ELSEVIER

Journal of Chromatography B, 666 (1995) 233–239

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Selective separation of human peripheral platelets, granulocytes and lymphocytes by surface affinity chromatography

Yoichi Shibusawa*, Kuniaki Suzuki, Hiroaki Kinoshita, Ushiho Matsumoto

Department of Analytical Chemistry, Tokyo College of Pharmacy, 1432-1, Horinouchi, Hachioji, Tokyo 192-03, Japan

First received 4 October 1994; revised manuscript received 2 December 1994; accepted 16 December 1994

Abstract

Selective separation of human peripheral platelets, granulocytes and lymphocytes was investigated by column liquid chromatography using methoxyethoxymethyl (MEM) bonded-phase columns (25 × 0.9 cm I.D.). Isotonic solutions containing mono- and disaccharides, methyl-α-D-pyranosides and a physiological saline at pH 7.4 were used as the mobile phase. Granulocytes and lymphocytes were separated on the MEM-Cellulofine GH-25 column by elution with 0.3 *M* D-mannose solution. The isolation of platelets and lymphocytes from human leukocyte-rich plasma was performed with a MEM-Sephadex G25 column and elution with 0.27 *M* sucrose solution. On the same column platelets could also be collected selectively by elution with 0.31 *M* methyl-α-D-mannoside at the high recovery of 100%. The isolated cells were viable for more than 90%.

1. Introduction

Chromatographic separations of human peripheral blood cells have been reported by using polyethylene glycol (PEG) and polypropylene glycol (PPG) bonded agarose columns [1–7]. With a phosphate buffered solution containing dextran as the mobile phase the blood cells were eluted from the column in the following order: erythrocytes, platelets, granulocytes and lymphocytes. The retention volumes of granulocytes and lymphocytes showed a marked dependence on the number of oxyethylene and oxypropylene units of the bonded PEG and PPG.

In previous studies [6,7], the capacity factor (k') of granulocytes and lymphocytes on the PEG and PPG bonded agarose columns increased considerably with increasing hydrophobicity of the column packings. Platelets were only retained on the column with the highest content of oxypropylene units (PPG 950-agarose) which was the most hydrophobic column among the PEG and PPG bonded-phase columns. On the other hand, the k' values of erythrocytes are very small even with the most hydrophobic column packings. Separation of erythrocytes and platelets could not be achieved on the PEG or PPG bonded columns because the capability of these columns to retain these cells is too small.

In the present study, methoxyethoxymethyl (MEM) groups–which are similar to the two oxyethylene groups of PEG–were introduced

* Corresponding author.

0378-4347/95/$09.50
SSDI 0378-4347(94)00580-X

into column packings, such as Cellulofine and Sephadex, used for size-exclusion chromatography. Cellulofine (cellulose beads) consists of a D-glucose polymer in which the D-glucose units are bound in $\beta(1\rightarrow4)$ linkages. Sephadex (cross-linked dextran beads) consists of a D-glucose polymer in which the linkage between the D-glucose units is $\alpha(1\rightarrow6)$. The selective separation of human peripheral blood cells was investigated by using MEM-bonded Cellulofine GH-25-c (MEM-Cellulofine) and Sephadex G25 (MEM-Sephadex) columns. Isotonic mono- and disaccharides and methyl-α-D-pyranosides solutions were used as the mobile phases. The adsorption and the elution behavior of human peripheral blood cells, especially platelets, granulocytes and lymphocytes, on the MEM-bonded columns were investigated.

2. Experimental

2.1. Materials

Sephadex G25 (coarse, particle size 100–300 μm) (Pharmacia, Uppsala, Sweden), Cellulofine GH-25-c (particle size 105–210 μm) (Tisso, Tokyo, Japan) were commercially available. (β-Methoxyethoxymethyl)triethylammonium chloride (MEMTEA-Cl) was obtained from Tokyo Chemical Industry (Tokyo, Japan). Other chemicals were all of reagent grade.

2.2. Instrumentation

A Model 5100 C osmometer (Wescor, Logan, UT, USA) was employed for the measurement of the osmotic pressure of the mobile phase. An LKB 2120 Varioperpex II peristaltic pump (LKB, Bromma, Sweden) was used to elute the human blood cells. An ISCO Model UA-5 absorbance monitor (Instrumentation Specialties, Lincoln, NE, USA) was used for detecting the absorbance of the eluates at 254 and 580 nm simultaneously. An LKB 2112 RediRac fraction collector (LKB, Bromma, Sweden) was used for fractionation of eluates. A Model D Coulter counter (Coulter Electronics, Harpenden, UK) was used for counting the number of blood cells.

2.3. Preparation of MEM-bonded column packings

(β - Methoxyethoxymethyl)triethylammonium chloride (MEMTEA-Cl) is known as a reagent which can protect the hydroxy groups of several compounds during chemical reactions. The hydroxy groups of the glucose in the Cellulofine and Sephadex were modified with MEMTEA-Cl as follows: 1 g of dry Cellulofine GH-25 or Sephadex G 25 was mixed with 10 ml of acetonitrile containing 0.3–5.0 g (0.0013–0.0221 *M*) of MEMTEA-Cl and refluxed for 6 h. After reaction, the coupled products were washed on a sintered glass filter funnel successively with water and sieved to obtain a narrow sieve fraction (usually $105\sim210$ μm). The MEM-Cellulofine GH-25 and MEM-Sephadex G25 were hydrolyzed with 20.2% hydrochloric acid-d_2 (DCl) and the amount of MEM groups was determined by NMR spectroscopy [6].

2.4. Determination of the MEM residue content bonded to support materials

MEM-Sephadex and MEM-Cellulofine were dried in vacuo (P_2O_5) overnight at 70°C. About 100 mg of dried gel was mixed with 20.2% DCl in D_2O and the mixture was heated for about 4 h on a boiling water-bath for complete hydrolysis and then evaporated to dryness under reduced pressure at 70°C. The hydrolysed product was dissolved in deuterium oxide (D_2O) and lyophilized. This deuterium exchange procedure was repeated three times. Finally, 100 mg of lyophilized powder were weighed and dissolved in 0.5 ml of D_2O for ^1H NMR measurement. The methoxy proton signal from the MEM group is observed at ca. 4.0 ppm. The peak area of the methoxy proton signal was determined from a calibration graph obtained with a series of concentrations of MEMTEA-Cl as a standard.

2.5. Collection and isolation of blood cells

Human blood was drawn from normal male adult donors by venous puncture and heparin was added–0.05 ml of a 1000 U/ml solution per 10 ml of blood. In the case of collection of platelets, 1% disodium EDTA solution was used as anticoagulant. Siliconized glassware was used in all procedures.

Platelets

A centrifugal isolation technique based on that of Leeksma and Cohen [8] was employed. A 10-ml volume of whole blood was mixed with 1 ml of 1% disodium EDTA in 0.9% physiological saline, and filtered through an absorbent gauze. The anticoagulated blood was centrifuged at 65 *g* for 20 min. The supernatant (platelet-rich plasma, PRP) was centrifuged at 250 *g* for 20 min and sedimented platelets were then washed twice with 0.9% saline.

Granulocytes

The sodium metrizoate–dextran T500 sedimentation technique of Bøyum [9] was used. A mixture of 10 ml of blood and 2 ml of 0.9% saline was carefully layered on 10 ml of a mixed solution of 32.8% sodium metrizoate–6% dextran T500 (1:2) and allowed to stand for 1–2 h until the sedimentation of erythrocytes was complete. The granulocytes layer was pipetted off, and centrifuged at 130 *g* for 10 min. The precipitated granulocytes were then washed three times with saline.

Lymphocytes

The sodium metrizoate–Ficoll sedimentation technique based on that of Thorsby and Bratlie [10] was used. A 5-ml volume of blood was defibrinated by rotating the test-tube vertically for 10 min with five glass spheres of 5 mm diameter and 5 ml of the defibrinated blood were mixed with an equal volume of 0.9% saline in a centrifuge tube and carefully layered on 7.5 ml of Lymphoprep (a mixture of 9.6% sodium metrizoate and 5.6% Ficoll; Nyegaard, Oslo, Norway). The tube was centrifuged at exactly 400 *g* for 30–40 min. The lymphocyte layer was pippeted off and the cell suspensions were washed three times with saline.

Leukocyte-rich plasma

The blood was centrifuged at 65 *g* for 15 min. After the erythrocytes were all sedimented, the supernatant (leukocyte-rich plasma, LRP) was centrifuged at 250 *g* for 20 min. Most of the sediment consisted of platelets, granulocytes and lymphocytes.

2.6. Chromatography of blood cells

Four kinds of mono- and disaccharides solutions, 0.31 *M* D-glucose, galactose, mannose and 0.27 *M* sucrose, three methyl-α-D-pyranosides, 0.3 *M* methyl-α-D-glucoside, galactoside and mannoside and 0.154 *M* sodium chloride isotonic solution were used as the mobile phases.

MEM-Sephadex G25 and MEM-Cellulofine GH-25 were suspended independently in the mobile phase which contained isotonic concentrations of the mono- and disaccharides and methyl pyranosides and columns were filled with the slurried packings. The columns were thoroughly washed with the mobile phase to equilibrate the chemically bonded MEM phase by the use of a peristaltic pump. The total amount of platelets, granulocytes, lymphocytes and leukocyte-rich plasma prepared as above was suspended in 0.6 ml of the eluent. A 0.5-ml volume of the cell suspension containing about 10^5–10^7 each of platelets, granulocytes, lymphocytes and leukocyte-rich plasma was loaded on the column (25 × 0.9 cm I.D.), and the column was eluted with each of the eluents. A flow-rate of 20 ml/h was maintained by the pump. The absorbance of the eluate was monitored continuously at 254 and 580 nm. The fractions were collected and each fraction was diluted with 5 ml of Isoton (aqueous electrolyte diluent for blood cell counting; Coulter Diagnostics, Hialeah, FL, USA) and the number of the cells was counted with a Coulter counter. All retention volumes and recovery data are the average of at least triplicate determinations.

2.7. Viability of cells after chromatography

The viability of the cells eluted from the column was checked by trypan blue dye exclusion. A 1-ml volume of isotonic dye solution (4 mg/ml) was added to the 1-ml fractions obtained by chromatography. The mixture was allowed to stand for 5 min until the dead cells were stained. The viable cells were counted under the microscope. In the case of platelets, the capability of aggregation was checked by microscopy. A 25-μl volume of thrombin solution containing 1.25 U of enzyme was added to a 0.2-ml volume of the chromatographic platelet fraction. The aggregation of platelets was observed by microscopy.

3. Results and discussion

3.1. Determination of bonded MEM groups in the column packings

MEM groups were directly coupled to the hydroxyl groups in the Sephadex and Cellulofine as shown in Fig. 1. The coupling conditions depend greatly on the amount of MEMTEA-Cl added to the reaction mixture. The contents of MEM groups in the MEM-Cellulofine and MEM-Sephadex range from 0.37 to 0.48, and from 1.25 to 1.95 mmol MEM groups in one gram of the dry powder, respectively. The packing with the highest MEM content is best for the retention of blood cells, especially platelets. Thus we used the packing having the highest content of MEM group for the chromatography.

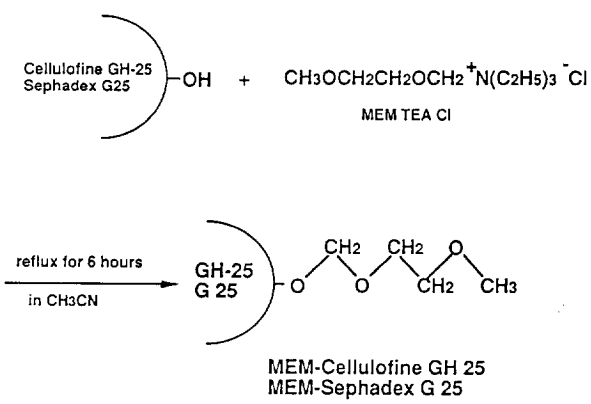

Fig. 1. Preparation of MEM-bonded Cellulofine and Sephadex column packings used for chromatography of human blood cells.

3.2. Chromatography of human peripheral blood cells

MEM-Cellulofine GH-25 column

Since erythrocytes are easily separated from human blood by centrifugation, in the present studies we examined the separation of human peripheral platelets, granulocytes and lymphocytes. Platelet, granulocyte and lymphocyte suspensions were chromatographed on a MEM-Cellulofine GH-25 column (25 × 0.9 cm I.D.). Eight kinds of isotonic solutions including 0.9% sodium chloride solution were used for the mobile phase. Table 1 shows the retention volumes and the recoveries of the platelets, granulocytes and lymphocytes eluted from the MEM-Cellulofine GH-25. The amount of MEM groups in the packings used in the chromatography is 0.48 mmol/g dry powder. In Table 1, "ads" indicates that no cells could be recovered.

The different retention volumes of the platelets, granulocytes and lymphocytes could not be obtained by using the physiological saline as a mobile phase, because only weak interactions between the column packing beads and these three kinds of cell surfaces would occur. On the other hand, when we used the mobile phase which containing 0.27 *M* sucrose, 0.31 *M* D-glucose and 0.30 *M* methyl-α-D-glucoside, the granulocytes and lymphocytes are adsorbed on the column. It can be seen that selective separation of the platelets is easily performed by using these three mobile phases. The recoveries of the platelets eluted with sucrose, D-glucose and methyl-α-D-glucoside were 100, 88.9 and 80.6%, respectively. The eluted platelets can be aggregated by adding thrombin solution to the chromatographic fraction.

With mobile phases containing the mono- and disaccharides and methyl-α-D-pyranosides, it was possible to recover over 20% of the lymphocytes, and 30% of the granulocytes from the column by eluting with both D-galactose and

Table 1
Retention volumes of human peripheral platelets, granulocytes and lymphocytes on MEM-Cellulofine column

Mobile phase[a]	Concentration (*M*)	Retention volume (ml)		
		Platelets	Granulocytes	Lymphocytes
NaCl	0.154	5.8 (32.0)[b]	5.5 (6.9)	6.1 (64.7)
Sucrose	0.270	4.2 (100.0)	ads[c]	ads
D-Glucose	0.310	4.4 (88.9)	ads	ads
D-Galactose	0.310	13.4 (64.3)	6.7 (34.6)	12.8 (22.9)
D-Mannose	0.310	13.9 (5.6)	5.5 (44.3)	15.2 (25.4)
Methyl-α-D-glucoside	0.300	4.7 (80.6)	ads	ads
Methyl-α-D-galactoside	0.300	14.7 (40.0)	5.3 (4.5)	ads
Methyl-α-D-mannoside	0.300	6.0 (8.4)	ads	7.1 (7.7)

Column: MEM-Cellulofine GH-25-c (25 × 0.9 cm I.D.).
All retention volumes and cell recoveries are the average of at least triplicate determinations. The recovery of the eluted cells was calculated from the combined number in each fraction compared with the cells loaded on the column.
[a] Mobile phase is isotonic solution at pH 7.4.
[b] Recoveries of cells from column indicated in parentheses.
[c] ads: no cells are recoverable.

D-mannose isotonic solutions; 0.31 *M* D-mannose gives the highest recovery of 44.3% of the granulocytes. The viability of the eluted granulocytes and lymphocytes was checked by their capability to exclude trypan blue dye. Over 90% of the cells are alive after surface affinity chromatography. In order to demonstrate the applicability of the MEM-Cellulofine column to the selective separation of granulocytes and lymphocytes, a mixture of these blood cells was chromatographed with D-mannose solution. Fig. 2 shows the elution pattern of the granulocytes and the lymphocytes using the mannose solution as the mobile phase. Granulocytes and lymphocytes obtained from 10 ml of blood were mixed together and eluted with the mannose solution from the MEM-Cellulofine column (25 × 0.9 cm I.D.) at a flow-rate of 20 ml/h. The number of cells is indicated in the histogram. The maximum absorbances of the elution curves at 254 and 580 nm coincide with the maximum peaks in the histogram of the cells in the fractions. Fraction 6–7 and 12–14, corresponding to center cuts of the first and second peaks in the chromatogram, contain over 95% granulocytes and lymphocytes, as confirmed by giemsa staining. These two types of cells well separated within ca. 1.5 h.

MEM-Sephadex G25 column

Table 2 shows the retention volumes and the recoveries of the platelets, granulocytes and lymphocytes from the MEM-Sephadex G25 column (25 × 0.9 cm I.D.). The packing with the highest content of MEM groups (1.95 mmol/g dry powder) prepared in this study was used for

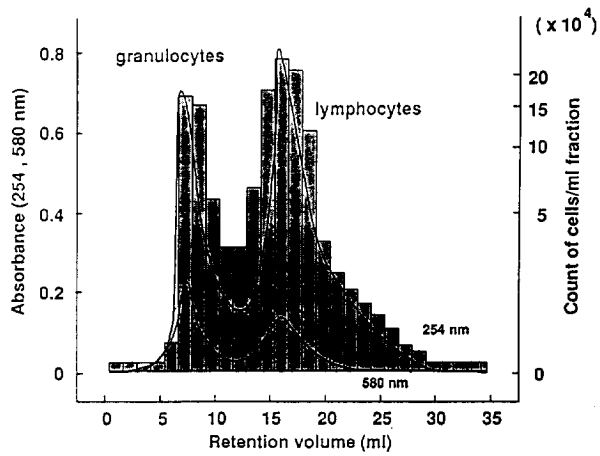

Fig. 2. Elution profile of human peripheral granulocytes and lymphocytes. Column: MEM-Cellulofine GH-25 (25 × 0.9 cm I.D.); mobile phase: 0.31 *M* D-mannose solution pH 7.4; flow-rate: 20.0 ml/h; sample: 1.5 ml cell suspension of granulocytes and lymphocytes.

Table 2
Retention volumes of human peripheral platelets, granulocytes and lymphocytes on MEM-Sephadex column

Mobile phase[a]	Concentration (M)	Retention volume (ml)		
		Platelets	Granulocytes	Lymphocytes
NaCl	0.154	5.5 (100.0)[b]	5.6 (30.8)	5.4 (17.9)
Sucrose	0.270	13.4 (72.9)	ads[c]	6.3 (41.4)
D-Glucose	0.310	4.2 (6.3)	ads	13.2 (7.3)
D-Galactose	0.310	ads	6.7 (34.6)	21.8 (8.5)
D-Mannose	0.310	13.9 (14.0)	5.5 (44.3)	5.1 (40.4)
Methyl-α-D-glucoside	0.300	14.6 (18.5)	ads	ads
Methyl-α-D-galactoside	0.300	13.6 (31.7)	5.3 (4.5)	14.4 (6.6)
Methyl-α-D-mannoside	0.300	6.1 (100.0)	ads	ads

Column: MEM-Sephadex G25 (25 × 0.9 cm I.D.).
All retention volumes and cell recoveries are the average of at least triplicate determinations. The recovery of the eluted cells was calculated from the combined number in each fraction compared with the cells loaded on the column.
[a] Mobile phase is isotonic solution at pH 7.4.
[b] Recoveries of cells from column indicated in parentheses.
[c] ads: no cells are recoverable.

chromatography. In Table 2, "ads" means that most blood cells adsorb onto the column. The retention volumes of the platelets, granulocytes and lymphocytes are nearly equal using physiological saline as a mobile phase. As with the MEM-Cellulofine column, it seems that the interactions between the MEM stationary phase or the surface of the Sephadex beads and the surface of the cells are only very small in the saline solution. The results indicate that the mono- and disaccharides and methyl-α-D-pyranoside in the mobile phase are necessary for the adsorption and separation of human blood cells. Using the methyl-α-D-glucoside and mannoside isotonic solutions, platelets were separated selectively, because the interaction between the surfaces of the granulocytes and the lymphocytes and the MEM-Sephadex G25 column packing beads are too strong to elute the cells from the column. The recovery of the platelets using the methyl-α-D-mannoside is 100%, which is higher than using a methyl-α-D-glucoside solution. It is found that the methyl-α-D-mannoside isotonic solution is excellent for the purification of platelets on the MEM-Sephadex column. The eluted platelets were also tested by checking their capability to aggregate upon the addition of thrombin solution. More than 90% of the cells were aggregated in this experiment.

The highest recovery of 41.4% of the lymphocytes is obtained by elution with the 0.27 M sucrose solution. To demonstrate the capability of the MEM-Sephadex column packings to separate and purify the platelets and lymphocytes from human blood, human leukocyte-rich plasma was eluted from the column by an isotonic sucrose solution. Fig. 3 shows the elution pattern of the human leukocyte-rich plasma on the MEM-Sephadex G25 column (25 × 0.9 cm I.D.) using a sucrose solution at pH 7.4. The sample solution (leukocyte-rich plasma) contained mainly platelets, granulocytes and lymphocytes. The eluted cells from the MEM-Sephadex column were identified on the basis of their retention volumes compared with the retention volumes of platelets and lymphocytes eluted independently. Separation was performed within 1.5 h and two types of cells were well separated from the leukocyte-rich plasma. Fractions 6–8 and 13–16, corresponding to center cuts of the first and second peaks, contain over 95% of platelets and lymphocytes. The viability of the blood cells eluted from the column was evaluated by confirmation of trypan blue dye exclusion under the

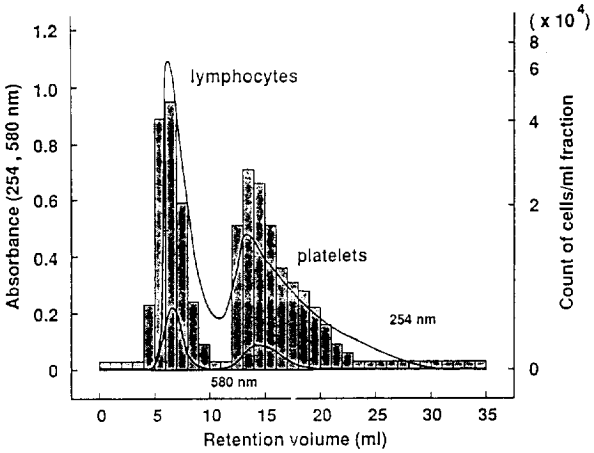

Fig. 3. Elution profile of human leukocyte-rich plasma. Column: MEM-Sephadex G25 (25 × 0.9 cm I.D.); mobile phase: 0.27 *M* sucrose solution pH 7.4; flow-rate: 20.0 ml/h; sample: 1.5 ml human leukocyte-rich plasma.

microscopy; over 90% of the platelets and lymphocytes are confirmed to be viable cells. It seems that the chromatographic separation procedures described in this study are very useful for the fractionation of granulocytes and lymphocytes, because contamination with erythrocytes is low compared with the conventional Ficoll gradient method. Furthermore, the chromatographic system does not require special apparatus, e.g. a cell separator, and does not need a special technique, e.g. the attachment of beads to the cell surface for the separation by a magnetic field.

The results of the above studies indicate that the use of MEM-bonded column packings (MEM-Cellulofine and MEM-Sephadex) and isotonic solutions of the mono- and disaccharides and methyl-α-D-pyranosides are very useful for the selective separation of human blood platelets, granulocytes and lymphocytes. It was found that the selective separation and purification of human blood cells was performed on the basis of the different affinities between the MEM stationary phase, the surface of the packings and the blood cell surfaces.

References

[1] U. Matsumoto and Y. Shibusawa, *J. Chromatogr.*, 187 (1980) 351.
[2] U. Matsumoto and Y. Shibusawa, *J. Chromatogr.*, 206 (1981) 17.
[3] U. Matsumoto, Y. Shibusawa and Y. Tanaka, *J. Chromatogr.*, 268 (1983) 375.
[4] U. Matsumoto, M. Ban and Y. Shibusawa, *J. Chromatogr.*, 285 (1984) 69.
[5] U. Matsumoto and Y. Shibusawa, *J. Chromatogr.*, 356 (1986) 27.
[6] Y. Shibusawa, U. Matsumoto and M. Takatori, *J. Chromatogr.*, 398 (1987) 153.
[7] U. Matsumoto, Y. Shibusawa and M. Yamashita, *J. Chromatogr.*, 504 (1990) 69.
[8] C.H.W. Leeksma and J.A. Cohen, *J. Clin. Invest.*, 35 (1956) 964.
[9] A. Bøyum, *Nature (London)*, 204 (1964) 793.
[10] E. Thorsby and A. Bratlie, in P.E. Terasaki (Editor), *Histocompatibility Testing*, Munksgaad, Copenhagen, 1970, p. 655.