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Liquid chromatography of guanidino compounds using a porous graphite carbon column and application to their analysis in serum

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Abstract

The retention mechanism of guanidino compounds on a porous graphitic carbon seemed to be mainly hydrophobic interaction, according to the retention factors in buffer solutions and the results of an analysis by computational chemical calculation using molecular mechanics (MM2). The baseline separation of ten guanidino compounds was achieved by the addition of a hydrophobic counterion. The retention mechanism may be dynamic ion-exchange. The stable system was applied to the analysis of guanidino compounds in serum from nephritic patients. The effluent was monitored by a post-column labeling detection method using ninhydrin. The detection limit of guanidino compounds was a few picomoles; however, that of creatinine was one hundredth of those of the other compounds. The reproducibilities of the peak height and area of the ten guanidino compounds using gradient elution were quite high, and the standard deviations were within a few percent ($n=5$), except for creatinine. The recovery of the compounds from serum was more than 90% ($n=5$). The reproducibility of retention times was within 1% ($n=5$). © 1998 Elsevier Science B.V.

Keywords: Isocratic elution; Column switching system; Porous graphitized carbon columns; Guanidino compounds

1. Introduction

Guanidino compounds have been suspected to be uremic toxins. The guanidino compound methylguanidine was shown to be related to the uremic polyneuropathy found in uremia [1]. Guanidinosuccinate is related to uremic bleeding diathesis, and was shown to inhibit excitatory neurotransmission in rat hippocampal brain slices, an effect that hypothetically could contribute to uremic encephalopathy [2]. The compounds guanidinosuccinate, methylguanidine, guanidine and creatinine were suggested to be the cause of chronic and generalized seizures after systemic and intracerebroventricular administration in mice [3,4]. The measurement of L-arginine (a physiological precursor of nitric oxide) is used in studies of the disposition of exogenous doses of L-arginine [5]. Methylguanidine and

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guanidinoacetate are measured by enzyme activity assays; however, the sensitivity of the assays is not satisfactory for the measurement of these compounds in blood [6]. In addition, quantitative analyses of other guanidino compounds and the measurement of the enzyme activity of their reaction processes are not yet well established. The effects of clinical dialysis have been studied by measuring the guanidino compounds by liquid chromatography (LC). The serum guanidino compound levels found in uremic children were comparable to those in adults [7]. A single hemodialysis lowered the level of most serum guanidino compounds temporarily, and the level remained more stable in continuous cycle peritoneal dialysis-treated patients [7]. A mechanistic explanation for the important differences in the present decrease in the different guanidino compounds was not yet available. No correlation was reported between the molecular mass or the acidity of the guanidino compounds and their percentage decrease. It was concluded that the guanidino compound distribution in the body tissues and their protein binding, eventually in combination with other factors, contribute to the differences in their dialysability [8]. Several guanidino compounds are included in the extensive list of possible uremic toxins [9], and further liquid chromatographic analyses of the guanidino compounds are thus required.

Guanidino compounds are very polar, and usually exist in ionic form. Their separation is usually carried out by ion-exchange LC [10–13] or reversed-phase ion-pair LC [14,15]. However, such separations have not been satisfactory. Pre-derivatization was applied using benzoin [16,17] and ninhydrin [18]. However, these separations and reproducibility levels have also not been satisfactory due to the instability of packing materials and the complicated separation systems; in addition, the highly sensitive post-column reaction detection is preferable to pre-column derivatization for the quantitative analysis of these compounds.

A porous graphitic carbon of small particle-size was developed by Knox and coworkers [19,20], and later by Obayashi et al. [21] and Ichikawa et al. [22]. Porous graphitic carbon is stable in strong acid and alkali solutions, and eluents at any pH can therefore be used. Porous graphitic carbon is extremely hydrophobic, and the retention time of ordinary compounds is quite long compared to that of the commonly used octadecyl-bonded silica gels. Porous graphitic carbon is thus suitable for the chromatography of very polar compounds, e.g., saccharides [23–25] and ions [26–28]. The retention mechanism is thought to be a mixture of hydrophobic and electrostatic interactions, and the electrostatic interaction seems to be more important than the hydrophobic interaction in the retention mechanism of polar compounds [29]. In the present study, therefore, the separation of guanidino compounds was studied using porous graphitic carbon columns. The retention mechanism was analyzed by computational chemical calculations using the molecular mechanics (MM2) program.

2. Experimental

2.1. Materials

The guanidino compounds guanidino succinate (GSA), guanidine hydrochloride (G), glycosylamine (GAA), β-guanidino propionic acid (GPA), taurocyamine (TAU), methylguanidine (MG), γ-guanidino butyric acid (GBA), arginine (ARG), creatinine (CTN) and creatine (CT) were purchased from Wako (Tokyo, Japan). The ion-pair reagents sodium lauryl sulfate (C12), sodium 2-naphthalene sulfonate (Na), sodium 1-undecanesulfonate (C11), sodium 1-decanesulfonate (C10), sodium 1-nonanesulfonate (C9), sodium 1-octanesulfonate (C8), sodium 1-heptanesulfonate (C7), sodium 1-hexanesulfonate (C6), sodium 1-pentanesulfonate (C5), sodium 1-butanesulfonate (C4) and sodium 1-propanesulfonate (C3) were purchased from Nacalai Tesque (Kyoto, Japan). Other chemicals and high-performance grade solvents were purchased from Wako.

2.2. Chromatography

The analytical system of the guanidino compounds was composed of a liquid chromatograph with a gradient elution and a post-column reaction detector. Two Model CCPM pumps were from Tosoh, Tokyo, Japan. The graphitic carbon columns were a BTR carbon column (porous graphitic carbon column, 50

mm×4.6 mm I.D., 3.5 μm; BioTech Research, Saitama, Japan) and a TSKgel Carbon-500 column (100×4.6 mm I.D., 6 μm; Tosoh).

Post-column derivatization was accomplished as follows. Aqueous ninhydrin solution (6 g/l) and 1 *M* sodium hydroxide were delivered from two reagent pumps (Model 885-PU, Japan Spectroscopic, Tokyo, Japan) at a flow-rate of 0.2 ml/min. The effluent from the carbon column was first mixed with 1 *M* sodium hydroxide solution, then mixed with aqueous ninhydrin solution. The mixture was heated in a reaction coil (perfluorocarbon tube of 10 m×0.5 mm I.D.) placed in a water bath at 56°C, and the reaction products were monitored at excitation (ex) 392 and emission (em) 500 nm with a spectrofluorometric detector (Model FS8010, Tosoh).

2.3. Computational chemical analysis

The computer used for the calculation was a Macintosh 8100/100 running the CAChe program including ProjectLeader from Sony-Tektronix (Tokyo). The molecules were first optimized by molecular mechanics calculation. Properties for the calculation were selected according to the manual from CAChe Scientific (Portland, OR, USA).

3. Results and discussion

3.1. Retention of guanidino compounds on a graphitic carbon

Graphitic carbon is supposed to be very hydrophobic and the polar compounds be retained on it by hydrophobic and electrostatic interactions [29]. Therefore, the retention of guanidino compounds on a graphitic carbon was studied by LC in buffer solutions and computational chemical analysis.

3.2. LC of guanidino compounds

The pH and metal ion (lithium, sodium and potassium) effects in citrate and phosphate buffers were studied using a TSKgel Carbon-500 column in citrate buffer from pH 3.00 to 12.00 and phosphate buffer from pH 3.00 to 12.90. The results are summarized in Table 1, where the results in sodium phosphate and sodium citrate buffers are given as the example.

The retention times of the guanidino compounds in the 50 m*M* sodium and potassium phosphate buffers were similar. GBA and GSA were retained in low pH solutions and CTN, ARG, MG and CT were retained in high pH solutions. In the 50 m*M* lithium, sodium and potassium citrate buffers, these guanidino compounds were retained in the pH 4.50 and 11.00 solutions; their retention in the pH 4.50 lithium citrate buffer were the highest. The smaller the size of metal ion, the longer the retention; however, increasing the concentration of buffer up to 200 m*M* did not affect their retention. Sodium acetate and rubidium acetate buffers were also examined, but their retention times were equivalent to those in the sodium citrate buffer. Strong retention of the compounds was observed in the pH 4.50 citrate buffer, and in the high pH phosphate buffer. The former retention mechanism may be due to electrostatic interaction, and the latter retention mechanism due to hydrophobic interaction. The retention mechanisms were further studied using computational chemical calculation. These guanidino compounds were scarcely retained on octadecyl- and octyl-bonded silica gels in the 50 m*M* sodium citrate buffer at pH 3.00 to 10.00. The separation of these compounds was possible with the pH 4.50 citrate buffer on the graphitic carbon column (where these compounds are completely ionized), but the total separation was difficult.

3.3. Computational chemical analysis of the retention mechanisms on graphitic carbon

The molecular interactions of guanidinino compounds on graphitic carbon were analyzed by a computational chemical calculation using molecular mechanics calculations (MM2) [30]. The model graphite-like layer was constructed with 272 carbon atoms, 444 bonds, 888 connectors and 173 organic rings. The even number of carbon atoms in two layers were bonded tightly to diminish the flexibility of the model adsorbent. The basic structure was identical to that of a previous structure used for a study of the retention selectivities on methylated and hydroxylated phases in gas chromatography (GC) [31]. One guanidino compound was put at the center

Table 1
Effects of buffer components and pH on the retention factors of guanidino compounds on a porous graphitic carbon column

Eluent	pH	GSA	G	GAA	GPA	TAU	MG	GBA	ARG	CTN	CT
1	3.00	0.85	0.77	1.52	2.24	0.55	0.80	6.65	2.89	3.50	0.41
	3.50	1.06	0.63	1.95	4.10	0.79	1.43	14.12	5.01	7.42	1.00
	4.00	0.95	0.82	1.80	4.54	1.04	1.91	18.46	5.90	10.50	1.08
	4.50	0.69	0.87	1.48	3.66	1.29	2.16	16.89	6.29	13.51	1.22
	5.00	0.49	0.85	1.38	2.35	1.59	2.15	9.01	5.78	11.82	1.45
	5.50	0.35	0.63	1.19	1.44	1.59	1.50	4.60	4.18	7.71	1.27
	6.50	0.38	0.41	1.14	1.18	1.53	1.00	2.78	2.79	6.30	1.24
	7.50	0.39	0.38	1.10	1.12	1.49	0.89	2.58	2.68	6.14	1.22
	9.00	0.34	0.61	1.04	1.09	1.40	1.54	2.50	3.69	5.67	1.31
	11.00	0.38	0.43	1.15	1.23	1.59	1.21	2.87	4.25	7.27	1.56
	12.00	0.33	0.45	1.10	1.22	1.48	1.34	2.91	4.70	6.97	1.74
2	3.00	1.07	0.18	0.88	1.12	1.10	0.32	3.82	0.54	1.54	0.91
	3.50	1.05	0.19	0.92	1.11	1.12	0.34	3.76	0.57	1.81	0.98
	4.00	0.96	0.21	0.93	1.07	1.13	0.36	3.45	0.60	2.50	1.01
	4.50	0.88	0.23	0.96	1.05	1.16	0.40	2.99	0.67	3.56	1.04
	5.00	0.74	0.22	0.91	0.96	1.11	0.39	2.46	0.67	4.63	0.99
	5.50	0.70	0.25	0.94	0.96	1.17	0.45	2.23	0.80	5.59	1.02
	6.50	0.56	0.27	0.91	0.91	1.15	0.51	2.04	1.03	6.03	1.02
	7.50	0.48	0.30	0.92	0.93	1.18	0.60	2.04	1.30	6.15	1.05
	9.00	0.37	0.27	0.95	0.97	1.23	0.66	2.30	2.83	7.29	1.18
	11.00	0.41	0.30	0.98	1.05	1.29	0.65	2.38	3.56	7.38	1.38
	12.00	0.33	0.33	0.97	1.18	1.18	1.55	2.38	3.77	7.46	1.55
	12.90	0.31	0.57	0.86	1.03	1.02	1.67	2.42	3.88	7.71	1.65

Column size: 100×4.6 mm I.D., 6 μm, concentration of buffer 50 m*M*, flow-rate 0.8 ml/min at 40°C. Eluent 1: potassium cutrate buffer, eluent 2 sodium phosphate buffer. See Section 2.2 for other chromatographic conditions. t_0=2.74 min.

of this adsorbent, and then the adsorption position was optimized by MM2 calculation.

The minimized final, hydrogen bonding, electrostatic and Van der Waals energies of this adsorbent are given in Table 2 with the properties of the guanidino compounds. The adsorption energies were obtained by subtracting the energies of a complex from the sum of energies of the adsorbent and a guanidinino compound, and the values are listed as ΔFE, ΔHB, ΔES and ΔVW, respectively, in Table 2. The adsorption of guanidino compounds on the model carbon phase decreased the energy values, especially the Van der Waals energy values to about 10 kcal/mol. However, the values of hydrogen bonding and electrostatic energy of the guanidino compounds were not affected by the adsorption. The decrease of energy values where cationic guanidino compounds were adsorbed were larger than those where anionic guanidino compounds were adsorbed. That is, guanidino compounds should be adsorbed strongly at low pH rather than at high pH. These results supported the chromatographic behavior of guanidino compounds in citrate buffer. The decrease of energy values for the adsorption of the guanidino compounds was similar to that of benzene and *n*-hexane adsorbed on the same carbon phase. The final energy decreased to 9.38 and 11.70 kcal/mol for benzene and *n*-hexane, respectively. The hydrogen bonding and electrostatic energy values were zero, and the Van der Waals energy contributed to their adsorption.

The computational chemical calculation indicated that the molecular interaction of guanidino compounds on graphitic carbon was due to hydrophobicity, even when the guanidino compound was very polar, and that the hydrogen bonding and electrostatic interaction are negligible. These results are different from those obtained regarding the retention of saccharides on amino and guanidino phases, where the influence of hydrogen bonding and electrostatic interaction are predominant [21,32]. Furthermore, when the solvent effect can be include

Table 2
Molecular interactions and properties of solutes

Solute	MW	FE	HB	ES	VW	ΔFE	ΔHB	ΔES	ΔVW
Carbon (adsorbent)		1761.52	0	0	260.11	–	–	–	–
Molecular form guanidino compounds									
ARG	174.2	−17.67	−4.06	−20.84	4.59	15.10	0.02	0.00	14.85
G	95.53	−22.98	0.00	−20.89	0.47	7.18	0.00	0.00	7.08
GAA	117.11	−17.07	−3.62	−16.96	2.99	11.16	0.02	−0.01	11.40
GPA	131.13	−26.57	−3.46	−27.15	3.32	12.63	0.01	0.07	13.06
GSA	176.1	−20.95	−7.35	−20.67	4.11	12.98	−0.05	−0.01	13.27
MG	109.6	−21.52	0.00	−23.23	2.24	8.92	0.00	0.00	8.82
TAU	167.18	−9.48	−0.43	−17.45	2.36	12.26	0.00	0.06	12.08
Cationic guanidino compounds									
ARG	–	−24.14	−4.07	−28.66	4.38	15.46	0.04	0.01	15.28
G	–	−30.17	0.00	−28.29	0.41	7.82	0.00	0.00	7.67
GAA	–	−23.18	−3.65	−24.75	3.04	11.90	0.01	−0.01	11.80
GPA	–	−32.58	−3.47	−34.98	3.53	13.79	0.01	0.02	13.72
GSA	–	−27.63	−7.70	−28.44	4.22	13.93	0.07	0.02	13.93
MG	–	−27.63	0.00	−31.03	2.28	9.56	0.00	0.01	9.52
TAU	–	−15.94	−0.45	−25.26	2.23	12.97	0.00	0.03	12.65
Anionic form guanidino compounds									
ARG	–	−11.37	−0.01	−18.57	4.31	14.68	−0.00	0.03	14.40
G	–	–	–	–	–	–	–	–	–
GAA	–	−12.73	0.00	−15.94	2.86	10.36	0.00	0.06	10.25
GPA	–	−21.59	0.00	−25.42	3.29	10.86	0.00	−0.00	10.48
GSA	–	−9.23	0.00	−16.06	3.93	12.25	0.00	−0.06	12.30
MG	–	–	–	–	–	–	–	–	–
TAU	–	−11.17	−0.01	−19.62	2.44	12.28	0.00	0.05	12.14

MW=Molecular mass; FE, HB, ES and VW are final, hydrogen bonding, electrostatic and Van der Waals energies, ΔFE, ΔHB, ΔES and ΔVW are stability energies (kcal/mol) calculated by MM2, respectively.

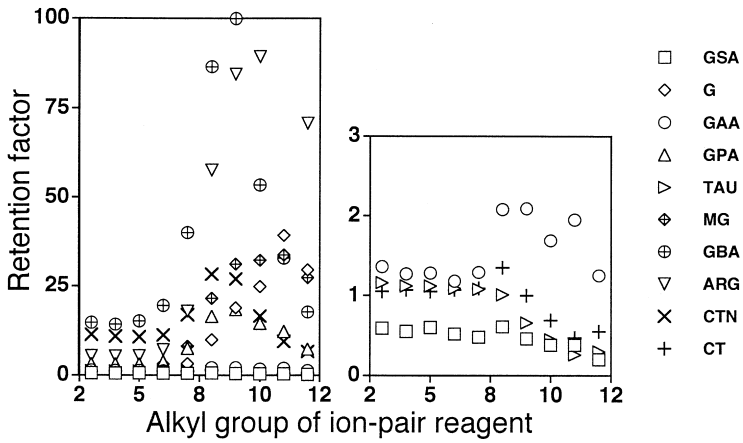

Fig. 1. Selectivity of ion-pair reagents in 50 m*M* sodium citrate buffer of pH 4.50. Column: 100×4.6 mm I.D. packed 6 μm graphitic carbon. Eluent: 50 m*M* sodium citrate buffer (pH 4.50) containing 5 m*M* ion-pair reagent, flow-rate: 0.8 ml/min at 40°C. Flow-rate of 0.6% ninhydrin and 1 *M* NaOH solutions: 0.2 ml/min.

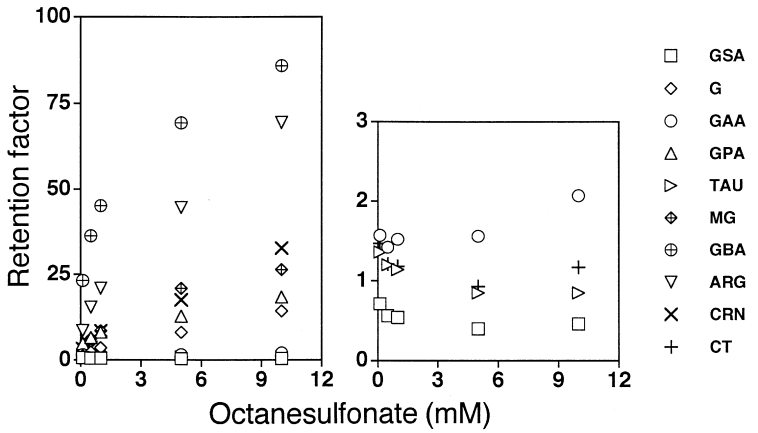

Fig. 2. Concentration effects of ion-pair reagents. Column: 100×4.6 mm I.D., packed 6 μm graphitic carbon. Eluent: 50 m*M* sodium citrate buffer (pH 4.50) containing an ion-pair reagent; flow-rate: 0.8 ml/min at 40°C, flow-rate of 0.6% ninhydrin and 1 *M* NaOH solutions: 0.2 ml/min.

in the calculation, the energy values will indicate the retention mechanism with high precision.

3.4. Ion-pair LC of guanidino compounds

Ion-pairing effects were studied mainly in the pH 4.50 citrate buffer for the total separation of guanidino compounds. Several ion-pair reagents (5 m*M*) were added to the 50 and 100 m*M* sodium citrate buffer at pH 4.50. The results from the use of 50 m*M* buffer solution are summarized in Fig. 1. In the 50 m*M* sodium citrate buffer at pH 4.50, ion-pair reagents smaller than hexylsulfonate were not effective for the retention, that is, the retention mechanism may be electrostatic between the guanidino compounds and the porous graphite carbon and not ion-pair. The decrease of retention times of paired-ions with the larger ion-pair reagents, decanesulfonate, undecanesulfonate and lauryl sulfate may be due to their critical micelle concentration (CMC). The CMC of sodium-1-decanesulfonate (33 m*M*) decreased to 20 m*M* in 50 m*M* sodium buffer (pH

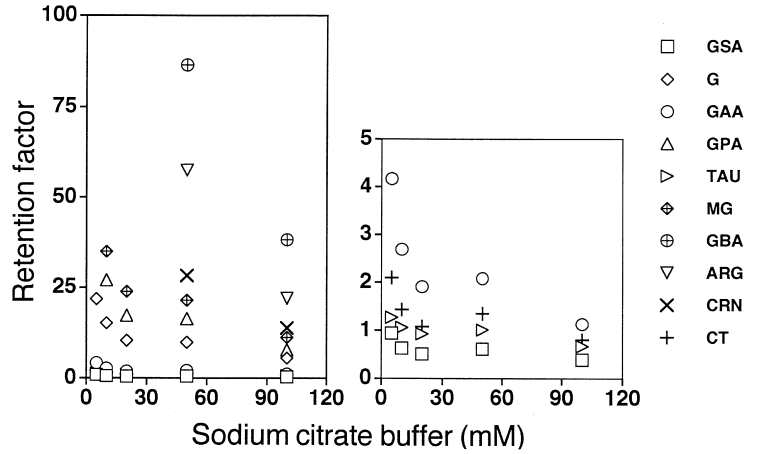

Fig. 3. Buffer concentration effects in ion-pair LC of guanidino compounds. Column: 100×4.6 mm I.D. packed 6 μm graphitic carbon. Eluent: sodium citrate buffer (pH 4.50) containing 5 m*M* octanesulfonate, flow-rate: 0.8 ml/min at 40°C. Flow-rate of 0.6% ninhydrin and 1 *M* NaOH solutions: 0.2 ml/min.

7.0) [33]. Therefore, that of sodium-1-dodecylsulfonate (8.10 m*M*) may be about 5 m*M* in 50 m*M* sodium phosphate buffer (pH 4.50). The hydrophobic counterions may act as dynamic ion-exchanger, however the formation of micelle may reduce the retention of guanidino compounds. Especially, the retention of polar compounds reduced their retention by the addition of hydrophobic counterions which intercepted their direct retention on the porous graphite carbon, as shown in Fig. 1.

Octanesulfonate was the most effective ion-pair reagent in the 100 m*M* sodium citrate buffer at pH 4.50. The retention factors of paired-ion with 2-naphthalene sulfonate were about the same as those with decanesulfonate, which has the same carbon number. The retention factors of arginine, methylguanidine and guanidine with 2-naphthalene sulfonate were lower than those with decanesulfonate, but were not effective for the separation of all guanidino compounds. Further analyses were therefore carried out using octanesulfonate as the ion-pair reagent.

The concentration effect of octanesulfonate was studied in 50 m*M* sodium citrate buffer at pH 4.50, and the results are shown in Fig. 2. Increasing the concentration of octanesulfonate basically increased the retention factors; however, increasing the concentration made the operation difficult. Therefore, 5 m*M* was selected as the final concentration of octanesulfonate for subsequent studies. The concentration effect of sodium citrate buffer was examined with a pH 4.50 solution containing 5 m*M* octanesulfonate, and the results are summarized in Fig. 3. The retention factors of guanidino compounds were increased in less than 20 m*M* of sodium citrate buffer, and the retention times of some compounds exceeded over 1 h (except the very polar compounds). The addition of organic modifier decreased the retention time. The addition of 5% (v/v) of methanol cut the retention time in half. The baseline separation of the ten guanidino compounds was successful using a gradient elution from eluent A (10 m*M* sodium citrate buffer at pH 4.50 containing 5 m*M* octanesulfonate) to eluent B (10 m*M* sodium citrate buffer at pH 4.50 containing 20% acetonitrile) on a BTR carbon column. A four-step gradient was used. The eluent for the first 2.5 min was 100% eluent A. That of the next 2.5 to 22 min period was a mixture of eluents A and B (75:25). The mixture of eluents A and B in third step (from 22 to 29 min) was 50:50. In the final step, 100% eluent B was used from 29 to 33 min. Examples of the chromatograms of standard guanidino compounds are shown in Fig. 4. The standard deviation of retention times of these guanidino compounds was less than 1%, except for creatine ($n=5$). In the quantitative analyses of these compounds, the standard deviation of the peak height and area were less than a few percent ($n=5$), except for creatinine, whose values were 4.77 and 4.91%, respectively ($n=5$). The detection limit was measured by the injection of 50 μl of sample solutions, and the value of guanidino compounds was 7.8 ng/ml ($S/N=3$), (except for creatinine), and their

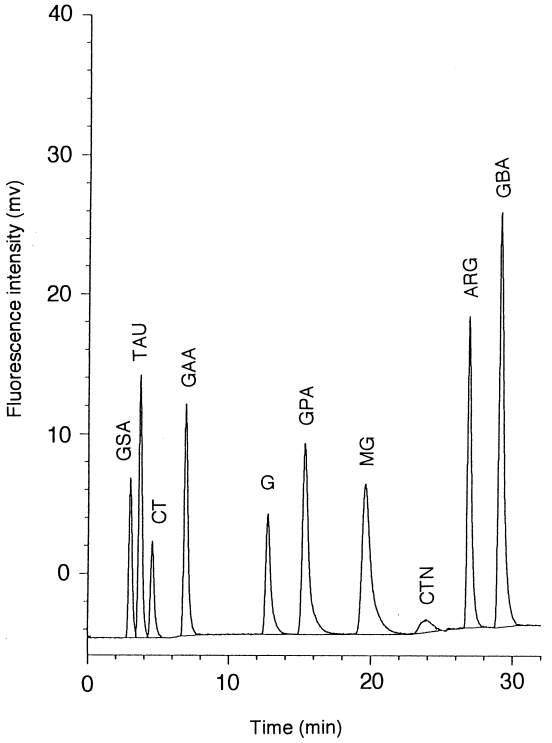

Fig. 4. The separation of ten guanidino compounds on a porous graphitic carbon column in ion-pair LC. Column: 50×4.6 mm I.D. packed 3.5 μm graphitic carbon. Eluent: four step-wise gradient from 10 m*M* sodium citrate buffer (pH 4.50) containing 5 m*M* sodium octanesulfonate to 10 m*M* sodium citrate buffer (pH 4.50) containing 20% (v/v) acetonitrile (see details in Section 2.2); flow-rate: 0.8 ml/min at 40°C. Flow-rate of 0.6% ninhydrin and 1 *M* NaOH solutions: 0.2 ml/min. Fluorescence detection: ex 392 and em 500 nm.

calibration curves were linear up to 2 μg/ml (r^2= 1.000). The detection limit of creatinine was 1 μg/ml, about one hundredth of those of the other guanidino compounds. The recovery of these guanidino compounds from serum was more than 90% (except for creatinine). The reproducibility of retention times, the peak height, peak area and recovery are summarized in Table 3.

3.5. Analysis of serum guanidino compounds

This newly developed stable system was applied to the analysis of guanidino compounds in serum from nephritic patients such as diabetic mellitum nephropathy (DMN), membraneproliferative glomerulonephritis (MPGN), chronic glomerulonephritis (CGN), continuous ambulatory peritoneal dialysis after renal transplantation (CAPD-RT), gouty kidney (GK), focal glomerular sclerosis (FGS), hypertension (HT), IgA glomerulonephritis (IgAGN), nephrotic syndrome (NS) and membranous nephropathy (MN). Blood samples collected from humans were centrifuged at 1000 *g* for 15 min at room temperature, and the sera were stored at −30°C. The 300-μl samples of sera were filtered with a Minicent-10 (Tosoh) by centrifugation at 3000 *g* for 20 min. Aliquots (50 μl) the of filtrates were injected into the analytical system described in Section 2.2.

Examples of the chromatograms of a healthy control subject, a CAPD-RT patient and a MPGN patient are shown in Fig. 5A–C. The quantitative data of five controls and sixteen patients are summarized in Table 4. GSA, G, GPA and MG were found in sera from the patients but not in sera from the controls. The amount of creatine, GAA and GBA were large in the sera from the controls, and that of creatinine and arginine were doubled in the sera from the patients. However, the existence and amount of GSA, G, GPA and MG were not related to the diseases of these patients. That is, their enzyme activity may not be related to these nephritis. The chromatographic results were not related to the values of blood urea nitrogen (BUN), creatinine (CTN) and uric acid (UA) of patients as measured by routine clinical methods. The values of BUN, CTN and UA are summarized in Table 5. The presently available enzyme activity methods, with their low sensitivity, can handle only methylguanidine and guanidinoacetate, and the chromatographic analysis of guanidino compounds is therefore important as a quantitative analysis in serum.

4. Conclusions

The retention mechanism of cationic compounds on a graphitic carbon may be electrostatic interaction for cationic guanidino compounds and hydrophobic interaction for molecular form compounds. The micelle formation of hydrophobic ion-pair reagents

Table 3
Reproducibility of chromatographic analysis of guanidino compounds using a gradient elution on a BTR Carbon column (50×4.6 mm I.D.) at 40°C

Guanidino compounds	Retention time[a] Mean±S.D. (min)	C.V. (%)	Peak height[b] C.V. (%)	Peak area[b] C.V. (%)	Recovery[c] (%)
GSA	3.62±0.05	1.03	3.14	3.42	88.53
TAU	4.39±0.04	0.95	1.93	1.79	105.13
CT	5.43±0.09	1.71	2.99	2.38	89.03
GAA	7.82±0.10	1.22	3.08	3.36	90.57
G	13.43±0.09	0.68	0.94	1.05	93.68
GPA	15.64±0.13	0.82	2.34	2.49	93.76
MG	19.97±0.22	1.08	2.24	2.25	91.13
CTN	23.12±0.15	0.65	4.77	4.91	85.94
ARG	27.90±0.10	0.34	2.88	3.13	92.01
GBA	30.06±0.08	0.26	2.24	2.52	89.12

[a] Chromatographic conditions as in Fig. 4.
[b] Twenty μl injection of 2 μg/ml solutions of guanidino compounds except for CTN (10 μg/ml) (n=5).
[c] Twenty μl injection of 2 μg/ml (CTN 10 μg/ml) standard guanidino compounds spiked with the control serum (n=5).

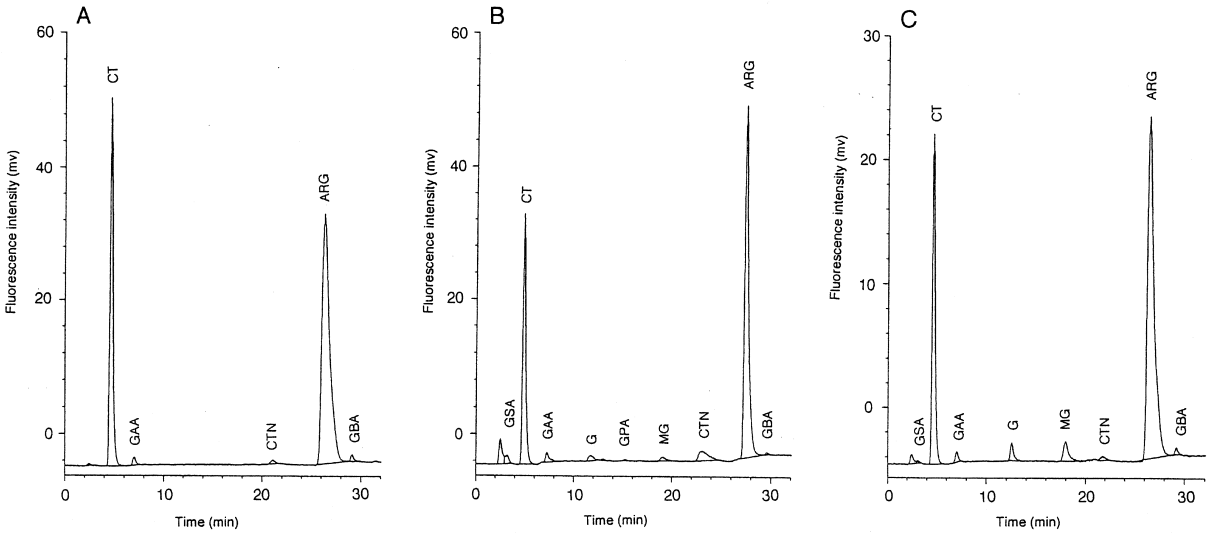

Fig. 5. Analyses of guanidino compounds in sera from nephritic patients. (A) Control (sample No. 5 in Table 3), (B) CAPD-RT (sample No. 9 in Table 3), (C) MPGN (sample No. 18 in Table 3). Chromatographic conditions: see Fig. 4.

Table 4
Guanidino compounds found in sera (μmol/l) measured on a BTR Carbon column (50×4.6 mm I.D.) at 40°C

No.	Age	Sex	GSA	TAU	CT	GAA	G	GPA	MG	CTN	ARG	GBA	Disease
1	26	M	N.D	N.D.	6.75	0.64	N.D.	N.D.	N.D.	95.51	62.61	0.38	Control
2	27	M	N.D	N.D.	14.85	0.75	N.D.	N.D.	N.D.	78.10	72.07	0.48	Control
3	28	M	N.D	N.D.	43.51	0.58	N.D.	N.D.	N.D.	75.19	73.64	0.44	Control
4	40	M	N.D.	N.D.	9.85	0.72	N.D.	N.D.	N.D.	83.90	71.65	0.34	Control
5	32	F	N.D.	N.D.	18.42	0.58	N.D.	N.D.	N.D.	102.74	142.17	0.33	Control
	Average		–		18.68	0.65	–	–	–	87.09	84.43	0.39	–
	S.D.		–		14.59	0.08	–	–	–	11.71	32.57	0.06	–
6	57	M	1.70	N.D.	3.57	0.50	N.D.	N.D.	N.D.	220.35	220.62	0.19	DMN
7	76	F	1.37	N.D.	2.56	0.39	0.22	N.D.	N.D.	141.97	164.65	0.09	MPGN
8	52	F	N.D.	N.D.	25.37	0.56	0.16	N.D.	N.D.	63.58	181.62	0.49	CGM
9	29	F	2.25	N.D.	12.51	0.63	0.26	0.24	0.75	467.12	203.07	0.14	CAPD-RT
10	67	M	N.D.	N.D.	46.97	0.83	N.D.	N.D.	N.D.	124.55	164.73	1.26	GK
11	84	M	1.22	N.D.	5.76	0.54	0.32	N.D.	N.D.	191.32	174.88	0.20	FGS
12	52	F	1.65	N.D.	12.53	0.44	0.35	0.19	N.D.	144.87	107.43	0.19	CGN
13	74	F	4.07	N.D.	6.79	0.54	0.36	N.D.	N.D.	292.93	137.95	0.35	CGN
14	77	M	N.D.	N.D.	9.94	0.63	0.16	N.D.	N.D.	110.03	121.90	0.39	HT
15	18	F	N.D.	N.D.	9.07	0.48	N.D.	N.D.	N.D.	75.19	113.36	0.20	IgAGN
16	50	F	N.D.	N.D.	10.84	0.62	0.14	N.D.	N.D.	72.29	114.93	0.42	IgAGN
17	50	M	N.D.	N.D.	8.67	0.66	N.D.	N.D.	N.D.	98.20	110.41	0.34	IgAGN
18	50	F	1.12	N.D.	8.97	0.62	1.56	N.D.	1.77	139.06	107.27	0.21	MPGN
19	79	M	0.91	N.D.	5.61	0.42	0.21	N.D.	0.14	101.32	92.64	0.29	NS
20	73	F	0.84	N.D.	11.65	0.60	0.14	0.16	N.D.	144.87	98.39	0.38	MPGN
21	63	M	N.D.	N.D.	7.53	0.46	N.D.	N.D.	N.D.	83.90	74.33	0.30	MN

N.D.=Not detected.

Table 5
Clinical analytical results of sera from patients

Sample No.	BUN (mg/dl)	CTN (mg/dl)	UA (mg/dl)
6	34.7	3.2	5.1
7	40.4	3.0	–
8	13.2	0.9	4.3
9	52.4	10.9	7.6
10	57.8	2.2	7.4
11	45.1	0.3	5.4
12	60.2	2.5	11.9
13	81.4	5.3	10.3
14	24.4	1.5	7.2
15	12.6	1.0	4.5
16	22.6	1.1	5.2
17	23.6	1.5	5.6
18	25.2	0.6	6.5
19	18.4	1.4	6.2
20	38.3	2.5	5.0
21	18.0	1.3	7.4

Sample numbers are the same as in Table 3.

may reduce the retention of guanidino compounds. The successful separation of guanidino compounds was achieved by the addition of octanesulfonate in citric buffer. A LC system equipped with a graphitic carbon column and a post-column labeling detector was a reliable system for analyzing trace levels of guanidino compounds in human sera as a routine analytical method, due to the chemical and physical stability of the graphitic carbon column and the sensitivity of the detector. However, it is very important to avoid injecting hydrophobic compounds into the graphitic carbon column in long-term operations, because hydrophobic compounds were retained on the graphitic carbon in the eluent used and could not be eluted by this eluent. A small pre-column packed with octadecyl-bonded silica gel was useful for eliminating small particles and hydrophobic compounds. Such a column can be washed using alkali solution containing an organic modifier.

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