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Development of protamine-bonded phase for separation of saccharides in liquid chromatography

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Abstract

A protamine-bonded polymer gel was synthesized by passing this protein through an activated carbamate polyamine polymer gel layer. This new packing material was suitable for versatile separation of monosaccharides, oligosaccharides, sugar alcohols and uronic acids in liquid chromatography. The column packed with this gel could be operated at room temperature and gave excellent recovery for the reducing monosaccharides such as 2-deoxyribose, 2-deoxyglucose, ribose and mannose for which the conventional alkylamine-bonded phase column gave poor recovery. This column was used for the analysis of saccharides in urine using a post-column reaction detection system.

Keywords: Stationary phases, LC; Protamine; Saccharides; Uronic acids; Sugar alcohols

1. Introduction

Separation of carbohydrates by high-performance liquid chromatography (HPLC) has been widely used because saccharides are usually found as complex mixtures in bio-organisms. Anion-exchange columns with borate buffer and propylamine-bonded silica gel columns with aqueous acetonitrile have been used for this purpose [1–5]. Ion-exchange liquid chromatography of monosaccharides and oligosaccharides on anion-exchange resins required longer analysis time. On the other hand, liquid chromatography on amine phase columns has provided fast analysis. However, the amine phase columns gave poor recovery for some reducing saccharides such as ribose, mannose and most of the 2-deoxysugars, since these sugars react with the primary amino group of the stationary phase to give N-glycosides or their Amadori rearrangement products [6–8]. The amide columns recently developed showed both fast analysis and high recovery of carbohydrates [9–11]. However, amide columns

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should be operated at higher temperatures, around 80°C, in order to avoid the separation of sugar anomers which make the chromatogram too complex.

We found that protamine, a naturally occurring strongly basic protein, composed mainly of arginine, readily binds to silica gel [12]. This property was applied to the preparation of protamine-coated silica gel for HPLC to give both fast separation and excellent recovery. However, the lifetimes of this column were relatively short.

In the present study, we have developed an organic polymer to which protamine bonded covalently, and its application to the separation of carbohydrates was investigated.

2. Experimental

2.1. Reagents and materials

An NH2P-50 column (250×4.6 mm I.D., 5 μm) packed with a pentaethylenehexamine-bonded vinyl alcohol copolymer gel [13] was purchased from Showa Denko (Tokyo, Japan). Protamine-free base grade IV from salmine testis was obtained from Sigma (St. Louis, MO, USA). *N,N'*-Disuccinimidyl carbonate (DSC) was purchased from Seikagaku Kogyo (Tokyo, Japan).

Monosaccharide and oligosaccharide standards were obtained from Wako (Osaka, Japan), Seikagaku Kogyo and Tokyokasei (Tokyo, Japan). Guanidine hydrochloride and potassium tetraborate were purchased from Nakarai Tesque (Kyoto, Japan). Ion-exchange resins were obtained from Dow Chemical (USA).

HPLC-grade acetonitrile was obtained from Wako. Deionized and distilled water was used throughout. All other chemicals and solvents were analytical grade or the highest grade commercially available.

2.2. Preparation of the protamine-bonded polymer gel column (PBP column)

Protamine is a basic protein of about 4200 Da, with a p*I* between 10 and 12, an arginine content of about 70%, and a N-terminal proline residue. The primary structure is shown in Fig. 1 [14]. The

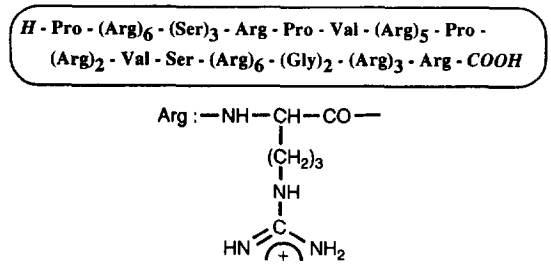

Fig. 1. Primary structure of protamine (salmon salmine A1).

protamine bonding process scheme is shown in Fig. 2. The PBP column was prepared according to the method of Nimura et al. [15]. A pre-packed polyamine-bonded gel column (250×4.6 mm I.D.) was washed with acetonitrile. A 0.5% (w/v) DSC solution in acetonitrile was delivered through the column at a flow-rate of 0.5 ml/min for 15 h to give an activated carbamate polymer gel column. After washing the column with acetonitrile, a 0.5% (w/v) solution (pH 8) of protamine-free base in water was delivered through the column at a flow-rate of 0.5 ml/min for 15 h to give a protamine-bonded polymer gel column. The column was then washed with water and 50% aqueous acetonitrile at a flow-rate of 1.0 ml/min for 3 h. The bonding process of protamine to the amine-bonded polymer gel was monitored using Sakaguchi's reaction [16]. This reaction reagent developed red-orange with guanidyl groups of protamine on PBP gel but did not react with amine-bonded polymer gel. However, the amount of bonded protamine could not have been quantitatively determined because PBP gel is an organic polymer.

2.3. Chromatography of carbohydrates

The liquid chromatography system consisted of a Model 655-12 pump (Hitachi, Tokyo, Japan), a Model 7125 injector (Rheodyne, Cotati, CA, USA) and an RI-2 refractive index detector (Japan Analytical Industries, Tokyo, Japan). The column temperature was controlled with a Taitec CL-80 Coolnit and a Taitec Thermominder Jr-1000 (Taitec Saitama, Japan). The chromatograph had a post-column reaction system and it consisted of two Model LC9A pumps, a DGU3A degassing unit, a CTO6A column oven, an RF535 fluorescence detector, a CRB6A reaction oven and a CR6A integrator (Shimadzu,

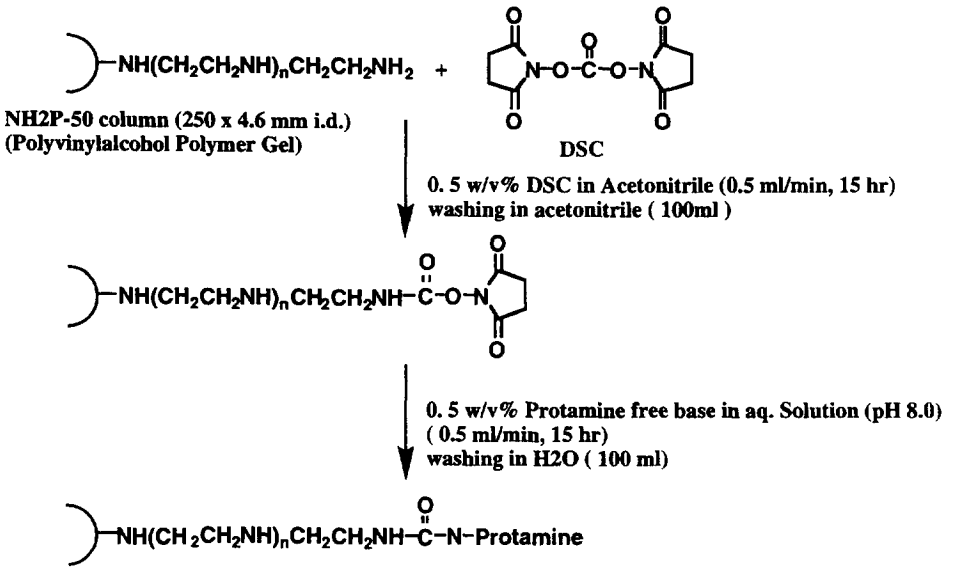

Fig. 2. Synthetic scheme for the preparation of the PBP column.

Kyoto, Japan). The stainless-steel reaction tube was 10 m×0.5 mm I.D., and the cooling tube was 1.5 m×0.5 mm I.D. The reaction temperature was 150°C. The flow diagram of a post-column reaction fluorescence detection system using a guanidine method was previously reported [17].

Urine collected was stored at −20°C. The urine was melted at room temperature and passed a double-bed column packed with H^+ form cation-exchange (Dowex 50W-X8) and OH^- form anion-exchange (Dowex 1-X8) resins to eliminate cations, anions and proteins. The sample clean-up column was made using Pasteur pipettes. The column bed size was 20×5 mm I.D.; 20 μl effluent from the sample clean-up column was directly injected onto the protamine column.

3. Results and discussion

The chromatographic profiles of monosaccharides, oligosaccharides, sugar alcohols and uronic acids separated on the protamine-bonded polymer gel column (PBP column) are shown in Figs. 3–7. These figures demonstrate that the PBP column recognizes the fine configuration differences among the saccharides. Fig. 3A shows the chromatogram of 2-deoxyribose, ribose, arabinose, xylose, fructose, mannose, galactose and glucose, obtained by eluting them with 87.5% (v/v) aqueous acetonitrile. This concentration of acetonitrile was found to be the optimum for the separation of reducing monosaccharides.

Fig. 3 compares the chromatogram of reducing carbohydrates separated on the PBP column (Fig. 3A) with those separated on a conventional alkylamine-bonded column (Fig. 3B). The eluent was modified in order to give the same retention time of fructose on the PBP and the NH2P-50 columns. The elution of monosaccharides was performed in an isocratic elution. The alkylamine-bonded column gave no peak for 2-deoxyribose and only reduced peaks for ribose, rhamnose, arabinose, xylose, mannose and galactose. On the other hand, the PBP column gave high peaks for 2-deoxyribose, ribose, arabinose, mannose and galactose as well as glucose. Table 1 compares the recovery of reducing carbohydrates from the PBP column with those from a conventional alkylamine-bonded column. The recoveries were normalized to that of fructose. The PBP column showed excellent recovery for almost all saccharides tested, while the amino column gave poor recovery for several saccharides.

Fig. 4 shows the separation of the disaccharide

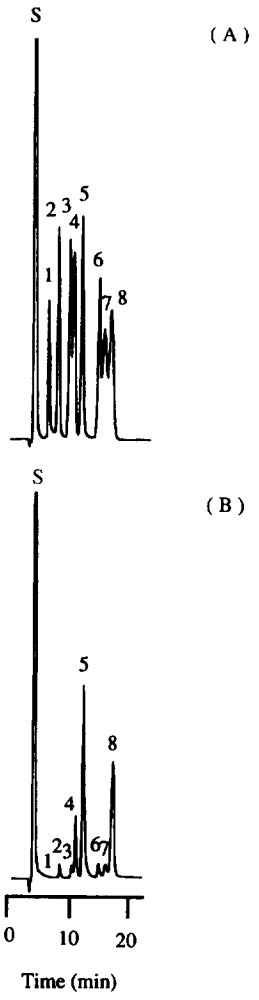

Fig. 3. Chromatograms of monosaccharides on (A) the PBP column and (B) the NH2P-50 column (250×4.6 mm I.D.). Eluent, (A) 87.5% (v/v) aqueous acetonitrile; (B) 82.5% (v/v) aqueous acetonitrile; flow-rate, 0.90 ml/min; column temperature, 30°C; detector, refractive index detector (range ×32). Each 100 μg sample was injected. Peaks: S=solvent; 1=2-deoxyribose; 2=ribose; 3=arabinose; 4=xylose; 5=fructose; 6=mannose; 7=galactose; 8=glucose.

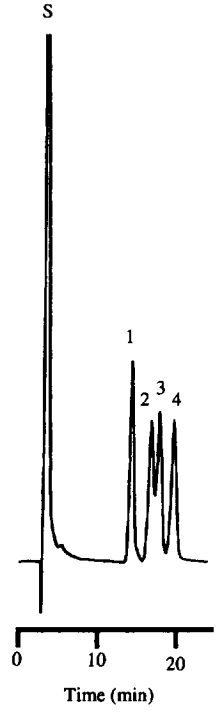

Fig. 4. Chromatogram of disaccharides. Eluent, 82.5% (v/v) aqueous acetonitrile; flow-rate, 1.0 ml/min. Further conditions were the same as described in the legend to Fig. 3. Peaks: S=solvent; 1=sucrose; 2=lactose; 3=maltose; 4=isomaltose.

mixture containing sucrose, lactose, maltose and isomaltose. Glucose and maltooligosaccharides were completely separated as shown in Fig. 5. Fig. 6 demonstrates that the PBP column could also be applicable to the separation of sugar alcohols such as glycerin, xylitol, sorbitol, mannitol and inositol. The separation of uronic acids and sialic acid were also examined. They were too strongly retained on the stationary phase and hardly eluted from the column but they were eluted by adjusting the pH of the eluent. Fig. 7 demonstrates that the PBP column is applicable to the separation of amino sugar and uronic acids such as glucuronic acid, N-acetyl glucosamine and N-acetylneuraminic acid.

Although conventional alkylamine-bonded columns also separate these saccharides, they often give poor recovery for aldoses other than glucose [6–8]. It has recently been claimed that amide-type columns, such as TSK gel Amide-80, give excellent recovery for the aldoses that show poor recovery on the alkylamine-bonded columns. However, anomers of reducing carbohydrates were separated on the amide-type columns, and the complicated chromatogram made the identification of individual carbohy-

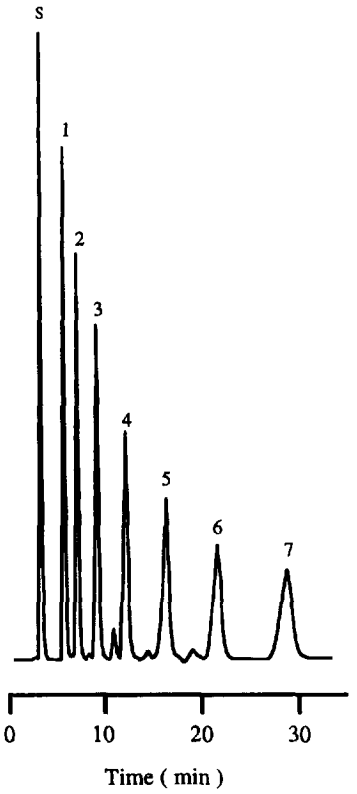

Fig. 5. Chromatogram of glucose–maltooligosaccharides mixture. Eluent, 72.5% (v/v) aqueous acetonitrile; flow-rate, 1.0 ml/min. Further conditions were the same as described in the legend to Fig. 3. Peaks: S=solvent; 1=glucose; 2=maltose; 3=maltotriose; 4=maltotetraose; 5=maltopentaose; 6=maltohexaose; 7=maltoheptaose.

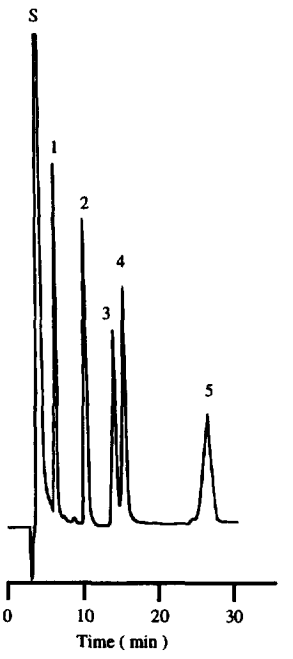

Fig. 6. Chromatogram of sugar alcohols. Eluent, 87.5% (v/v) aqueous acetonitrile; flow-rate, 1.0 ml/min. Further conditions were the same as described in the legend to Fig. 3. Peaks: S=solvent; 1=glycerin; 2=xylitol; 3=sorbitol; 4=mannitol; 5=inositol.

drates more confusing. Although anomers are known to give a single peak on the amide column by eluting with alkaline solution, such treatment may degrade the amide-type column. Otherwise, amide-type columns should be operated at higher temperatures, about 80°C, to obtain a single peak for anomers.

Fig. 8 shows that the PBP column gives a single peak for each reducing carbohydrate in a wide temperature range. The column was operated in the range 4–30°C without resolution of anomers. No anomer resolution was observed even at 4°C, whereas broadening of the peaks was observed for some monosaccharides. This PBP column is suitable for operation at room temperature using mobile phases at neutral pH. The basicity of the guanidyl group of protamine may accelerate the mutarotation to make the anomers indistinguishable.

The retention times and the number of theoretical plates were reproducible in a 20-day series with 80% (v/v) aqueous acetonitrile solution as the eluent even though the theoretical plate number of the PBP column was about 75% that of the original NH2P-50 column.

Chemically bonded silica gels are generally known to lack chemical stability because they are poorly resistant to basic materials. The polymer gels are free from this limitation owing to their elevated chemical stability. The PBP column showed excellent separation of carbohydrates in comparison with separation using amine phase columns. The elution order of saccharides from the PBP column was almost the same as that from amine phase columns such as the alkylamine-bonded silica gel column, except that the

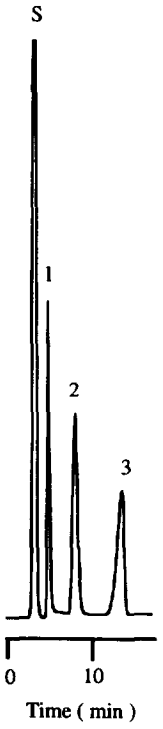

Fig. 7. Chromatogram of amino sugar–uronic acids mixture. Eluent, 80% (v/v) aqueous acetonitrile, pH 7–10 m*M* potassium dihydrogenphosphate; flow-rate, 1.0 ml/min. Further conditions were the same as described in the legend to Fig. 3. Peaks: S=solvent; 1=glucuronic acid; 2=N-acetylglucosamine; 3=N-acetylneuraminic acid.

Table 1
Recovery of reducing carbohydrates ($n=3$) on the PBP column and the NH2P-50 column

Analyte	Recovery (%)	
	PBP column[a]	NH2P-50 column[b]
2-Deoxyribose	70±0.8	0
Ribose	83±0.9	6±0.3
Rhamnose	87±0.7	45±1.3
Arabinose	92±0.2	6±0.5
2-Deoxyglucose	84±1.0	0
Xylose	95±0.2	29±0.3
Fructose	100	100
Mannose	104±0.9	7±0.3
Galactose	98±0.8	7±1.0
Glucose	102±1.2	75±10

Flow-rate, 0.90 ml/min. Further conditions are the same as described in the legend to Fig. 3.
[a] Eluent, 87.5% (v/v) aqueous acetonitrile.
[b] Eluent, 82.5% (v/v) aqueous acetonitrile.

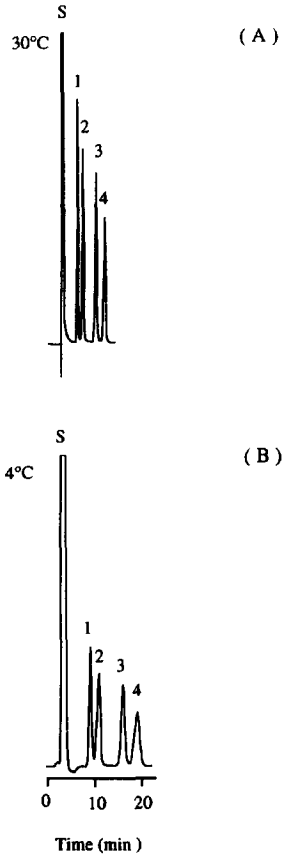

Fig. 8. Effect of temperature on separation of sugar anomers on the PBP column. Eluent, 80% (v/v) aqueous acetonitrile; flow-rate, 1.0 ml/min; column temperature, (A) 30°C; (B) 4°C. Further conditions are the same as described in the legend to Fig. 3. Peaks: S=solvent; 1=fructose; 2=glucose; 3=sucrose; 4=maltose.

reversal elution order was observed for glucose and galactose and for maltose and lactose. These results show that the retention mechanism of the PBP column is similar to that of the conventional amine phase columns [18–21]. However, the highly hydrophilic nature of the guanidyl groups of the protamine molecule caused the reversal of the elution order of some saccharides. The high recovery from the PBP column may be caused by the properties of the guanidyl groups; these are an ionized form under ordinary conditions and do not react with reducing carbohydrates, while the amino groups of alkylamine-bonded support readily react with the carbohydrates to give N-glycosides. The effect of the

pH and other solvents on the PBP column is similar to the effect on the propylamino column. Acetonitrile–water mixtures were found to be appropriate eluents.

The protamine-bonded column was applied to the analysis of urine. A post-column reaction detection system using guanidine as the reaction reagent was used for the high-sensitivity detection of saccharides.

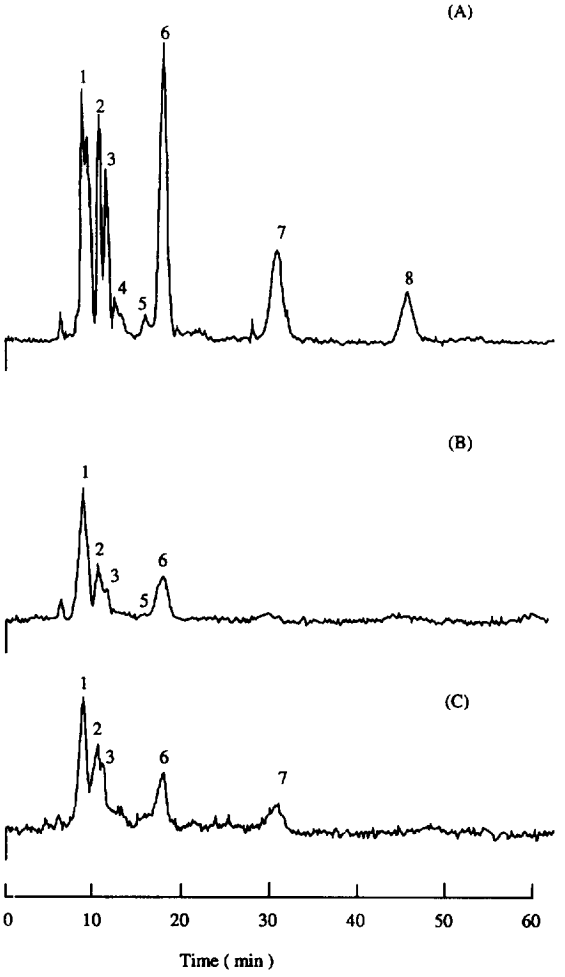

Fig. 9. Chromatograms on a PBP column (250×4.6mm I.D.) of saccharides in urine from patients with (A) lung cancer; (B) hepatocellular carcinoma; (C) multiple myeloma. Eluent, 85% (v/v) aqueous acetonitrile; flow-rate, 1.0 ml/min; column temperature, 40°C; detector, fluorescence detection; λ_{ex}=314 nm; λ_{em}=433 nm; reagent, 50 m*M* potassium borate containing 20 m*M* guanidine hydrochloride (pH 10.5); flow-rate of reagent, 0.5 ml/min. Peaks: 1=ribose; 2=arabinose; 3=xylose; 4=fructose; 5=mannose; 6=glucose; 7=unknown; 8=lactose.

The chromatographic system had a post-column reaction detector and was the same as that previously reported [17]. The sensitivity of the post-column detection was 1 pmol when a sodium hydroxide–borate solution was used as the eluent for the chromatographic separation [22]. However, the protamine-bonded column required 85% (v/v) aqueous acetonitrile solution as the eluent for the separation of carbohydrates. Therefore, the reagent solution for the detection was modified in accordance with the solubility of borate. The detection limit of ribose, arabinose, xylose, fructose, mannose, glucose and galactose was about 500 pmol; ribose, deoxyribose, arabinose and mannose were detected at almost the same sensitivity with glucose owing to the improved recovery.

Fig. 9 shows chromatograms of hydrophilic and neutral compounds in urine from patients with lung cancer, hepatocellular carcinoma and multiple myeloma. The peaks were tentatively identified based on their retention time. The quantitative results are summarized in Table 2. The excellent recovery of carbohydrates from the PBP column and the simple clean-up procedure developed in the present study afforded the analysis of carbohydrates in the urine of cancer patients, although the urine of patients with diabetes, cancer, renal dysfunction, etc., contains various compounds which interfere with the carbohydrate chromatography. The present method is expected to be applicable to various clinical investigations.

4. Conclusions

The PBP column is prepared easily and provides excellent separation of various carbohydrates; this separation is as good as that achieved using a conventional amine phase column. The column shows far better recovery of aldoses in comparison with a conventional amine phase column owing to the guanidyl group of protamine, which has low nucleophilic reactivity compared to a primary amine. This column has a long lifespan owing to the existence of the chemical stability of the copolymer gel based on covalently bonded protamine. Furthermore, this column is expected to be utilized in the fields of biochemistry and clinical chemistry.

Table 2
Saccharides in urine from five patients

Patient No.[a]	Concentration (μg/ml)						
	Ribose	Arabinose	Xylose	Fructose	Mannose	Glucose	Lactose
1	175.4	134.1	111.7	61.71	40.21	778.5	74.51
2	287.3	89.50	78.46	–	18.18	259.9	–
3	110.9	46.29	27.76	–	–	89.71	–
4	287.1	143.5	73.45	148.2	–	358.9	407.0
5	185.6	44.90	14.21	–	–	153.3	84.07

[a] 1, Lung cancer; 2, hepatocellular carcinoma; 3, malignant lymphoma; 4, acute myelo leukemia; 5, multiple myeloma.

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