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POST-COLUMN DERIVATIZATION OF GUANIDINO COMPOUNDS IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING NINHYDRIN

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SUMMARY

A new method for the high-performance liquid chromatography of guanidino compounds using ninhydrin as the fluorescence reagent is described. Use of organic solvent is not required since ninhydrin is highly soluble in aqueous media, and the problem of precipitation formation occurring in the phenanthrenequinone method was avoided. Creatine was also assayed in the present procedure. Separation of ten guanidino compounds was completed within 30 min using a small-size (38 × 4.2 mm I.D.) strong cation-exchange column.

INTRODUCTION

The level of guanidino compounds such as guanidinoacetic acid [1—4] and methylguanidine [2—7] rises significantly in the plasma of uremic patients. Liquid chromatography has recently been used for the separation and determination of these guanidino compounds. Several workers [8, 9] have reported the assay of plasma guanidines employing a modified automatic amino acid analyser. However, this analytical method is rather time-consuming and the colorimetry shows poor sensitivity.

Yamada and Itano [10, 11] have reported that micro-amounts of arginine and arginine-containing peptides can be detected fluorimetrically using 9,10-phenanthraquinone (PQ). This reaction was applied to the microdetermination of guanidines by Sakaguchi et al. [12]. Yamamoto and co-workers [13, 14] have recently developed a high-performance liquid chromatographic (HPLC) method for the fluorimetric determination of guanidino compounds in physiological fluids using PQ as the reagent for the post-column derivatization. Although this method is highly sensitive, PQ is practically insoluble in water, so the derivatization reagent should be prepared by dissolving PQ

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in dimethylformamide. The derivatization reagent often causes precipitation which blocks the chromatographic tubing after it is mixed with the column effluent.

The present paper describes a new HPLC system using ninhydrin [15—19], which is highly water-soluble, for the development of fluorescence from guanidino compounds. A rapid separation of guanidino compounds using a small-size column is also described.

EXPERIMENTAL

Chemicals

Guanidinosuccinic acid, guanidinobutyric acid, guanidinopropionic acid, guanidinoacetic acid, and methylguanidine hydrochloride were all obtained from Sigma (St. Louis, MO, U.S.A.). Creatine, creatinine, L-arginine hydrochloride, sodium citrate, sodium hydroxide, sodium chloride, perchloric acid (60%), boric acid, and ninhydrin were purchased from Wako Pure Chemical (Osaka, Japan). All chemicals used were of analytical reagent grade.

Elution buffers and reagent solutions

The eluent buffer solutions are listed in Table I. The pH of each eluent buffer is adjusted with perchloric acid or 1.0 *N* sodium hydroxide. All eluent buffers, 0.75 *N* sodium hydroxide solution and 0.6% ninhydrin solution are prepared using glass-redistilled water, and passed through a 0.22-μm microfilter (Fuji Photo Film, Tokyo, Japan) prior to use.

TABLE I

COMPOSITION OF ELUENTS

	Eluents					
	First (5 min)	Second (8 min)	Third (2 min)	Fourth (14 min)	Fifth (2 min)	Sixth (3 min)
pH	3.5	5.0	6.0	11.4		
Na^+ concentration (*N*)	0.15	0.35	0.15	0.85		
Sodium citrate $2H_2O$ (g/l)	14.7	34.3	34.3	34.3		
Perchloric acid (60%) (ml/l)	10.5	11.0	3.0	—		
Boric acid (g/l)	—	—	—	6.2		
Sodium hydroxide (g/l)	—	—	—	4.0		
					0.2 *N* NaOH	H_2O

Chromatographic system

Fig. 1 shows the flow diagram of our chromatograph. A single plunger pump (Sanuki Industry, Tokyo, Japan) served to deliver the eluent with the constant flow-rate of 0.7 ml/min. The eluent-selecting valve was controlled by an SGR-1A step gradient programmer unit (Shimadzu Seisakusho, Kyoto, Japan), and pumped through a valve universal injector (Sanuki Industry, Tokyo, Japan). An ISC-05/S0504 packed column (strong cation-ex-

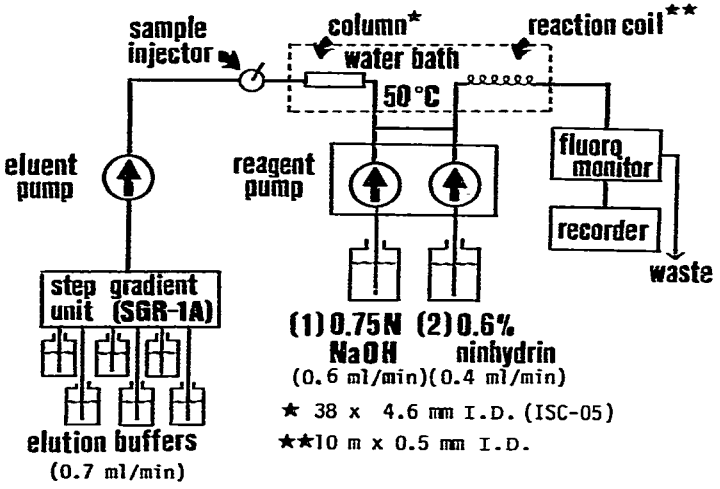


Fig. 1. Flow diagram of the HPLC system.

change resin; 5-μm particle size; 38 mm × 4.2 mm I.D.; Shimadzu Seisakusho) was used for the separations. It was operated at 50 ± 0.05°C using a C-600 thermo-unit (Taiyo Scientific Industry, Tokyo, Japan). A double plunger pump (Sanuki Industry) served to deliver the alkaline solution and the ninhydrin solution at constant flow-rates of 0.6 ml/min and 0.4 ml/min, respectively.

The column effluent was first mixed with 0.75 *N* sodium hydroxide in a T-junction, and then mixed with 0.6% ninhydrin solution in another T-junction. The mixture was then allowed to flow through a PTFE-tubing reaction coil (10 m × 0.5 mm I.D.) where it was heated to 50 ± 0.05°C in a water-bath.

The fluorescence intensity of the effluent was measured using an RF-500 LC spectrofluoromonitor (Shimadzu Seisakusho). The excitation and emission wavelengths were 395 nm and 500 nm, with slit widths of 20 nm and 40 nm, respectively. The excitation lamp was a xenon discharge lamp (Wacom R & D Corp.).

Operation of the chromatograph for analysis

The guanidino compounds were separated with a strong cation-exchange column using a stepwise pH gradient. The first buffer was pumped through the column for 5 min, then the second buffer for 8 min, the third buffer for 2 min and the fourth buffer for 14 min; then the column was washed successively with 0.2 *N* sodium hydroxide for 2 min and with water for 3 min. Ten guanidino compounds (from taurocyamine to methylguanidine) were analysed within 30 min using this system.

Preparation of samples

To 200 μl of plasma were added 100 μl of 30% trichloroacetic acid solution and the mixture was centrifuged at 1000 *g* for 10 min. Then the pH of the supernatant solution was adjusted to 2.0 with 0.4 *N* sodium hydroxide and 150 μl of this deproteinized sample were used for the analysis.

RESULTS AND DISCUSSION

Effects of pH and ionic strength on the retention times of guanidino compounds

Fig. 2 shows the effect of the pH and ionic strength of 0.35 *N* sodium citrate buffer on the retention times of various guanidino compounds. Guanidinosuccinic acid (GSA), creatine (CT) and guanidinoacetic acid (GAA) were not favorably separated by gradient lowering of the pH (Fig. 2A). On the other hand, these three guanidino compounds were satisfactorily resolved by decreasing the ionic strength of the eluent buffer as shown in Fig. 2B. Accordingly, 0.15 *N* sodium citrate buffer (pH 3.5) was used for the separation (Table I).

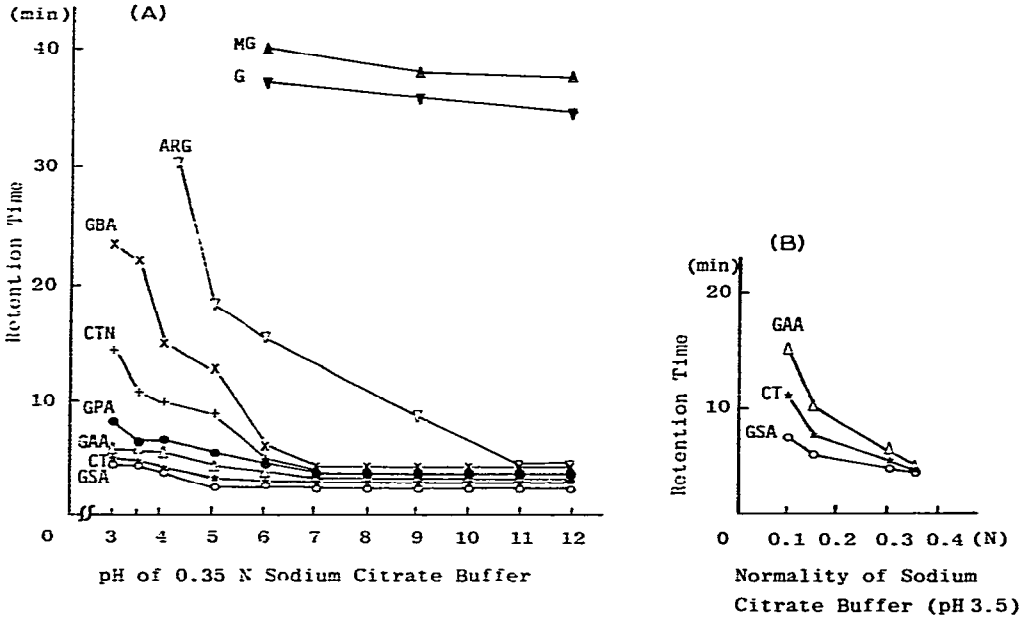


Fig. 2. (A) Effect of the pH of the elution buffer on the retention times of various guanidino compounds. (B) Effect of the ionic strength of the elution buffer on the retention times of various guanidino compounds.

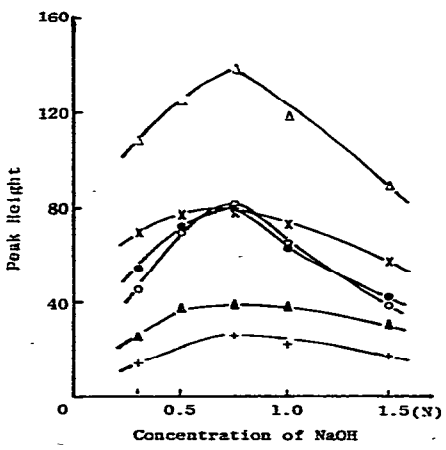
The retention time of guanidinopropionic acid (GPA) decreased markedly around pH 3.0—4.0 and was constant above pH 5.0. The values for creatinine (CTN) and guanidinobutyric acid (GBA) sharply decreased around pH 5.0—6.0 and were approximately constant above pH 6.0. Guanidine (G) and methylguanidine (MG), strongly basic guanidino derivatives, were not eluted within 30 min with 0.35 *N* sodium citrate buffer (pH 11.4). G and MG were eluted with 0.85 *N* sodium citrate buffer, containing 0.62% (w/v) boric acid (pH 11.4), showing the retention times of 15 min and 18 min, respectively. The conditions for the stepwise gradient elution were set as shown in Table I on the basis of these results. All the guanidino compounds tested were eluted with sodium citrate buffers within 29 min, and the column was washed with 0.2 *N* sodium hydroxide and water after the separation.

Fluorescence properties of ninhydrin derivatives of guanidino compounds

Ninhydrin has been reported to combine with guanidine, monosubstituted guanidines, and N,N-disubstituted guanidines to give highly fluorescent addition products in strongly alkaline media [15—17]. This reaction has also been used for the determination of creatine [18]. The five-membered ring of ninhydrin is cleaved immediately after the addition of alkali to produce *o*-carboxyphenylglyoxal [18], which then condenses with guanidines. Accordingly, amino acids do not interfere with the reaction when the ninhydrin solution is previously made alkaline. The present system first delivers sodium hydroxide solution and then ninhydrin solution to the column effluent. Ninhydrin is converted by the action of alkali into *o*-carboxyphenylglyoxal [16] which does not react with amino acids.

The excitation maxima of ninhydrin derivatives of guanidines in the effluent from the column were at 305 and 395 nm, and the single emission peak had a maximum at 500 nm. The fluorescence at 395 nm was approximately twice as intense as that excited at 305 nm. These data agreed well with those reported by Conn and Davis [16]. The excitation and emission wavelengths of the fluoromonitor were therefore set at 395 nm and 500 nm, respectively.

Reaction conditions for the post-column derivatization were examined by injecting 50 µl of standard solution of guanidino compounds. Fig. 3 shows the fluorescence intensity of the derivatized guanidino compounds against the alkali concentration. All the guanidino compounds gave the maximum fluorescence intensity at the sodium hydroxide concentration of 0.75 *N*. Fig. 4 demonstrates that the maximum fluorescence intensity was shown at the ninhydrin concentration of 0.6%. The effect of temperature on the reaction of the derivatized guanidino compounds with ninhydrin was studied in



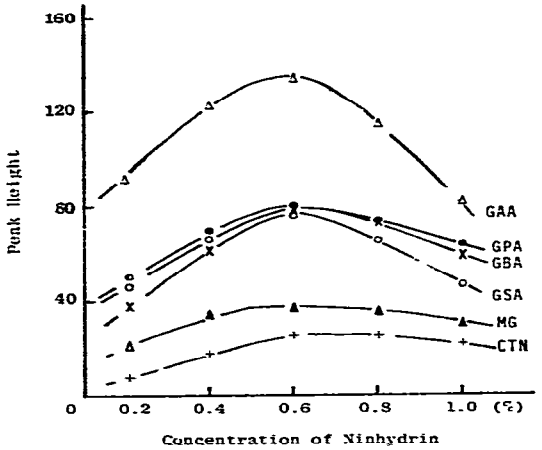
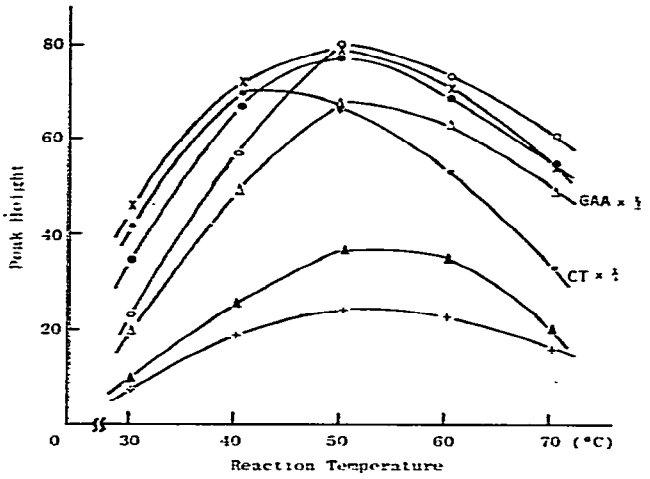


Fig. 3. Effect of alkali concentration on the fluorescence intensity of various guanidino compounds (1 nmol each). ○, GSA; △, GAA; ×, GBA; +, CTN; ●, GPA; ▲, MG (for abbreviations, see text).

Fig. 4. Effect of ninhydrin concentration on the fluorescence intensity of various guanidino compounds (1 nmol each). For abbreviations, see text.

the range of 30°C to 70°C. Fig. 5 shows that the optimum temperature for these compounds except creatine was 50°C. Creatine gave a slightly lower peak at 50°C whereas it gave maximum peak height at 40°C. However, this does not affect the assay because the ninhydrin reagent is highly sensitive to creatine.



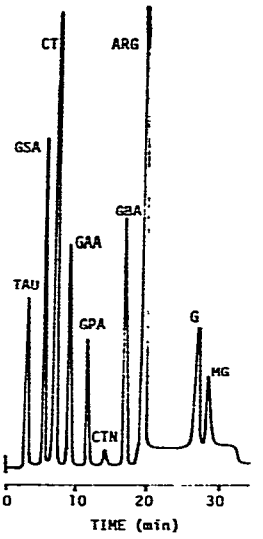


Fig. 5. Effect of reaction temperature on the fluorescence intensity of various guanidino compounds (1 nmol each). ○, GSA; *, CT; △, GAA; X, GBA; +, CTN; ●, GPA; ▲, MG (for abbreviations, see text).

Fig. 6. Chromatogram of a standard mixture of guanidino compounds. TAU = taurocyamine (1 nmol); GSA = guanidinosuccinic acid (1 nmol); CT = creatine (0.5 nmol); GAA = guanidinoacetic acid (0.5 nmol); GPA = guanidinopropionic acid (0.5 nmol); CTN = creatinine (5 nmol); GBA = guanidinobutyric acid (1 nmol); ARG = arginine (2 nmol); G = guanidine (2 nmol); MG = methylguanidine (0.5 nmol).

Chromatographic separation and quantitative response

A typical separation of a standard solution of guanidino compounds is demonstrated in Fig. 6. GSA, CT, GAA, GPA, CTN, GBA, arginine (ARG), G and MG were all completely resolved, and the entire analysis required 35 min including the column-washing procedure.

The limits of detection for guanidino compounds, determined by the peak height at twice the noise level, are as follows: GSA, 5 pmol, CT, 1 pmol; GAA, 10 pmol; GPA, 5 pmol; CTN, 1 nmol; GBA, 1 pmol; ARG, 50 pmol; G, 5 pmol; MG, 5 pmol.

Standard curves for these guanidino compounds are depicted in Fig. 7. Fluorescence responses are linear for these seven guanidino compounds up to at least 5 nmol. The lower limit of the assay varies from 10 pmol (GSA, GPA, GBA and MG) to 50 pmol (GAA).

Excellent reproducibility was observed for the determination of all the guanidino compounds listed in Table II.

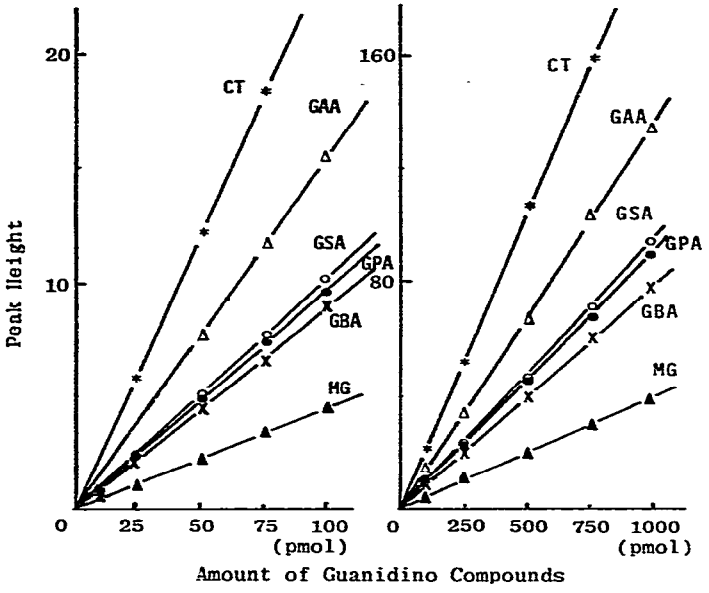


Fig. 7. Standard curves for guanidino compounds. For abbreviations, see text.

TABLE II

DAY-TO-DAY PRECISION OF THE PRESENT METHOD FOR AQUEOUS SOLUTIONS OF VARIOUS GUANIDINO COMPOUNDS

Injection volume was 50 μl. $n = 8$ in all cases.

Compound*	Concentration (nmol per 50 μl)	C.V. (%)
GSA	0.136	0.57
CT	1.360	2.54
GAA	0.170	3.29
GPA	0.105	1.57
CTN	3.570	1.65
GBA	0.344	2.16
ARG	4.900	1.36
G	0.342	1.59
MG	0.059	2.63

*For abbreviations, see text.

Excellent recoveries of these compounds from human serum were observed with satisfactory reproducibility, as listed in Table III.

Analysis of human plasma samples

Fig. 8 shows a chromatogram of a plasma sample from a person in normal health. The following compounds were assayed: CT, 0.24 mg/dl; GAA, 15 μg/dl; CTN, 1.20 mg/dl; ARG, 2.06 mg/dl.

Fig. 9 shows a chromatogram of a plasma sample from a chronic glomerulonephritis patient. The following guanidines were identified by comparing

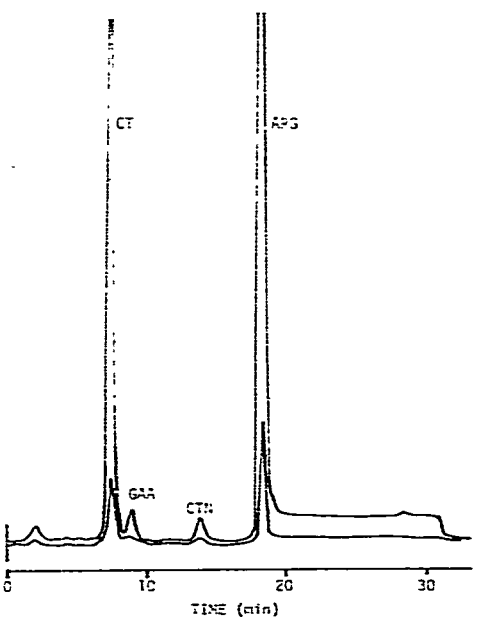
TABLE III

RECOVERIES OF GUANIDINO COMPOUNDS FROM HUMAN SERUM

150 μl of deproteinized samples were injected. n = 9 in all cases.

Compound*	Added (nmol)	Within-day	
		Recovery ($\bar{X}$) (%)	C.V. (%)
GSA	0.272	92.1	2.30
CT	1.00	90.9	3.11
GAA	0.340	95.5	3.17
GPA	0.289	96.3	0.06
CTN	18.0	96.2	1.20
GBA	0.081	91.3	0.25
ARG	5.00	96.7	1.32
G	3.00	96.6	1.35
MG	2.00	93.9	1.74

*For abbreviations, see text.



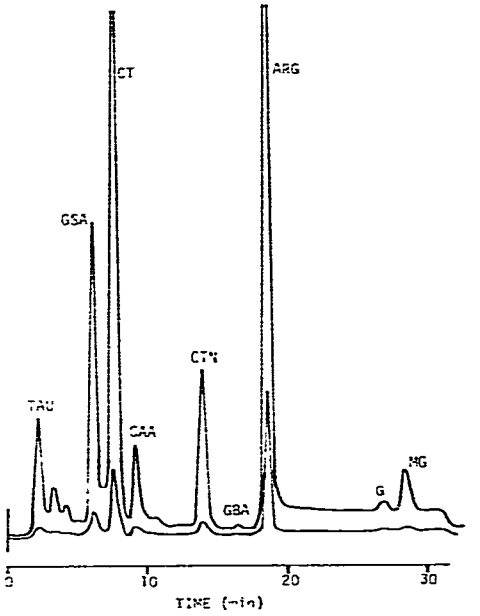


Fig. 8. Chromatogram of a plasma sample from a person in normal health. For abbreviations, see text.

Fig. 9. Chromatogram of a plasma sample from a chronic glomerulonephritis patient. For abbreviations, see text.

retention times with those of standard solutions: GSA, 0.475 mg/dl; CT, 0.26 mg/dl; GAA, 49 μg/dl; CTN, 10.55 mg/dl; ARG, 2.56 mg/dl; G, 43.3 μg/dl; MG, 98.2 μg/dl.

The present method is as sensitive as the PQ method. Creatine, which is

not detected by the PQ method, can be sensitively detected. The present method, therefore, offers more-detailed metabolic information.

Ninhydrin is readily soluble in water and its aqueous solution can be used as the fluorescent reagent. Accordingly, the present method does not require any organic solvent and no precipitation occurs during the chromatography. In addition, the use of a short column, ISC-05, facilitated more rapid separation of guanidino compounds compared with the conventional methods. Conclusively, the ninhydrin method is more practical and widely applicable than the PQ method and it is recommended to be adopted in clinical laboratories.

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