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SEPARATION OF NONHISTONE HIGH MOBILITY GROUP (HMG) FROM HUMAN LYMPHOCYTES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The high mobility group (HMG) of nonhistone proteins have been investigated using two high performance liquid chromatographic techniques (HPLC). Reversed-phase HPLC under conditions of 50 mM triethylamine adjusted to pH 2.2 with phosphoric acid (solvent A) and 95% acetonitrile in water (solvent B) was used to separate proteins primarily on the basis of differences in the overall hydrophobicity. Size exclusion HPLC under conditions of two different solvents (A, 0.1% trifluoroacetic acid TFA; B, 1.0% sodium dodecyl sulphate, SDS) was used to separate proteins. HMG proteins from human lymphocytes were separated into the HMG 1, HMG 2, HMG 14 and HMG 17 components. RP-HPLC is a proper method to resolve all the human lymphocyte HMG-proteins. Size exclusion HPLC was employed to resolve the HMG-protein subunits and determine their molecular weights. Ideal SE-HPLC is not capable of resolving HMG 1 from HMG 2 or HMG 14 from HMG 17 due to their molecular weight similarities. The purity of protein fractions were examined by acetic acid-urea-triton X-100 gel electrophoresis.

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INTRODUCTION

High mobility group (HMG) proteins have been identified in a variety of cell types from different organisms.[1-5] It has been suggested that they can substitute histone Hl in the nucleosome spacer region, therefore altering the local ordered structure of chromatin,[6-10] although the precise function of such behavior is unknown.

The HMG proteins have a distinctive amino-acid composition. They have a high percentage of both acidic (about 20%) and basic amino acid (25%) residues. In addition, they contain characteristically high percentages of proline (7% or more) residues. The presence of acidic and basic amino acid residues (greater than 50%) will give HMG proteins a higher polar character even though they possess an overall neutral net charge.

HMG proteins from mammalian cells are divided into four main groups: HMG 1, HMG 2, HMG 14 and HMG 17.[3] HMG 1 and 2 have approximate molecular weights of 26-28,000 whereas HMG 14 and 17 have approximate molecular weights of 10-12,000. Other forms of the HMG proteins have been isolated from various cell types.[11]

HMG 1 and 2 bind to single-stranded DNA and may alter the superhelical structure of DNA.[12,13] HMG 14 and 17 on the other hand have been implicated in changing the chromatin structure of transcriptionally active genes.[14]

The purification of HMG 1, 2, 14, and 17 from human lymphocytes by RP-HPLC and SC-HPLC is described in this paper. We report the use of 18 columns as the primary purification step; although the possibil-

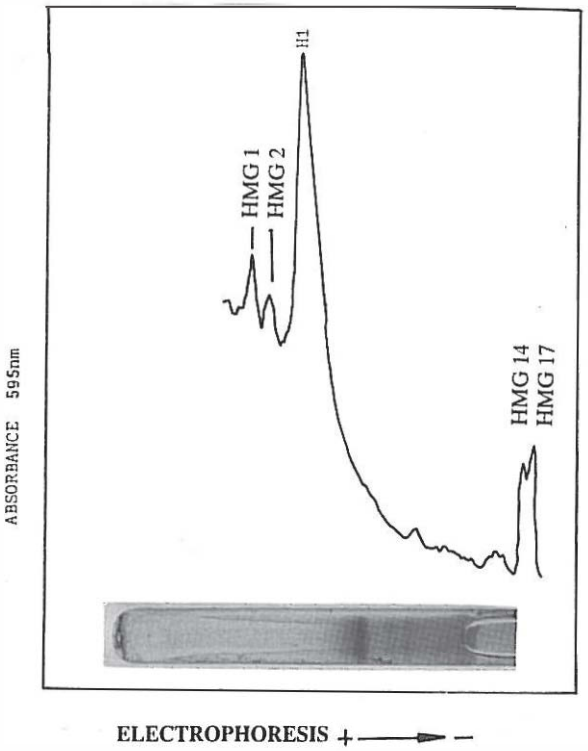

Fig.1. Analysis of acetone extract proteins from perchloric acid-extract of calf thymus by 15% acetic acid/urea/Triton X-100 (AUT) polyacrylamide gel electrophoresis.

ity of separation of HMG proteins on SE-HPLC matrix has also been investigated. It is hoped that improved separation of HMG proteins may clarify the biological role of these proteins in human lymphocytes and their possible involvement in carcinogenic behavior of these cells.

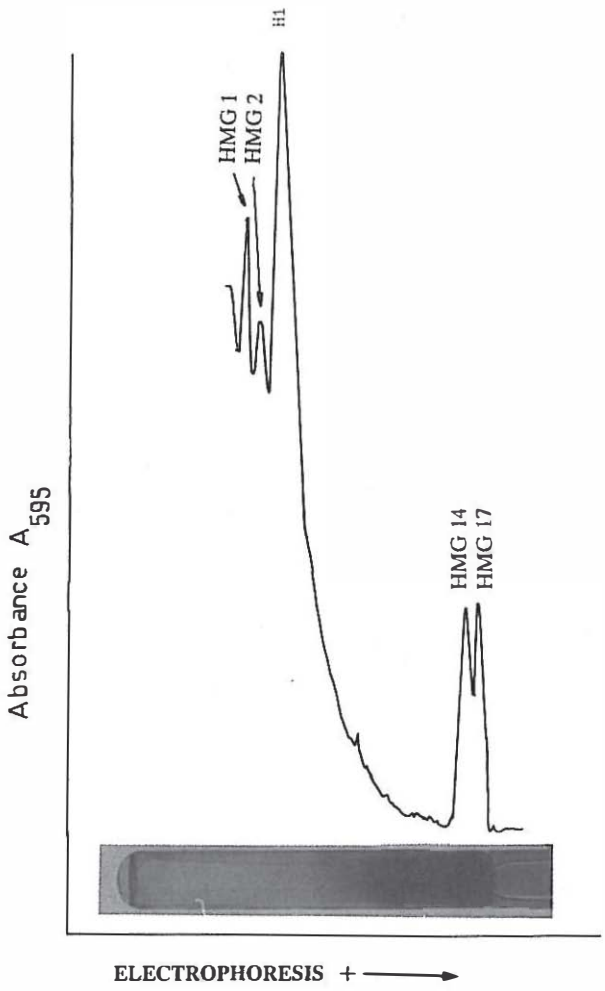

Fig. 2. Analysis of acetone extract proteins from perchloric acid-extract of human lymphocytes by 15% acetic acid/urea/triton X-100 polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Calf thymus tissue was obtained fresh from a local abattoir. Trifluoroacetic acid (TFA) and dithiothreitol (DTT) were purchased from Sigma Chemical Company (St. Louis, MO.). Acetonitrile (HPLC grade) was purchased from Merck Company (Darmmstart, FRG). All other chemicals were reagent grade and purchased from a variety of sources.

Separation of Human Lymphocyte: Twenty milliliter samples of venous blood were collected from fasting healthy individuals. Lymphocytes were separated from other blood cells using a Ficoll-metrizamide gradient.[15]

Nuclei Isolation: For the isolation of nuclei, the cells were washed with a buffercontaining0.25 mM sucrose, 50 mM Tris-HCL (pH 7.5), 5 mM $MgCl_2$, 25 mM KCl, 1 mM phenylmethyl sulfonyl fluoride (PMSF). The cells were resuspended by vortexing in the same buffer containing 0.20% Triton X-100 in order to remove the

Table I. Polypeptide Standards

Number	Polypeptides	Molecular Weight
1	Insulin	5,370
2	Lysozyme	14,400
3	Somatotropin	22,000
4	Alpha-Chymotrypsin	25,000
5	Carbonic Anhydrase	29,000
6	Ovalbumin	42,700
7	Trypsin Inhibitor	53,000
8	Serum Albumin	66,000
9	Bovine Albumin	68,000
10	Alkaline Phosphatase	100,000
11	Glucose Oxidase	160,000

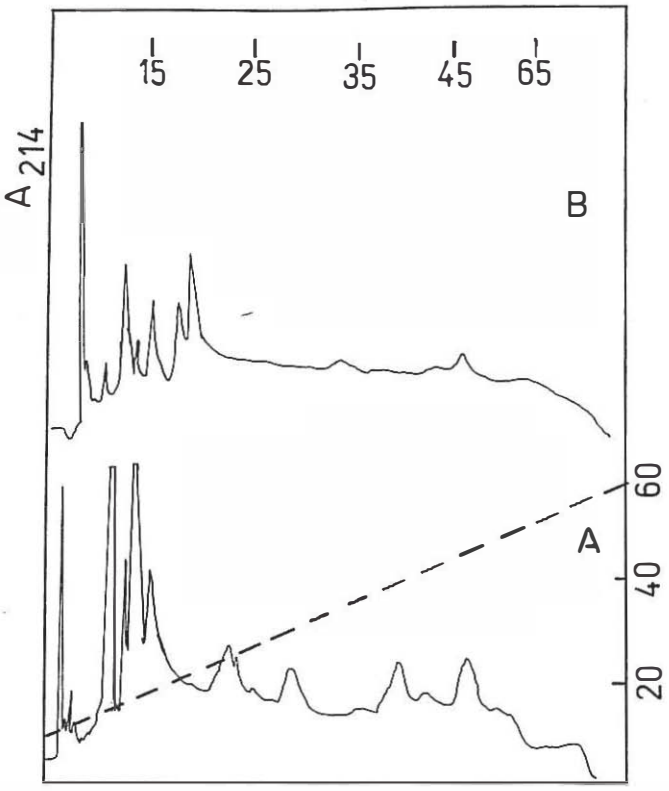

Fig. 3. Reversed phase liquid chromatography of non-histone proteins from calf thymus (A) and human lymphocytes (B). Protein samples were dissolved in solvent A (50mM triethylamine pH 2.2 with H_3PO_4) injected onto the RP-HPLC column and eluted with 10-60% linear gradient of solvent B (95% CH_3CN, 5% H_2O).

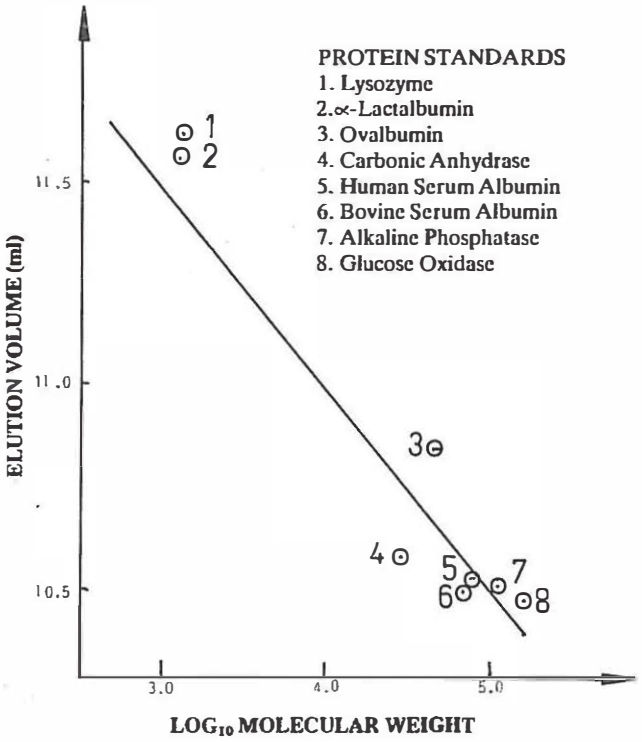

Fig. 4. Relationship between elution volume and logarithm of molecular weight on a column TSK-G2000SW using a mobile phase of 0.1% trifluoroacetic acid.

cytoplasmic membrane, then incubated for 10 min, on ice, and centrifuged for 15 min. at 3000 x g. The nuclear pellet was washed twice with the same buffer without the detergent.

Calf thymus nuclei were prepared using a rapid purification method. About 100g of fresh calf thymus tissue was homogenised in one liter of the above buffer (without detergent) using a Waring blender for five min. Nuclei were pelleted for 15 min. at 3,000 x g, resuspended in one liter of the same buffer containing 0.2% Triton X-100, repelleted and subjected once more to a suspension precipitation step using 5 mM $MgCl_2$, 10 mM Tris-HCl(pH 7.5), 1 mM PMSF.

Isolation of HMG Proteins: HMG proteins were isolated from calf thymus and human lymphocytes according to the method of Goodwin, et al.[16] with slight modifications. The washed nuclei were resuspended in a salt solution (0.35 NaCl, 10 mM Tris pH 7.5, 1 mM PMSF) and lysed in a blender (full speed, 2 min). The homogenate was stirred at 4°C for one hour and centrifuged at 7,000 xg for 15 min. Incubation at 4°C helped to stabilize the HMG proteins. The supernatant was saved and the pellet was subjected to two more washings. The pooled supernatant solution was adjusted to 2% trichloroacetic acid and incubated at 4°C for 2 hr in order to precipitate low mobility group proteins (LMG proteins). The LMG proteins were centrifuged at 8,000 xg for 20 min. HMG 1 and 2 were selectively separated by raising the concentration of TCA to 10% and incubating at 4°C for 2hr before centrifugation (8,000 xg for 20 min). HMG 14 and 17 and the remaining HMG 1 and 2 proteins were removed by raising the concentration of TCA to 25% and collecting the precipitate as described above. In some cases, HMG 1, 2, 14 and 17 were collectively precipitated by raising the acid concentration of the 2% TCA soluble material to 25% TCA. The precipitated materials were collected by centrifugation (10,000 xg for 20 min), washed with cold acetone, freeze dried and stored at 20°C. In order to compare the effectiveness of TCA precipitation, Sander's method[17] for precipitation of HMG was repeated.

High Performance Liquid Chromatography: In all HPLC experiments two LKB pumps model 2150, chromatography system with a variable UV detector model 2151, liquid handling controller model 2152, Rheodyne sampler injector model 7125 (Rheodyne Inc., Cotati, CA) were used. The detector signal was recorded on a Hewlett-Packard model 3390A recorder. Separations were performed on a reversed phase Lichrosorb RP-18 column (4.0 X 250 mm, 5 micron)

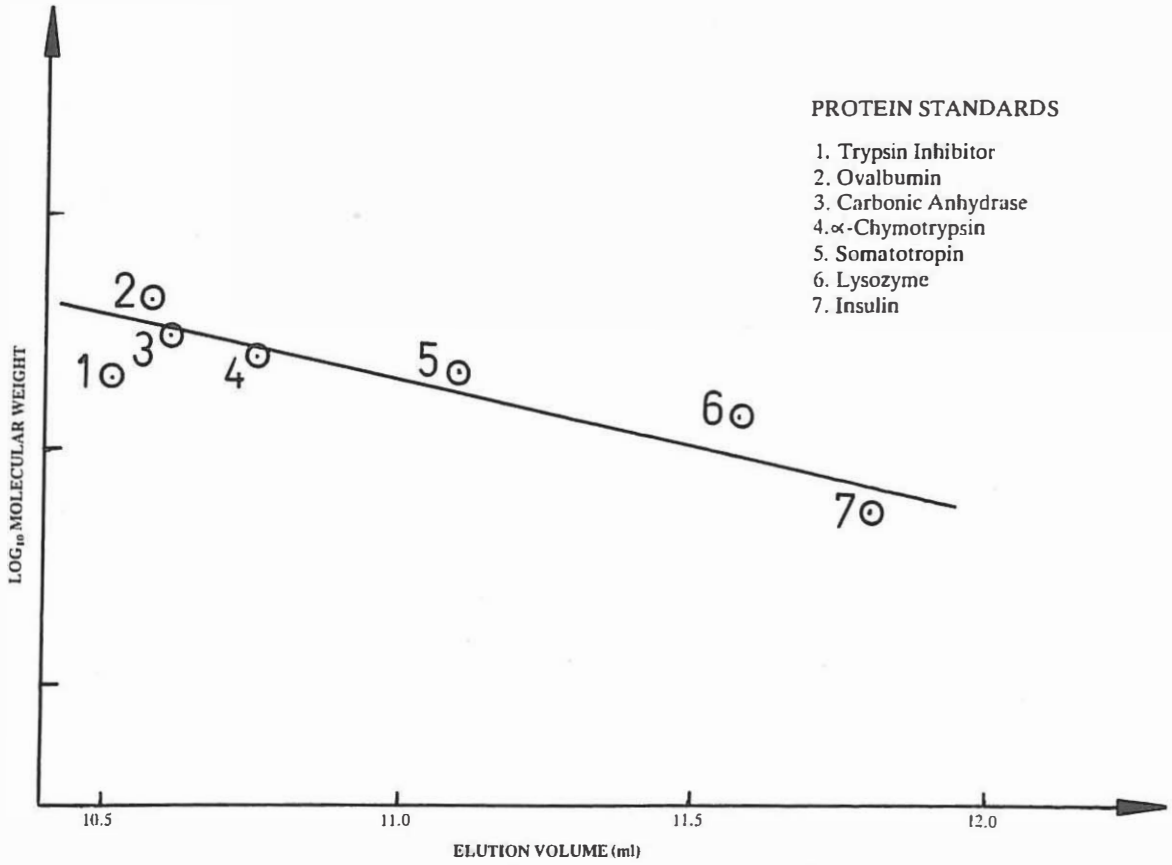

Fig. 5. Relationship between elution and logarithm of molecular weight on a column TSK-G2000SW using a mobile phase of 1.0% SDS.

and a size exclusion TSK G2000-SW (7.5 X 600mm, 10 microns) (Toya Soda Corp., Japan) column.

Reversed phase separation of HMG subfractions were performed under a gradient solvent delivery program. Solvent contained 50 mM triethylamine adjusted to pH 202 with ortho-phosphoric acid, and solvent B contained 95% CH_3 CN and 5% H_2O. See legned of Fig. 3 for details. Samples were dissolved in solvent A, filtered through a Millipore filter (Millex-GS, 0.22 microns) in order to remove the insoluble entities prior to injection. Both solvents were prepared by filtration through 0.45 micron filter and degassed.

The separation of HMG proteins was carried out by HPLC with an aqueous gel permeation column. SE-HPLC was performed using TSK-G2000SW column and to different elution profiles: (1) 1.0% SDS in water (pH 6.8) and (2) 0.1% TFA w/v in water. Eluted proteins from the columns were detected at 214 nm.

Polyacrylamide Gel Electrophoresis: The proteins obtained from acid precipitation and RP-HPLC and SE-HPLC were resolved by acetic acid-ureapolyacrylamide gel electrophoresis[19] with some modification. The gels contained 15% acrylamide, 0.25% bisacrylamide, 1 M acetic acid, 3 M urea and 0.2% Triton X-100. For the preparation of 100 ml of the gel, 50 ml 30% acrylamide-0.5% bisacrylamide, 6 ml glacial acetic acid, 18 g urea, 0.5 ml TEMED, 1 ml 20% w/v Triton X-100 and 28 ml of water were gently mixed and after degassing, 2 ml of 20% w/v ammonium persulfate solution was added. Following gel polymerisation, the sample wells were washed and filled with 3 M urea, 1 M acetic acid solution. The reservoir buffer contained 27.5 ml glacial acetic acid and 100 ml of 2 M glycine solution per liter (0.5 M acetic and 0.2 M glycine). The pH of the reservoir buffer was 3.5 without further adjustment. Electrophoresis was conducted at a constant current. For 22 cm long slab gels and with a surface area of 16 X 0.05 cm^2, 25 mA was applied for 6 hr.

Gels were stained directly, (0.2% coomassie blue G-250, 50% ethanol, 10% acetic acid and 0.05% 2-mercaptoethanol) for one hour and destained in 20% ethanol, 5% acetic acid.

Before and after the reversed phase and size exclusion HPLC, the protein samples were collected, lypholized and redissolved in water. Prior to RP-HPLC and electrophoresis, the protein samples were reduced

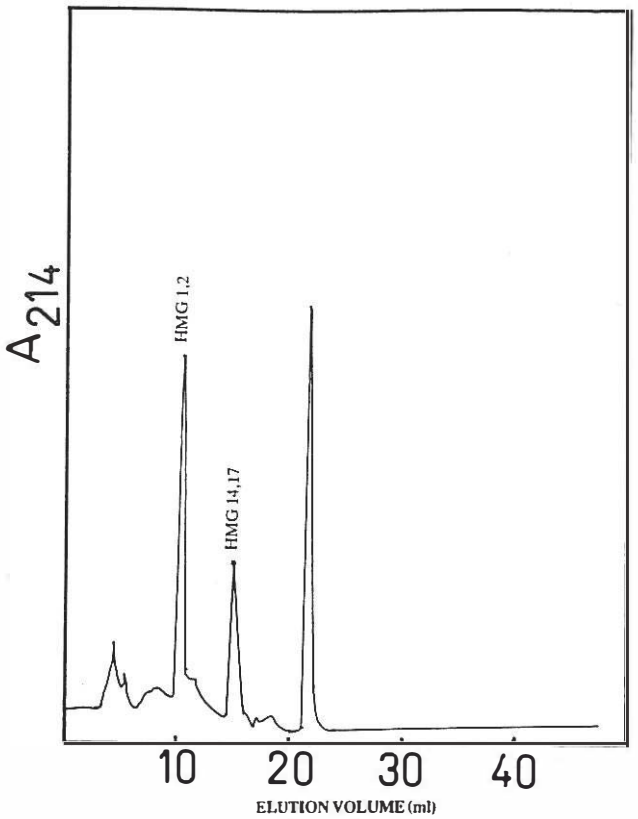

Fig. 6. Separation of HMG proteins from human lymphocytes on a column of TSK-G2000SW in 1.0% SDS. A solution of 20ul containing 50ug of total protein was injected. The flow rate was 1.0ml/min. Protein designation was determined with reference to the standard curve from Mr of known proteins.

with 0.1 M DTT (for 15 min, at 35°C).

SDS-gel electrophoresis (15% acrylamide and 0.25% bisacrylamide) was performed according to Laemmlie's method.[19] Protein was determined as described by Lowry, et al.[20] with bovine serum albumin (Sigma Chemicals, ST. Louis, MO.) as a standard.

RESULTS AND DISCUSSION

The separation of H1, HMG 1,2,14, and 17 proteins from calf thymus and human lymphocytes by acetic acid-urea-Triton X-100 gel electrophoresis is presented in Fig. 1 and 2, respectively. It is evident from these gels that TCA precipitation method gives good recovery of the major 1,2, 14, and 17 components. The cofractionation of HMG 1 and 2 on one hand and HMG 14 and 17 on the other hand is not surprising since there are common structural features and extensive sequence homology between HMG 1 and 2,[21] as well as betweem HMG 14 and 17.[22]

The chromatographic profile of the proteins present in different elution peaks shown in Fig. 3 indicates that RP-HPLC on Lichrosorb C_{18} column using a 10-66% acetonitrile linear elution gradient with triethylamine under acid conditions gave good separation of the HMG protein species from calf thymus and human lymphocytes. HMG 1 and 2 subfractions isolated from human lymphocytes by 5% perchloric acid extraction had the same elution characteristics as the HMG proteins isolated by salt extraction even though the acid extracted preparation contained high concentrations of histone HI protein as a contaminant.

In order to prevent intramolecular interactions, the reducing agent DTT was added to the crude extract prior to RP-HPLC separations and electrophoresis. The occurrence of oxidised forms of the HMG 1 and 2 proteins is in agreement with the results of several research groups.[23-25]

In the absence of a reducing agent, the HMG 1 and 2 subfractions may produce intramolecular disulfide bonds. In the oxidised form, an internal disulfide bond would prevent either HMG 1 or 2 proteins from totally relaxing when denatured by urea. Consequently, the oxidised form of the HMG 1 or 2 proteins would be expected to migrate in the electrophoretic field slightly faster than the reduced form of these same proteins during acetic acid-urea electrophoretic separations.

Reversed phase HPLC has been used extensively for separation of proteins and peptides.[26-29] The use of RP-HPLC for the analysis of human lymphocyte HMG proteins has a number of important advantages over other currently employed methods for separation of these proteins. Short elution times and very high efficiency of recovery of protein fractions from RP-column are some special features of RP-HPLC method. In addition, unfractionated extracts can be separated into a number of different pure HMG subfractions in single step. Since the separation of proteins and peptides is based primarily on differences of hydrophobicity, the presence of histones and other highly charged molecules in the starting samples does not interfere with or give artificical distortions to the resolution of HMG subfractions on RP-HPLC.

The advantage of triethylamine as the eluting buffer over other solvents mixtures is the higher rate of protein recovery. The Lichrosorb C_{18} column is a silica based column with maximum of 40-60% binding of C_{18} hydrophobic groups to the column and therefore the remaining silica gel hydrophilic groups ought to be recovered by an ion pairing agent. Triethylamine was selected for this purpose. Others have used TFA as eluting solvent for the separation of HMG proteins,[25] but the extremely acidic nature of the eluting solvent may reduce the efficiency of the column and irreversibly denature the protein samples.

One of the disadvantages of RP-HPLC is the use of organic mixtures for elution of proteins. Biological activity of native protein may be altered by introduction of the organic solvents. In order to reduce the

effect of organic solvents and separate human lymphocyte HMG subfractions, SE-HPLC was employed. The TSK-G2000SW is a porous silica based column (600 X 7.5 mm) which allows the separation of HMG subfractions on the basis of molecular weight differences. Fig. 4 and 5 shows the utility of the TSK-G2000SW column as a simple device for the estimation of the molecular weight of unknown entities. Two different solvent systems (A:.0.1% TFA in water, and B: 1.0% SDS) were employed. Polypeptides listed in Table I were used as standards in the construction of calibration plots relating elution volume to the logarithm of molecular weights. Proteins and peptides dissolved either in 0.1% TFA (Fig. 4) or 1.0% SDS (Fig. 5) and injected onto a column of TSK-G2000SW equilibrated and eluted with the respective solvents emerged as sharp peaks.

Under acidic conditions (0.1% TFA), fractionation is not only dependent on the size of the polypeptide but also on the charge. Anionic proteins were retained on the column. Trifluoroacetic acid is known to act as a weak hydrophobic ion pairing agent with proteins.[30,31] However at high concentrations, it may pair up with hydrophobic regions of some proteins and act as a hydrophobic pairing agent.[32] In order to eliminate the undesirable ion pairing effect of TFA, 1.0% SDS was used as the main solvent system for the separation of human lymphocyte HMG subfractions and the molecular weight determination was performed using the 1.0% SDS calibration data (Fig. 6). HMG 1 and 2 were found to have molecular weights of 26-28,000 and HMG 14 and 17 were found to have molecular weights of 10-12,000. Due to the molecular weight similarities between HMG 1 and 2 and also between HMG 14 and 17, it was not possible to fractionate these proteins under ISE-HPLC conditions. Nonideal SE-HPLC,[33] under altered pH and ionic strength conditions may prove to be an alternative for the separation of HMG protein subfractions under nondenaturing conditions. Further studies using nonideal conditions may allow maximum separation of human lymphocyte HMG protein subfractions.

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