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Protein determination by high-performance gel-permeation chromatography: applications to human pancreatic juice, human bile and tissue homogenate

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Abstract

A high-performance gel-permeation chromatography (HPGPC) method to determine the proteins of human pancreatic juice, bile, and tissue homogenate has been developed. A diol-type silica gel column (35×8 mm I.D., 5 nm average pore diameter) was used under a column temperature of 8°C. The eluent was acidic phosphate buffer with a high concentration of sodium chloride, nonionic detergent of polyoxyethylene (20) cetyl ether (Brij 58), glycerol and 2-propanol. The UV wavelength used for the protein detection was 210 nm. Analytical time was within 3.5 min. Good correlation coefficients were obtained with this HPLC method at a column temperature of 8°C and a spectrophotometric bicinchoninic acid (BCA) method. A photometric pyrogallol–red molybdate complex method was found to correlate well with this HPLC method and with the BCA method only for tissue homogenate. Since this HPGPC protein assay method is simple, convenient, rapid, reproducible, and reliable, it is expected to be generally applicable to clinical and also to biochemical research. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Proteins

1. Introduction

The analysis of proteins in pancreatic juice is important for the study of pancreas transplantation [1], clogging of pancreatic stents [2], pancreatic stone formation [3], entero-pancreatic circulation of trypsin [4,5], and possible diagnosis of pancreatitis and pancreatic cancer [6]. Bile protein analysis is also important in the research on gallstone formation [7], gallduct cancer [8], and protein excretion [9].

Previously, we devised a high-performance gel-permeation chromatographic (HPGPC) serum protein assay method [10]. The serum protein content was determined using a 10-nm pore diameter diol-type silica gel column (35×8 mm I.D.) [10]. Hence, the major proteins of human serum were albumin [relative molecular mass (M_r) 66 000; 50–78%] and immunoglobulin G (M_r 155 000; 11–26%), we used a 10-nm pore diameter diol-gel for human serum

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protein determination. The method seemed to be generally applicable to the endocrine-type body fluids, such as serum [10]. However, there are several exocrine-type body fluids where the M_r of major proteins are similar to or smaller than albumin, i.e., major pancreatic juice proteins are hydrolase enzymes such as trypsin, chymotrypsin and α-amylase (M_r ca. 23 000–51 000) [11], and 80–90% of the human milk proteins are casein (M_r ca. 24 000–38 000; 30%), lactoferrin (M_r 77 000; 10–30%), and α-lactalbumin (M_r 14 100; 20–30%) [12].

In order to apply this HPGPC serum protein assay method to the exocrine-type body fluids of pancreatic juice and bile, and also to tissue homogenates, we decided to use the narrower 5-nm pore diameter diol-type HPGPC column, because the 5-nm diol-gel is expected to exclude more of the smaller M_r proteins than the 10-nm diol-gel is. The protein values obtained by the high-performance liquid chromatography (HPLC) method were compared to the values of photometric bicinchoninic acid (BCA) and biuret methods, and also to a newly available pyrogallol–red molybdate complex (PRM) method of Wako. The column temperature and column length were optimized to 8°C and 3.5 cm, respectively. The separation analysis by HPGPC with a 30-nm pore diameter diol-gel column (30×0.8 cm I.D.) was performed. Electrophoretic analysis was performed using pancreatic juice samples.

2. Experimental

2.1. Chemicals and reagents

Bovine albumin (BSA; Cohn fraction V), human albumin (HSA; Cohn fraction V), bovine γ-globulins (Cohn fraction II), human γ-globulins (Cohn fractions II and III), lysozyme from hen-egg white (M_r 14 400), fibronectin related peptide [Cys (C)–Gln (Q)–Asp (D)–Ser (S)–Glu (E)–Thr (T)–Arg (R)–Thr (T)–Phe (F)–Tyr (Y); M_r 1249], and Gly–Phe (GF; M_r 222) were purchased from Sigma (St. Louis, MO, USA). Trypsin from bovine pancreas was from Boehringer Mannheim (Mannheim, Germany) (now Roche Diagnostics). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) molecular mass standards (broad range) kit was from Bio-Rad (Richmond, CA, USA). Aprotinin from bovine lung (M_r 6512) and insulin from bovine pancreas (M_r ca. 5800) was from Takara Shuzo (Kyoto, Japan). Brij 58 (polyethyleneglycol 1000 monocetylether) was from Nacalai Tesque (Kyoto, Japan). Sodium dihydrogenphosphate dihydrate, orthophosphoric acid (85%), copper(II) sulfate pentahydrate, potassium tartrate, glycerol and a protein assay rapid kit (PRM method) were from Wako (Osaka, Japan). A BCA protein assay kit was from Pierce (Rockford, IL, USA). HPGPC columns of Develosil 50 Diol-5 (two 35×8 mm I.D. columns of No. 1 Mfg. No. 0105055 and No. 2=Mfg. No. 1406054, one 50×8 mm I.D. column, 5 nm average pore diameter, 5000 nm average particle size) and Develosil 300 Diol-5 (300×8 mm I.D., 30 nm average pore diameter, 5000 nm average particle size) were from Nomura (Aichi, Japan). Standartips of 0.1 and 1.0 ml were from Eppendorf-Netheler-Hinz (Hamburg, Germany). Ekicrodisc 13 (200 nm average pore diameter) was from Gelman Sciences (Ann Arbor, MI, USA).

2.2. Specimens

Pure human pancreatic juice [P1, 56 years, female, anomaly of gallduct divergence; P2, 69 years, female, pancreatic cancer of moderately differentiated adenocarcinoma (T1bN1M0 stage III, serum CEA 3.7 ng/ml, serum CA19-9 230 U/ml); P3, 84 years, female, gallduct stone; P4, 54 years, male, gallduct stone; P5, 45 years, female, gallbladder stone] were collected without using gut hormones of secretin-cholecystokinin/pancreozymin. Patient P5 showed no pancreatitis or other pancreatic diseases or her blood showed no biochemical anomaly. Two of the pancreatic juice samples (P6, 70 years, male, pancreatic cancer of tail-portion, T3N1M0 stage III, serum CA 19-9>10 000 U/ml; P7, 64 years, female, gallduct cancer, T2N2M1 stage IVB, serum CEA 2.8 ng/ml, serum CA 19-9 1444 U/ml) were obtained with the use of gut hormones of secretin-cholecystokinin/pancreozymin. All the pure pancreatic juice samples were collected from the main pancreatic duct with a cannula inserted selectively into the main duct using the endoscope prior to the endoscopic retrograde cholangiopancreatography (ERCP). To date, pure pancreatic juice from humans

can only be obtained by the method described above, i.e., prior to the ERCP.

Similarly, pure gallduct bile samples were collected from the patients, i.e., B1 from gallduct cancer (the same patient P7), B2 from gallduct cancer [T2(?)N0M0 stage II] with gallduct stone, B3 from pancreatic cancer (caput portion, T3N1M0 stage III), B4 from chronic pancreatitis with alcoholic liver cirrhosis, and B5 from gallduct stone. All the human body fluids were collected with the consent of the patients. All the pancreatic juice and gallduct bile samples were kindly donated by the Saisei-kai Maebashi Hospital (Maebashi, Gunma, Japan). All the specimens were stored at −80°C.

Rats and mice (aged 8 weeks) were purchased from Japan Clea (Tokyo, Japan). After anesthetization with ether, the tissues from rats and mice were quickly removed and stored at −80°C. About a 0.1-g portion of the tissue was cut into pieces and homogenized in cold (+4°C) 1 m*M* sodium phosphate buffer (pH 7.0) containing 10% (v/v) glycerol. A glass–glass homogenizer (Econo-Grind Homogenizer 25–100 lambda; Randoni Glass Technology, Monrovia, CA, USA) was used. After homogenization, tissue homogenate was poured into the Eppendorf tube. The homogenate in the tube (ca. 0.6 ml) was cooled on an ice-bath (sodium chloride–water of ca. −20°C), and ultrasonicated for 3 min with a 1-s pulse input and a 1-s vacation by the ultrasonic processor (Model VC 70T, 130 W, 20 KHz, amplitude at 40, with stepped microtip; Renover Science, Tokyo, Japan). The ultrasonicated homogenate was then filtered through Ekicrodisc 25 (0.2 μm HT-Tuffryn; Gelman Japan, Tokyo, Japan), and the filtrate was used as a homogenate sample and stored at −80°C until use. In the case of rat liver and kidney (right), perfusions were performed with 0.9% (154 m*M*) sodium chloride solution.

2.3. High-performance liquid chromatography

A Model LC-10AT pump (Shimadzu, Kyoto, Japan) was used. The injector was a Model U6K (diaphragm type; Waters, Milford, MA, USA). Detection was carried out using a Model 757 absorbance detector (Perkin-Elmer, Foster City, CA, USA). The recorder was a Model 561 (Hitachi), and a Chromatopac C-R6A data processor (Shimadzu) was also used. A line filter (GL Science, Tokyo, Japan) was inserted between the injector and the column. The injector, line filter and detector were at room temperature (23–27°C) in the air-conditioned room.

Eluent (1.0 l) was prepared as follows: first, 15.6 g of sodium dihydrogenphosphate dihydrate was dissolved in 0.5 l of distilled water and pH adjusted to 2.0 by orthophosphoric acid. Then, 87.5 g sodium chloride, 300 ml of glycerol, 250 ml of 2-propanol, and 10 ml of Brij-58 (dissolved in a water-bath at 60°C) were added, mixed thoroughly (usually 30 min or more). Therefore, final concentrations were as follows: sodium phosphate (0.094 *M*), sodium chloride (1.42 *M*), glycerol (28%, v/v), 2-propanol (24%, v/v), and Brij-58 (0.9%, v/v). The final pH of the eluent as measured by the glass electrode was 2.11. Since the obtained Brij-58 showed a little turbid nature, the eluent was filtered using Whatman No. 3 filter paper. The flow-rate was 1.0 ml/min. The column inlet pressures were 11.06 MPa (1609 p.s.i., 113 kg/cm^2) for the 35-mm long column and 12.46 MPa (1850 p.s.i., 130 kg/cm^2) for the 50-mm long column, at a column temperature of 8°C. A column oven with cooler (Model CTO-10 ACvp; Shimadzu) was used with the aid of 1.2 kg of pre-frozen (−20°C) cooling bags. Detection of protein was performed at 210 nm.

For protein component analysis, a 30-cm long column with 30 nm average pore diameter was used with the following eluent. Eluent (1.0 l) was prepared as follows: first, 15.6 g of sodium dihydrogenphosphate dihydrate was dissolved in 0.5 l of distilled water. Then, 87.5 g sodium chloride, 400 ml of glycerol, 100 ml of 2-propanol, and 10 ml of Brij-58 were added, and mixed thoroughly. Therefore, final concentrations were as follows: sodium phosphate (0.1 *M*), sodium chloride (1.5 *M*), glycerol (40%, v/v), 2-propanol (10%, v/v) and Brij-58 (1%, v/v). Finally, apparent pH was adjusted to 3.40 with 10-fold diluted orthophosphoric acid using a glass electrode. The eluent was filtered by using Whatman No. 3 filter paper. The flow-rate was 0.2 ml/min, and the column inlet pressure was 10.25 MPa (1523 p.s.i., 107 kg/cm^2), at a column temperature of 15°C with a column oven with cooler (Model CTO-10 ACvp; Shimadzu).

After analysis, the U6K injector was thoroughly

washed with a washing solution, i.e., 0.1 *M* sodium phosphate buffer (pH 2.1)–methanol (50:50, v/v). The plug seal adjustment screw of U6K should be removed, and stored in water when not in use. In order to prevent the drift of the baseline, polishing the top and bottom surfaces of the valve stems of the U6K with a file and sharpening stone is effective.

In order to minimize the column difference, the HPGPC columns of Develosil 50 Diol-5 (two 35×8 mm I.D. column Nos. 1 and 2, and one 50×8 I.D. column) were washed with this washing solution (with 50-fold volume of the column) at a column temperature of 72°C.

Accumulated lipid compounds on the HPGPC columns are also washed out by eluting the pH 2.11 eluent at a column temperature of 72°C. When returning to the higher pH (or lower proton concentration) eluent, first wash out the pH 2.11 eluent with distilled water and then equilibrate to the desired eluent.

2.4. HPLC protein assay

Fresh pancreatic juice (0.01 ml), bile (0.01 ml), or tissue homogenates (0.01 ml) was dissolved in 0.99 ml of the neutral HPGPC eluent for serum (adjusted to pH 6.8) (Ref. [10]; Section 2.4). Tissue homogenate samples were further diluted 500-fold. If pancreatic juice protein concentration is low, a 0.04-ml volume of pancreatic juice was dissolved in a 0.36 ml of the neutral HPGPC eluent (10-fold diluted), or 0.02 ml was dissolved in 0.98 ml of the neutral HPGPC eluent (50-fold diluted). If bile protein concentrations are high, these samples were further diluted 1000-fold or 2000-fold. These 100-, 50- or 10-fold diluted pancreatic juice samples, 2000-, 1000- or 100-fold diluted bile samples, or 500-fold diluted tissue homogenate samples were filtered by Ekicrodisc 13, and a 0.005-ml portion of the filtrate was injected into the HPLC system. Standard BSA (2.0 mg/ml; BSA of Sigma dissolved in aqueous 0.9% NaCl, 0.02% sodium azide and 1 m*M* EDTA solution) was 10-, 20-fold or 100-, 200-fold diluted with the neutral HPGPC eluent [10]. One and 0.5 μg amounts for 0.2 AUFS or 0.1 and 0.05 μg for 0.05 AUFS (in 0.005 ml volume) were injected into the HPGPC system (external standard), and the two-point linear calibration curves were obtained. Analysis times was less than 3.5 min and 5.0 min using 35-mm and 50-mm long columns, respectively.

2.5. Spectrophotometric protein assays

In order to compare the values obtained by the HPLC method with the spectrophotometric values, pancreatic juice, bile and tissue homogenate protein contents were independently assayed by the BCA method [13]. Although not as reliable as compared to the BCA method, the biuret method and the newly available PRM method were also used. The assay methods of BCA and biuret were as described in Ref. [10]. The PRM method was performed according to Wako's instruction kit. Other dye-binding methods (such as using Coomassie brilliant blue) were not tested, since they had not worked well on the samples containing lipids or non-ionic detergents (unpublished observation). A Model U-3200 (Hitachi) spectrophotometer was used. BSA was used as a standard protein.

2.6. SDS–PAGE

SDS–PAGE of pancreatic juice was performed according to the method of Weber and Osborn [14]. A polyacrylamide concentration of 10% (w/v) was used. Proteins were detected with Coomassie brilliant blue R-250.

2.7. Micro-sequencing

Micro-sequencing of the pancreatic juice sample (P5) was performed after dialysis and concentration. A 2.0-ml volume of P5 pancreatic juice was dialyzed and concentrated at 4°C for 5 h in 0.9% (w/v) aqueous NaCl solution containing 15% (w/v) polyethyleneglycol 20 000 to 0.4 ml. Concentrated P5 (0.16 ml) and human serum albumin (as control or simulation; similar protein amount) were bound to two glass fiber disks by the paraphenylene diisothiocyanate (DITC) [15] and 1-ethyl-3-(3-dimethylaminopropy1)-carbodiimide (EDC) methods [16], and micro-sequenced. The phenylthiohydantoin (PTH)-amino acid produced was analyzed by the newly developed PTH-amino acid analysis method using reversed-phase HPLC [17]. Other pancreatic juices and biles of B3 and B4 were similarly

analyzed. Other biles were directly bound to the glass filters, and the filters were delipidated by dimethylformamide, methanol and chloroform–methanol solutions using ultrasonication.

2.8. Statistics

Both parametric and non-parametric statistics were used, i.e., Pearson's correlation coefficients (r) and Spearman's rank correlation coefficients (rS) for correlating the variables, respectively.

3. Results and discussion

3.1. The optimum conditions for HPGPC with 5-nm pore diameter diol gel

In order to exclude small M_r proteins from the HPGPC column, we chose 5-nm pore diameter diol-type silica gel and studied the optimum analytical conditions for this HPGPC column. As previously described in Ref. [17], ODS silica gel with a narrow pore diameter (6 nm) required a high-ionic-strength eluent. Therefore, we found that the acidic sodium phosphate solution (pH 2.11) with ca. 1.5 M NaCl affords a stable baseline in the case of 5-nm pore diameter diol gel. This eluent had higher proton and NaCl concentrations than the pH 5.3 eluent for the 10-nm pore diameter diol gel of Ref. [10]. Since proton and ionic strengths became high, we had to reduce the strong hydrophobic interactions between the proteins and glyceropropyl (diol) residues of the silica-gel support by adding 2-propanol and a non-ionic detergent as mentioned previously [18]. Furthermore, we reduced the column temperature to prevent possible heat- and acid-induced protein–protein aggregation in this HPGPC eluent containing 2-propanol. Finally, we obtained the stable and reproducible HPGPC conditions as described in Section 2.3. The choice of Brij-58 may be important, since our trial to use Brij-58 from Sigma instead of that from Nacalai was not successful, e.g., the melting temperature of Nacalai's Brij-58 was 40°C, but that of Sigma's was 42°C.

Typical exclusion patterns of some proteins and peptides are shown in Fig. 1. A stable baseline is obtainable at such a high sensitivity as 0.02 AUFS.

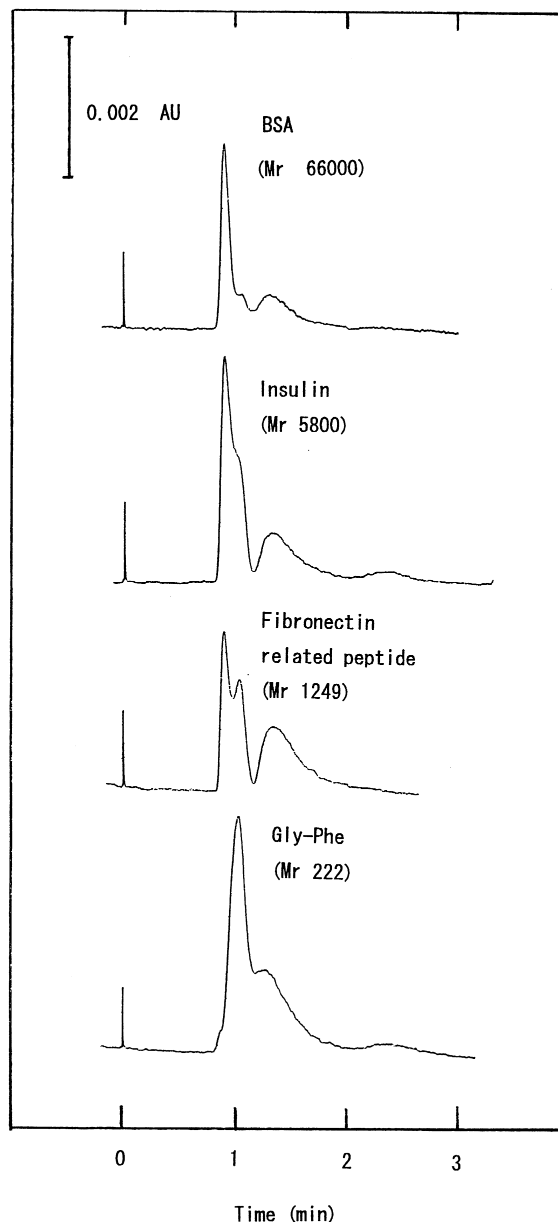

Fig. 1. Typical exclusion patterns of proteins and peptides using 5-nm average pore diameter diol-type silica gel. A 20-ng amount of BSA, 35 ng of insulin, 20 ng of fibronectin related peptide, and 60 ng of Gly–Phe were injected into the HPGPC system with a 35-mm long column (No. 1) at 8°C. Protein was detected at 210 nm at 0.02 AUFS. Chart speed was 20 mm/min. A broad peak which appeared at a retention time of 1.3 min was mainly due to the solvent for sample dissolution. Other conditions as described in Experimental.

As expected, the 5-nm pore diameter diol-gel fully excluded BSA (M_r 66 000; excluded BSA appears at a retention time of 0.9 min). Insulin (M_r 5800) and fibronectin related peptide (M_r 1249) are partially excluded, but a small peptide of glycyl–phenylalanine (M_r 222) is fully included (at a retention time of 1.0 min).

3.2. Applications to human pancreatic juice and bile

Typical application examples of human pancreatic juice and bile measured at a column temperature of 8°C are shown in Fig. 2 and in the upper panel of Fig. 3, respectively. Peak heights of BSA are linear in the HPGPC system [10], and a two-point calibration curve is obtained. Protein concentrations of the samples were calculated from obtained calibration curves, and the results are summarized in Table 1. As references, protein values measured by spectrophotometric methods are also shown in Table 1. As shown in Table 1, the results from different column lengths of 3.5 and 5.0 cm were similar. Analysis times were within 3.5 min for the 3.5-cm column and 5 min for the 5.0-cm column. Therefore, the 3.5-cm column was chosen and used in the following experiments. The effects of column temperatures on protein determinations were compared, and the results are also shown in Table 1. Lower temperatures than 15°C were desirable, since correlation coefficients between protein values of HPGPC method and those of the photometric BCA method increased at lower temperatures, i.e., $r=0.975$ ($n=7$) at 22°C, $r=0.993$ ($n=5$) at 15°C, and $r=0.997$ ($n=3$) at 8°C for the 3.5-cm column, and $r=0.972$ ($n=7$) at 18°C, $r=0.995$ ($n=5$) at 15°C, and $r=0.991$ ($n=3$) at 8°C for the 5.0-cm column. Further, better correlation coefficient was obtained in the case of bile samples at 8°C (Table 1), i.e., $r=0.918$ ($n=5$; $y=0.61134x+1.66778$) was observed at 8°C, but $r=0.895$ ($n=5$; $y=0.62807x+1.77104$) was observed at 15°C. Moreover, HPGPC protein values for B3 and B4 were closer to BCA protein values at 8°C than at 15°C. Inspections of bile proteins by HPGPC separation with the 30-nm pore diameter gel using the optimum condition for this HPGPC column as described in

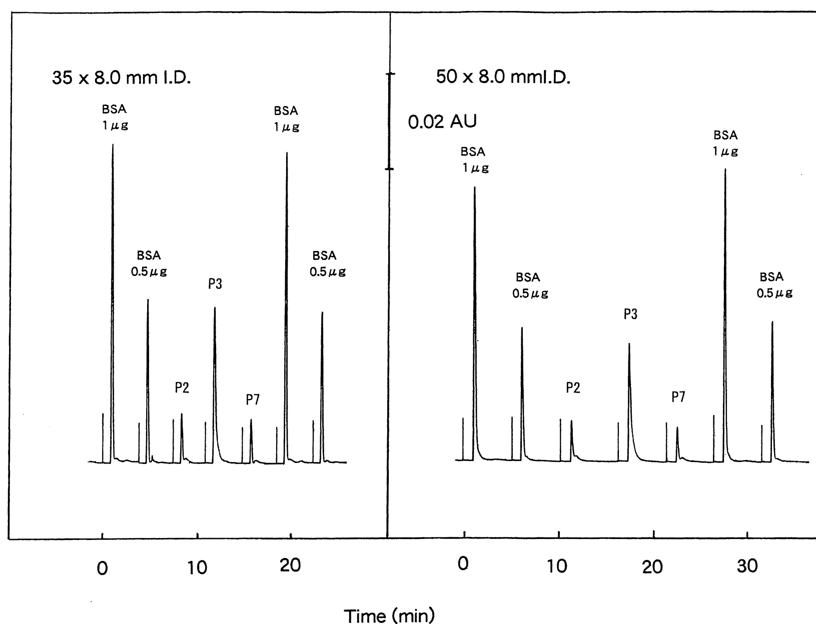

Fig. 2. Typical example of pancreatic juice protein analysis by the HPGPC method with 35×8 (No. 1) and 50×8 mm I.D. columns (5-nm average pore diameter) at 8°C. Protein was detected at 210 nm at 0.2 AUFS. A 0.005-ml volume of 100-fold diluted samples P2 and P3, and of 50-fold diluted sample P7 were injected into the HPLC system. A 0.005-ml volume of standard BSA (0.2 and 0.1 mg/ml) was also injected. Other conditions as described in Experimental.

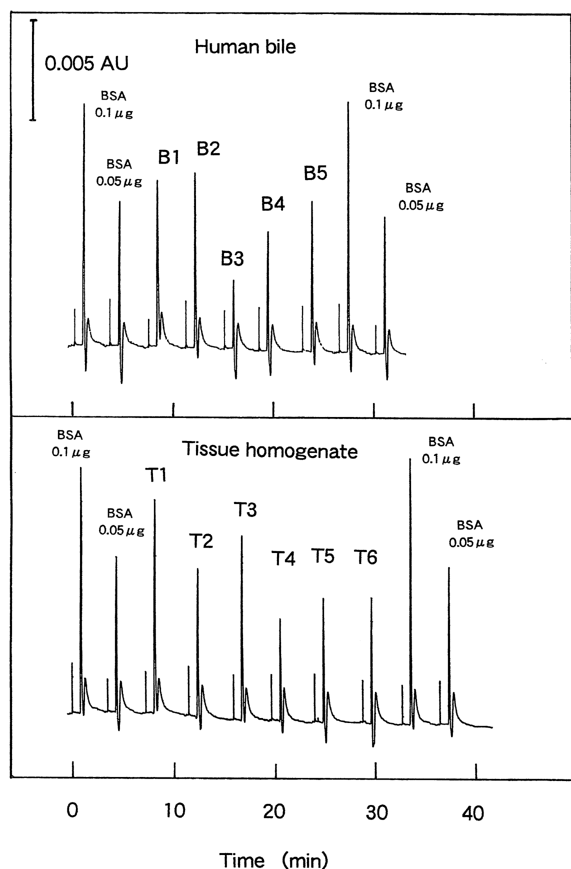

Fig. 3. Typical example of human bile and rodent tissue homogenate protein analysis by the HPGPC method with a 35×8 mm I.D. (No. 1) column (5-nm average pore diameter) at 8°C. Protein was detected at 210 nm at 0.05 AUFS. Upper panel: human biles. A 0.005-ml volume of 2000-fold diluted B1, 1000-fold diluted B2 and B5, and 100-fold diluted B3 and B4 were injected into the HPGPC system. Lower panel: tissue homogenates of rats and mice. A 0.005-ml volume of 500-fold diluted tissue homogenate samples were injected. Tissue homogenate samples were T1; female rat kidney 1, T2; male mouse kidney, T3; male mouse liver, T4; female mouse cerebellum, T5; female mouse cerebrum, and T6; male mouse abdominal skin. A 0.005-ml volume of standard BSA (0.02 and 0.01 mg/ml) was also injected. Other conditions as described in Experimental.

Experimental showed that high-molecular-mass components were richer in B3 and B4 than the other biles (data not shown). A low temperature of 4°C was also tested, however increased column inlet pressure made analysis inconvenient. Therefore, analysis at 8°C was chosen as an optimum temperature in the following studies. The use of a relatively low temperature of 15°C for ovalbumin analysis by reversed-phase HPLC has already been published by us [19]. Thus, contact of proteins and 2-propanol should occur at a relatively low temperature of less than 15°C either in the reversed-phase column or in the HPGPC column.

The reason why a difference is found for B1 was not fully understood at this time, however proteins of B1 bile (bile from late stage of gallduct cancer) seemed extremely labile as detected by a 30-nm pore diameter column (Fig. 4). Another sample showing extreme lability was the tissue homogenate of rat pancreas, however other specimens used in this study were stable in the sample buffer, i.e., neutral HPGPC eluent for serum (adjusted to pH 6.8) (Ref. [10]; Section 2.4). It was also found that the PRM method showed low values for B1 and B2 (both from gallduct cancer patients), although protein values for B3, B4 and B5 were similar to the HPGPC and BCA methods (Table 1). Thus, biles from gallduct cancer patients may contain some component(s), which interfere with the photometric PRM method. Although the biuret method is not applicable to bile due to bilirubin, it may be applicable to pancreatic juices.

The spectrophotometric BCA method gave an extremely higher protein value for pancreatic juice sample P5 than that of HPGPC method (Table 1), although the pancreas of patient (P5) was normal. In order to clarify this discrepancy, protein component analysis was first performed with a 30-nm pore diameter column. Considering the injected volume of pancreatic juice P5 and the observed peak area, the validity of the protein value obtained from the HPGPC method was suggested (data not shown). Then, SDS–PAGE was performed using the logic of the principle of contradiction. Two gels (gel A and B) were run. In each well of gel A and gel B was loaded the same amount (0.005 mg) of protein, which was calculated from the HPGPC method for gel A and from the BCA method for gel B. The result is shown in Fig. 5. As shown in Fig. 5, the protein amount of sample P5 as detected by Coomassie brilliant blue R-250 seemed to be consistent with the protein value obtained by the HPGPC method. Furthermore, it was also found that this sample P5 contained essentially a single major protein of M_r 66 000 (Fig. 5, lane 5). Therefore, proteins of the pancreatic juice sample P5 were covalently bonded

Table 1
Comparison of protein values (mg/ml) of pancreatic juice and bile samples among varying column temperatures and column lengths of HPGPC and spectrophotometric protein assays[a]

Sample	HPGPC						Photometry		
	Length (35 mm)			Length (50 mm)			BCA	Biuret	PRM
	22°C	15°C	8°C	18°C	15°C	8°C			
Pancreatic juice									
P1	12.8	15.2	–	12.3	16.5	–	18.4	–	–
P2	1.8	1.8	3.1	1.8	2.4	3.4	2.4	3.3	1.4
P3	8.1	10.3	9.8	8.2	9.2	8.8	11.3	9.2	5.6
P4	9.2	–	–	9.2	–	–	16.4	–	–
P5	0.17	–	–	0.18	–	–	2.0	–	–
P6	3.3	3.7	–	3.5	3.5	–	2.6	–	–
P7	0.7	1.9	1.4	1.5	1.4	1.5	1.1	1.3	0.7
Bile									
B1		22.8	24.6				14.0	ND	3.3
B2		10.8	13.0				13.0	ND	2.2
B3		0.4	0.3				0.2	ND	0.2
B4		1.2	0.8				0.9	ND	0.4
B5		10.0	10.8				10.5	ND	8.4

[a] Application of the BCA method to samples P1 and P6 were difficult, i.e., 10-fold diluted P1 of 0.005, 0.01 and 0.02 ml gave the protein values of 24.2, 18.4 and 17.0 mg/ml, and 10-fold diluted P6 of 0.01, 0.02, and 0.05 ml gave 1.85, 2433, 3.44 mg/ml, respectively. –, Not measured due to sample consumption. ND, Not determinative. PRM, Pyrogallol–red molybdate method of Wako.

to the glass fiber disks by the EDC method, and micro-sequenced as described in Experimental. The result indicated quantitatively that the major protein (M_r 66 000) of sample P5 possessed the *N*-terminal sequence of Asp (D)–Ala (A)–His (H)–Lys (K)–Ser (S)–. This is the same sequence as that of the HSA protein. Thus, it was concluded that the major protein of M_r 66 000 appearing in the pancreatic juice sample P5 is human albumin. Other pancreatic juice samples P7 and P1 also contained albumin as analyzed by micro-sequencing, although sample P1 contained mainly trypsin, chymotrypsin, and α-amylase. These observations are consistent with the immunological results of De Reggi et al. [3] and Appelt et al. [20]. Since the P5 sample mainly contained albumin, a higher protein value obtained by the BCA method seemed to be due to some interfering non-protein substance(s) in the pancreatic juice sample P5. Thus, the HPGPC method is shown to be the most reliable method for the pancreatic juice and bile protein determinations, since the prior separation of small molecules minimizes the occurrences of interferences.

3.3. Application to rodent tissue homogenates

Although the results from human serum and human body fluids are important for the clinical laboratory, it seems fruitful to understand the changes occurring in the body fluids from the knowledge of the original tissue(s). Thus, we applied this HPGPC method to tissue homogenates of rodents. Typical results are shown in Fig. 3 (lower panel), and summarized in Table 2. Relatively good correlations detected among the HPGPC, BCA and PRM methods (Table 2). The biuret method showed lower correlation to the HPGPC method ($rS=0.841$, $n=19$), and all the protein values were the highest among the four methods (Table 2). Therefore, the biuret method seems to be applicable neither to tissue homogenates nor to bile. Photometric BCA and PRM methods gave usually larger protein values than the HPGPC method. However, similar protein values were obtainable in the case of kidney and perfused kidney among HPGPC, BCA, and PRM methods. Therefore, some special component of liver, such as bilirubin and/or cholic acid, may

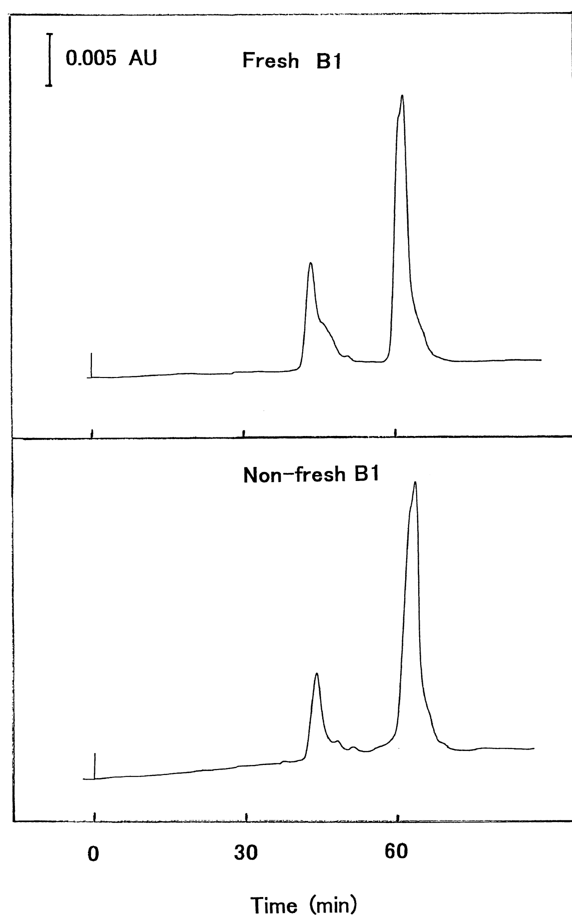

Fig. 4. Analysis of labile bile B1 proteins by the 30-nm average pore diameter HPGPC column. A 0.025-ml volume of 500-fold dilute bile sample B1 was injected into the HPGPC system. The column used was 300×8 mm I.D., and the column temperature was at 15°C. Protein was detected at 210 nm at 0.05 AUFS. Upper panel: freshly diluted B1. Lower panel: after standing the freshly diluted B1 at room temperature for 15 h. Other conditions as described in Experimental.

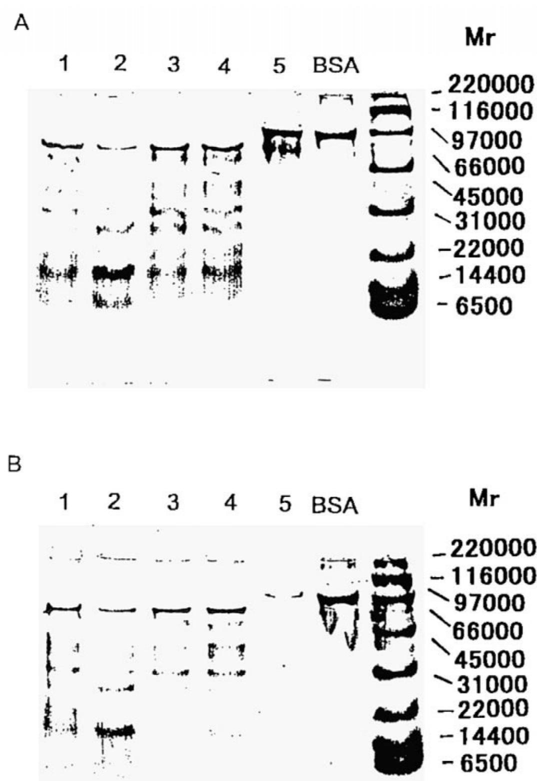

Fig. 5. Comparison of pancreatic juice protein values obtained from the HPGPC method (measured at 22°C) and the photometric BCA method by using SDS–PAGE. (A) Lanes 1–5, a 0.005-mg amount (calculated from the HPGPC method) of pancreatic juice protein of samples P1–P5, respectively, was loaded and electrophoresed. (B) Lanes 1–5, a 0.005-mg amount (calculated from the BCA method) of pancreatic juice protein samples P1–P5, respectively, was loaded and electrophoresed. Lane BSA (0.002 mg) and the lane of M_r standard protein (each 0.002 mg) were also electrophoresed. SDS–PAGE was performed according to the method of Weber and Osborn [14]. A polyacrylamide concentration of 10% (w/v) was used. Proteins were detected with Coomassie brilliant blue R-250.

interfere with photometric BCA and PRM methods in the case of liver and perfused liver. Thus, reliable protein values for both of liver and perfused liver may only be obtainable with the HPGPC method. Similar situations may be present in such another tissues as heart, stomach, and brain.

Comparison of the two protein assay columns Nos. 1 and 2 was performed, and the result is shown in Table 3. Satisfying correlations were obtained between No. 1 and No. 2 HPGPC columns. It is noteworthy that the newly obtained columns should be washed by the washing solution, i.e., 0.1 *M* sodium phosphate buffer (pH 2.1)–methanol (50:50, v/v), with 50-fold volume of the column, at a column temperature of 72°C. This washing procedure was also found to be effective to recover the separation abilities of the ODS column between PTH-Asp (D) and PTH-Gln (Q) and between PTH-

Table 2
The result of protein determinations of tissue homogenates[a]

Tissue	Protein concentration (mg/ml)			
	HPGPC at 8°C (35-mm column)	Photometric		
		BCA	PRM	Biuret
Rat kidney 1 (T1)	8.0	10.4	13.0	14.4
Mouse kidney (T2)	4.7	5.8	6.8	8.8
Mouse liver (T3)	6.6	9.5	9.2	12.0
Mouse cerebellum (T4)	2.4	3.6	3.4	7.5
Mouse cerebrum (T5)	3.6	3.6	4.8	5.9
Mouse abdominal skin (T6)	3.6	3.0	3.5	4.4
Rat cerebrum	8.1	13.0	14.5	29.2
Rat cerebellum	6.0	8.5	8.5	–
Rat esophagus	3.2	7.3	4.5	12.7
Rat stomach	5.4	8.4	8.8	12.5
Rat small intestine 1	7.1	9.8	6.6	11.0
Rat small intestine 2	6.8	8.8	5.5	–
Rat large intestine	5.3	5.6	4.6	10.0
Rat thigh muscle	4.1	5.1	6.2	8.0
Rat diaphragm	2.8	2.9	3.3	5.5
Rat spleen	27.6	32.0	27.5	–
Rat heart	7.9	13.3	12.9	18.8
Rat lung	3.5	4.4	4.5	11.6
Rat bone marrow	6.3	8.0	7.5	–
Rat liver	18.8	29.2	33.3	68.0
Rat perfused liver	12.0	22.2	18.7	37.2
Rat kidney 2	17.4	20.8	21.6	33.1
Rat perfused kidney	18.8	17.8	21.4	35.6

[a] The No. 1 column was used. Other conditions as described in Experimental. Correlation between HPGPC and BCA was rS=0.956, r=0.946 (y=1.20044x+1.08331); between HPGPC and PRM was rS=0.938, r=0.926 (y=1.19608x+1.01496); between BCA and PRM was rS=0.934, r=0.965 (y=0.98265x+0.08649). The significances of the correlations were less than 1%. –, Not measured.

Lys (K) and PTH-L-Ile (I) of Ref. [17]. The reason of this improved minimization of column differences is not fully understood at this time, however such a high ionic-strength eluent of acidic phosphate buffer with methanol may be working as a metal washing-out reagent of the silica gel columns, and may afford the stable analytical systems [17,19,21].

Thus, the HPGPC method as described in this report is a unique but a generally applicable method with confidence. Furthermore, this precise protein determination method is also expected to be helpful to the enzyme kinetic studies, i.e., to determine such an affinity parameter as k_{cat}/K_m (k_{cat}: molecular activity=V_{max}/mole of enzyme; K_m: Michaelis-Menten constant).

4. Conclusions

1. A general high-performance gel-permeation chromatography (HPGPC) method was developed to determine protein in human pancreatic juice, human bile and tissue homogenates of rodents.
2. A diol-type silica gel column (35×8 mm I.D., 5 nm average pore diameter) was used under the optimum column temperature at 8°C. The eluent was acidic phosphate buffer with a high concentration of sodium chloride, nonionic detergent of polyoxyethylene (20) cetyl ether (Brij-58), glycerol, and 2-propanol.
3. Analytical time was within 3.5 min.

Table 3
Comparison of two protein-assay 3.5-cm columns[a]

Sample	Column No. 1	Column No. 2
Pancreatic juice		
P2	3.1	2.8
P3	9.8	9.0
P7	1.4	1.2
Bile		
B1	24.6	27.6
B2	13.0	15.1
B3	0.3	0.4
B4	0.8	1.0
B5	10.8	11.7
Tissue homogenate		
Rat kidney 1 (T1)	8.0	8.2
Mouse kidney (T2)	4.7	4.6
Mouse liver (T3)	6.6	5.7
Mouse cerebellum (T4)	2.4	2.6
Mouse cerebrum (T5)	3.6	3.5
Mouse abdominal skin (T6)	3.6	2.9
Rat cerebrum	8.1	6.8
Rat cerebellum	6.0	5.0
Rat esophagus	3.2	3.8
Rat stomach	5.4	5.4
Rat small intestine 1	7.1	5.3
Rat small intestine 2	6.8	5.0
Rat large intestine	5.3	4.6
Rat thigh muscle	4.1	4.4
Rat diaphragm	2.8	2.9
Rat spleen	27.6	26.0
Rat heart	7.9	6.9
Rat lung	3.5	4.2
Rat bone marrow	6.3	6.8
Rat liver	18.8	16.8
Rat perfused liver	12.0	11.0
Rat kidney 2	17.4	18.8
Rat perfused kidney	18.8	19.1

[a] Assayed at 8°C. Protein concentration; mg/ml. Correlations between the two columns were rS=0.983 ($P<0.001$), $r=0.988$ ($P<0.001$) ($y=1.01834x-0.2985$; $n=31$).

4. The HPGPC method is reliably applicable to human pancreatic juice, human bile, and rodent tissue homogenates. Other photometric methods show limited applicability, i.e., the BCA method may be applicable to human bile and rodent tissue, the PRM method may only be applicable to rodent tissue, and the biuret method may only be applicable to human pancreatic juice.
5. The presence of albumin in human pancreatic juice is chemically demonstrated.
6. A method of protein component analysis using a 30-nm average pore diameter 30-nm long HPGPC column is also described, which is useful to assess the protein stability of body fluids and tissue homogenates.
7. This HPGPC protein assay method is simple, convenient, rapid, reproducible, and reliable, it is expected to be generally applicable to clinical and biochemical research.

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