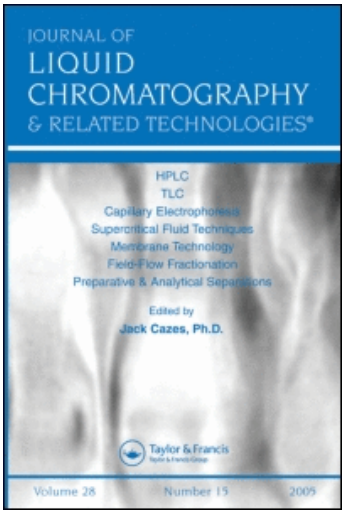

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Urinary Protein and Albumin Determinations by High-Performance Gel-Permeation Chromatography

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URINARY PROTEIN AND ALBUMIN DETERMINATIONS BY HIGH-PERFORMANCE GEL-PERMEATION CHROMATOGRAPHY#

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ABSTRACT

Sensitive high-performance gel-permeation chromatographic protein assay method was developed by using non-ionic detergent of Brij-58 which was UV transparent as compared to Nonidet P-40. A column (70 x 8.0 mm I.D.) packed with Develosil 100 Diol-5 (pore size 10 nm) was used for the protein determination. A commercially available column (300 x 8.0 mm I.D.) packed with Develosil 300 Diol-5 (pore size 30 nm) was used for the urinary albumin determination. Eluent used was a 0.1 M sodium phosphate buffer (pH 5.6) containing 0.3 M sodium chloride, 1% (v/v) Brij-

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58, 50% (v/v) glycerol. Flow-rates for protein assay and albumin assay were 1.0 and 0.5 ml/min, repectively. Proteins were eluted at the position of the exclusion limit in the case of protein assay (100 Diol-5). Albumin was eluted from 300 Diol-5 column as a symmetric peak. Bovine serum albumin (BSA) was used as an external standard for both protein and albumin assays. Analysis times were within four min for protein assay, and 32 min for albumin assay, respectively. Improved sensitive measurement of BSA at 5-20 ng level was achieved as compared to Nonidet P-40 by use of UV 210 nm. This method was successfully applied for various urine samples, such as healthy random urine of before and after sports, and the 24-h urines from insulin-dependent diabetes mellitus (IDDM) patients. Comparisons with clinical urinary protein and albumin tests were performed using IDDM urine. Three cases out of four tests for sports showed increased protein and albumin content after physical exercise. Thus, this HPLC method was proven to be applicable to the protein and albumin measurements in human urine.

INTRODUCTION

Previously, we developed a high-performance gel-permeation chromatographic (HPGPC) protein separation method using a non-ionic detergent of Nonidet P-40 in the eluent, and this method was successfully applied to the purification of human serum biotinidase in a high yield (1). However, strong UV absorbance at 280 nm by Nonidet P-40 decreased the sensitivity for the protein detection.

In this study, we further studied to improve the sensitivity of the HPGPC protein separation method by using more UV-transparent non-ionic detergent, Brij-58. It was found that Brij-35 (at 1% v/v) was not applicable to urine analysis, because column-inlet pressure increased during analysis. Further, the base-line separation between protein peak and smaller moleculer weight compounds was not obtainable when urine analyses by using Brij-35 were continued, which suggested the low recovery of proteins or lipids from the column. On the other hand, when Brij-58 (at 1% v/v) was used instead of Brij-35 as a component of the eluent, satisfactorily repeatable and applicable analysis of

urine was achieved. For protein analysis, a special column was devised; i.e., diol-type silica gel with pore size 10 nm was packed into a 70 x 8 mm I.D. stainless steel tube in order to elute the proteins at a retention time of exclusion limit. We firstly report in this paper about the protein and albumin content measurements in human urine.

MATERIALS AND METHODS

Chemicals and reagents: Bovine serum albumin (BSA) and gamma-globulins (bovine Cohn fraction II) were purchased from Sigma Chemical Co., St. Louis, MO, USA. Brij-58, Brij-35, and detergent starter kit (containing MEGA-8, MEGA-9, n-octyl-beta-D-thioglucoside) were from Wako Pure Chemicals Co., Osaka, Japan. Nonidet P-40, Triton X-100, and glycerol were from Nacalai Tesque Co., Kyoto, Japan. Sodium dodecyl sulfate (SDS) was from Bio-Lad, Richmond, CA, USA. A BCA (bicinchoninic acid) protein assay kit was from Pierce Chemical Co., Rockford, IL, USA. Develosil specially-packed column of Develosil 100 Diol-5 (70 x 8 mm I.D.: 10 nm mean pore-size, 5 μ meter mean particle diameter) and Develosil column of Develosil 300 Diol-5 (300 x 8 mm I.D.: 30 nm mean pore-size, 5 μ meter mean particle diameter) were from Nomura Chemical Co., Seto-City, Aichi, Japan.
Specimens: Human urine (24-h urines) from patients of insulin-dependent diabetes mellitus (IDDM) was kindly donated from National Children's Hospital (Tokyo, Japan). Other 24-h and radomly sampled healthy urines were from volunteers in this institute.

Human urine samples were filtered through Ekicrodisc 13 (0.2 μm VERSAPOR; Gelman Sciences Japan, Ltd., Tokyo, Japan), and stored at -80 °C.

High-performance liquid chromatography: A model 655A-11 pump (Hitachi, Tokyo, Japan) was used. Injector was a model U-6K (diaphragm type; 2 ml sample loading loop, Waters, Milford, USA). Detection was carried out by a model lambda-Max spectrophotometer (model 481, Waters; detected at 210 nm or 280 nm). Recorder was Hitachi 561 (Hitachi), and Chromatopac C-R6A data processer (Shimadzu) was also used. A line filter (GL Sciences, Tokyo, Japan) was inserted between the injector and the column. Eluent (2 l) was made as follows; 1 l of 0.2 M sodium phosphate buffer (pH 5.6) containing 0.6 M sodium chloride was first made. To this buffer, 1 l of glycerol and 20 ml of Brij-58 was then added and mixed thoroughly (usually 30 min). Thus, final concentrations were as follows: sodium phosphate (0.1 M), sodium chloride (0.3 M), glycerol (50%, v/v), and Brij-58 (1%, v/v), respectively. Flow-rates were 1.0 ml/min for protein assay (7-cm long column) and 0.5 ml/min for albumin assay (30-cm long column), and column inlet pressures were 100 kg/cm^2 and 120 kg/cm^2, respectively, at the column temperature at 35 °C. Detection for urine protein was performed at 210 nm.

HPLC urine protein assay: Filtered urine (0.04 ml) was dissolved into 0.36 ml of this eluent (10-fold diluted). This 10-fold diluted urine was further 5-fold diluted with the above eluent; i.e., finally 50-fold diluted urine was made. Standard BSA (2 mg/ml; BSA standard solution of Pierce BCA protein assay kit) was 1000-fold diluted by this eluent, and 0.02 ml (40ng of BSA) was injected into the HPGPC system with the 7-cm long column (external standard; Fig. 1, left panel). Usually, 0.01 ml of 50-fold diluted urine sample was injected into the HPLC system and analyzed.

HPLC urine albumin assay: The 10-fold diluted urine was further 10-fold diluted with the HPGPC eluent; i.e., 100-fold diluted

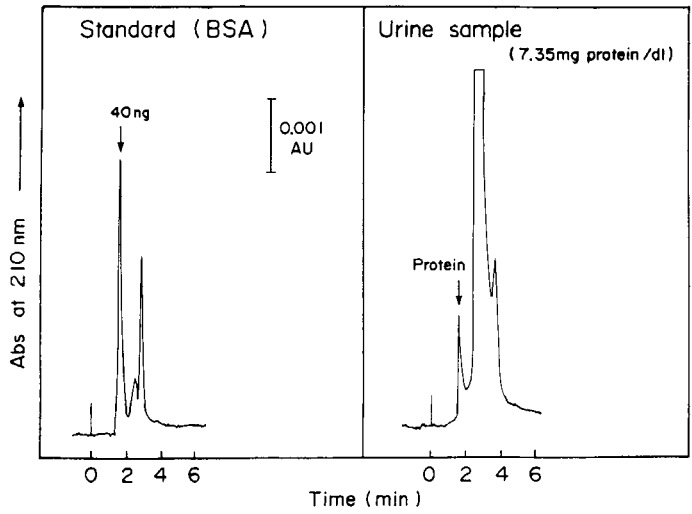

Fig. 1. Typical protein analysis of urine by HPGPC method with 10 nm pore-size and 70 x 8 mm I.D. column. Left panel; 0.02 ml of BSA solution (0.002 mg/ml) was injected into the HPLC system. Right panel; 0.01 ml of 50-fold diluted urine (IDDM patient's urine) was injected into the HPLC system. Other conditions are as described in Materials and Methods.

urine was made for albumin analysis. BSA was 100-fold diluted as above, and 0.01 ml (200 ng; Fig.3, left panel) was injected into the HPGPC system with the 30-cm long column as an external standard. Usually, 0.1 ml of 100-fold diluted urine sample was injected into the HPLC system and analyzed.

Another urine protein and albumin assays: In order to compare the values obtained by HPLC method, urine protein and albumin contents in IDDM urine samples were measured by photometric and immuno assay method, respectively, at the clinical laboratory of National Children's Hospital, Tokyo, Japan. Urine protein was

measured by a photometric kit (Micro TP-Test Wako, Pyrogallol-red Mo (VI) method, Wako Pure Chemical Co., Osaka, Japan). Urine albumin was measured by a turbidic immuno assay kit (ALB-TIA "Seiken", Nippon Roche Co., Tokyo, Japan).

Urine biotinidase assay: Biotinidase activity in human urine was assayed by using biotinyl-6-aminoquinoline as substrate as previously reported (3), and biocytin hydrolyzing activity was also determined by measuring the liberated L-lysine from biocytin by HPLC-amino acid analyzer (OPA method; Hitachi L-6200, Hitachi Co., Tokyo) (4).

RESULTS AND DISCUSSION

In a previous paper, we developed a high-recovery protein separation system by using diol-type silica gel column; i.e., high-performance gel-permeation chromatographic system (HPGPC system) with the eluent containing non-ionic detergent of Nonidet P-40 (1). Further, peak symmetry of carbonic anhydrase in the HPGPC system was considerably improved by increasing the glycerol concentration in the eluent from 2.5 % (v/v) to 30 % (v/v) (2).

Since Nonidet P-40 showed high UV absorbance and sensitivity for protein was not sufficient for urine analysis, we further tested another non-ionic detergent which showed low UV absorbance. We found only Brij-58 was satisfying for the urine protein analysis; i.e., 1) SDS solution of 2% in the phosphate buffer eluent of reference 2 did not dissolve gamma-globulin at all, 2) MEGA-8, MEGA-9, and Tween 20 gave no straight base-lines, 3) Tween 40 and 60 showed low solubility to phosphate buffer, 4) n-octyl-thio-D-glucoside showed high UV absorbance at 210 nm. Brij-58 was satisfactory and repeatable to urine analysis when glycerol

concentration of 50% (v/v) was used. On the other hand, Brij-35 showed an increase in column-inlet pressure even at 50% (v/v) glycerol eluent. Since high sensitivity was necessary for urine protein and albumin analysis, more stable base line was preferable; i.e., we found that a better and straight base line was obtainable at the pH of the eluent of 5.6 instead of 6.0, which was used in previous reports (1, 2).

In order to elute the proteins on the position of the exclusion limit, we chose 10 nm pore-size diol-type silica gel column (70 x 8.0 mm I.D.). As shown in Fig. 1 (left panel), BSA eluted as a triangle-shaped peak. This suggested that the elution of BSA occurred at the exclusion limit (5). Peak height of standard BSA was linearly correlated with injected amount (from 10 to 40 ng) of BSA (Fig. 2) intersecting the origin (0, 0).

Repeatability was tested by injecting 20 ng of BSA nine-times; retention time was 1.73 ± 0.0075 min (coefficients of variation; CV = 0.43 %), peak height (at 210 nm, 0.01 AUFS) was 39 ± 3.2 mm (CV = 0.82 %). Linear correlation curve was obtainable between 0 to 4 ug of BSA (data not shown). Thus, the wide range of linearlity from the origin for BSA was a unique feature of this chromatographic method as compared to the other photometric methods such as Lowry's method (6). This linear character of calibration curve is expected to be usefull for the application onto automated and convenient calculation with a data processor.

A typical example of urine protein analysis was shown in Fig. 1 (right panel). Urinary protein content was measurable within four min.

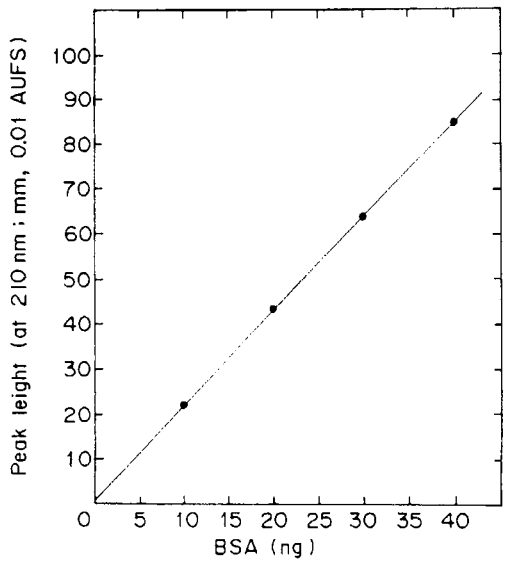

Fig. 2. Correlations between weight amount of BSA injected into HPLC system (70 x 8 mm I.D. column with 10 nm pore-size) and observed peak height (in mm) at 210 nm. 0.005, 0.01, 0.015, and 0.02 ml of diluted BSA (0.002 mg/ml) was injected into the HPLC system.

With 30 cm long commercial column packed with 30 nm pore-size diol-type silica gel, urinary albumin was measurable within 32 min as shown in Fig. 3. BSA was eluted at a retention time of 17 min.

In order to assess whether major urine proteins elute at the position of the exclusion limit of the protein assay column (10 nm pore-size, 70 mm long) or not, we collected the triangle-shaped peak of the exclusion limit (see Fig. 1, right panel) using one of the IDDM urine (protein concentration; 21.8 mg/dl). Then, a portion of the collected fraction was re-

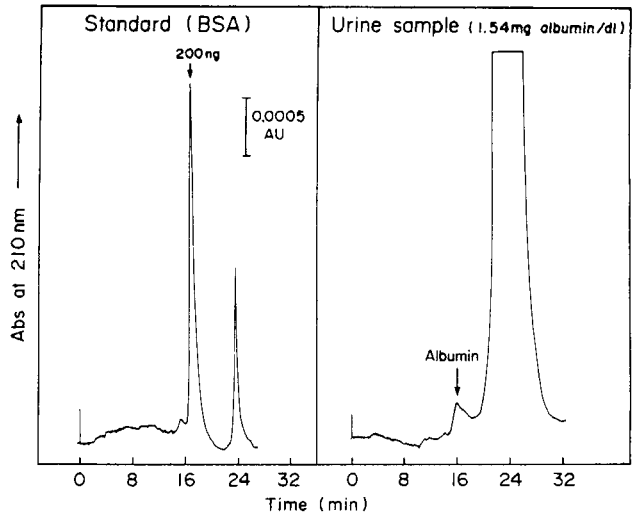

Fig. 3. Typical albumin analysis of urine by HPGPC method. Left panel; 0.01 ml of BSA solution (0.02 mg/ml) was injected into the HPLC system with 30 nm pore-size and 300 x 8 mm I.D. column. Right panel; 0.1 ml of 100-fold diluted urine (IDDM patient's urine) was injected into the HPGPC system. Other condition are as described in Materials and Methods.

analyzed by injecting into the above described albumin analyzing system with 30-cm column of 30 nm pore-size (Fig. 4, panel A), and compared to the elution pattern of the dialyzed urine of the patient (Fig. 4, panel B). As shown in Fig. 4, fractionated peak exhibited essentially the same elution pattern to the original dialyzed urine. This result indicates that most of the urine proteins are eluted at the exclusion limit of the 7-cm protein analyzing column as expectedly. Interestingly, small molecular size compounds of the urine, which eluted after the inclusion limit (about 20 min; e.g., Fig 3, right panel), was

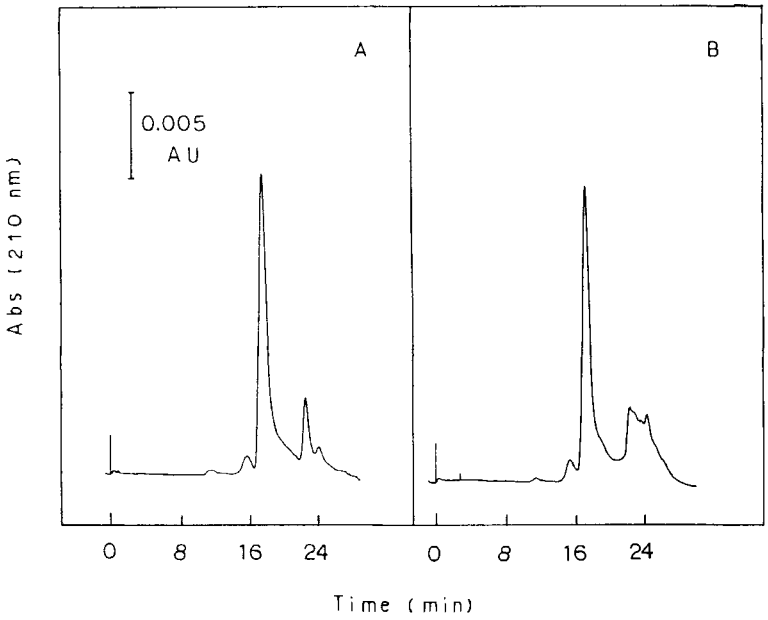

Fig. 4. Re-analysis of the collected peak fraction at the exclusion limit of protein analyzing system. An IDDM urine (protein concentration; 21.8 mg/dl) was 10 fold diluted with the HPGPC eluent. A 0.2 ml portion of the diluted urine was injected into the protein analyzing sytem with 7-cm column, and fractioned the triangle-shaped peak of the exclusion limit (1 ml). Then, a portion (0.18 ml) of the collected fraction was injected into the albumin analyzing system with 30 cm column (panel A). This re-analyzed chromatogram was compared with the chromatogram of original dialyzed urine (panel B). In panel B, 0.02 ml of the dialyzed urine was injected and analyzed as a reference. Dialysis of the urine (0.2 ml of urine) was performed at ambient for 24 h against 1000-fold volume (200 ml) of the HPGPC eluent.

decreased after a simple collection of the triangle-shaped peak at the exclusion limit of the protein analyzing column (7-cm long; 10 nm pore-size). Thus, this protein analyzing system with 7-cm long column is a useful tool to dialyze the urine sample within 4 min.

Then, the HPLC-protein and albumin analysis method was applied onto the 35 samples of IDDM 24-h urine, and compared to the results from clinical photometric protein and immuno turbidic albumin assay methods. Average protein excretions as measured by HPLC and photometric clinical methods were 17.2 ± 20.1 (mean ± SD) mg/day and 46.8 ± 56.5 mg/day, respectively. There was no significant correlation between them. Interestingly, we found a significant correlation ($r = 0.60$; $p < 0.01$) between protein excretion (HPLC value) per day and duration (years) of IDDM (submitted for publication), although clinical protein value showed no such correlation. This suggests that the HPLC protein assay method is preferable to analyze IDDM urines which may contain more complex interfering materials from diabetes. Average albumin excretions as measured HPLC and immuno clinical method were 16.9 ± 22.5 mg/day and 5.3 ± 10.3 mg/day, respectively. There was a correlation between HPLC and clinical immuno albumin measuring methods; i.e., $r = 0.705$ ($p < 0.01$). HPLC albumin excretion values were correlated with the duration of IDDM with $r = 0.63$ ($p < 0.01$), however clinical values at this time were not ($p < 0.05$, submitted for publication). HPLC method showed more reliable results to indicate the correlations which were present between duration of IDDM and excretion of albumin and protein (7) as compared to other clinical methods. This may be the result of the HPLC analysis method, which separates proteins from the possible interfering small molecular

TABLE I

Effect of sports (physical exercise) on the urinary protein and albumin excretions in the urine.

Sports / Excretion	Before	After	
	Cycling for 1h		
Protein	1.60 mg/dl	3.20 mg/dl	
Albumin	0.44 mg/dl	0.79 mg/dl	
Biotinidase *	ND	ND	
	Tennis for 75 min		
Ptrotein	2.00 mg/dl	3.75 mg/dl	
Albumin	0.50 mg/dl	2.30 mg/dl	
Biotinidase *	13.3	ND	
	Swimming for 2h		
		0h	2h
Protein	5.90 mg/dl	7.00 mg/dl	10.8 mg/dl
Albumin	0.36 mg/dl	0.72 mg/dl	1.44 mg/dl
Biotinidase *	N.D.	33.3	116.7
	Tennis for 45 min (7y child)		
Protein	2.06 mg/dl	1.59 mg/dl	
Albumin	0.29 mg/dl	0.15 mg/dl	
Biotinidase *	N.D.	30.9	

* Biotinidase; Specific activity was expressed as pmol biocytin hydrolyzed/min per mg of urine protein (4). N.D.; Not detectable. Protein and albumin content were measured by HPLC.

weight compounds in the IDDM urine. Thus, these HPLC protein and albumin measuring methods were shown to be applicable to disease urines of the IDDM patients.

Effects of sports on urinary excretion of protein and albumin were also tested, since such a test may be helpfull for estimation of physiological state of kidney (8). The results were shown in Table I. As a reference, the excretion of biotinidase activity was also shown (Table I). Although a child (7 y) seemed to show different physiology from adults, three adults exhibited the increased protein and albumin excretions after sports. Biotinidase excretion into urine seemed not to be directly related to physical exercise. Thus, this HPLC protein and albumin assay method was also expected to be applicable to the field of sports medicine.

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