful immunochemical identification of the various kappa or lambda chain-containing fractions. An optimization procedure needs to be devised individually for each patient's urine (Fig. 7).

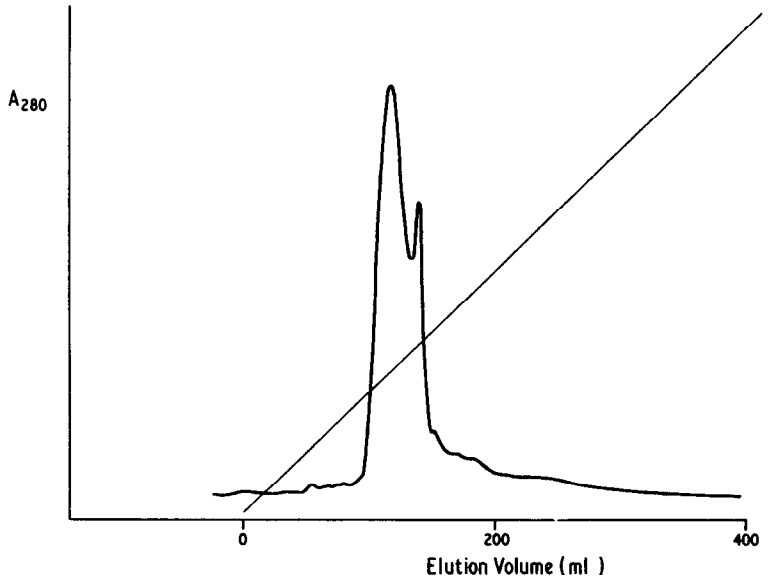

Fig. 7. Isolation of lambda free light chains from urine. Light chain-rich fraction from Sephadex G-25 separated on a Mono Q HR 16/10 column. Eluent A, 0.25 m*M* Bis-Tris-propane (pH 7.5); B, A + 0.35 *M* sodium chloride (pH 9.5); flow-rate, gradient, chart speed, and detection as in Fig. 2. Both peaks are lambda light chain; crossed immunoelectrophoresis against anti-whole human serum demonstrated no contamination.

Freeze-drying

The final products from the HR 16/10 columns were concentrated to 10 ml by ultrafiltration and exchanged into 50 m*M* ammonium hydrogen carbonate on a Sephadex G-25 column with a flow-rate of 1 ml/min. The final product was then freeze-dried.

Recovery and yield

Recovery from Mono Q HR 16/10: total protein 95%; β_2-m 95.2%; light chain (with a single peak) 96%. Yield from Mono Q HR 16/10: β_2-m 81.5%; RBP 67.3%; α_1-m, first step on Mono Q 43%, second step 54%.

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