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ION-EXCHANGE CHROMATOGRAPHIC SEPARATION AND FLUOROMETRIC DETECTION OF GUANIDINO COMPOUNDS IN PHYSIOLOGIC FLUIDS

YUKIO YAMAMOTO, TADATOMI MANJI, AKIRA SAITO, KENJI MAEDA and KAZUHIRO OHTA

The Biodynamics Research Institute, 1-3-2 Tamamizu-cho, Mizuho-ku, Nagoya 467 (Japan)

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SUMMARY

A high-performance liquid chromatographic procedure has been developed for the separation and fluorometric detection of guanidino compounds in physiologic fluids. All guanidino compounds were separated on a 17 × 0.46 cm cation-exchange column using a stepwise pH gradient. The chromatographic system was designed to enable the use of the specific reagent 9,10-phenanthrenequinone as a means of monitoring the guanidino compounds of physiologic fluids. This new analytical method is so sensitive that it enables the analysis at the picomole level. Our automatic guanidino-compound analyzer was successfully applied to the quantitative determination of all guanidino compounds in physiologic fluids from normal controls and uremic patients.

INTRODUCTION

Guanidino compounds such as methylguanidine [1—5] and guanidino succinic acid [3, 6—8] have been demonstrated to be elevated significantly in the sera of uremic patients compared with normal controls. The analysis of these guanidino compounds has been carried out using the colorimetric method after separation with ion-exchange [5, 9—13], paper [14—17] and thin-layer chromatography [18] and electrophoresis [19]. These methods require a large amount of time and effort, and have the limitation that only some guanidino compounds can be analyzed. Guanidino compounds have also been determined by gas—liquid chromatography [20—26]; however, this method involves a complex preparation procedure for derivatizing the volatile compounds before injection into the gas-chromatographic column. It has recently been reported that the automatic quantitative analysis of the various guanidino compounds in plasma has been performed with a modified automatic amino-acid analyzer [27—30]. However, this analytical method is still time-consuming and the colorimetric determination used lacks sensitivity.

The purpose of this study was to develop a rapid and sensitive method for the quantitative analysis of all guanidino compounds in physiologic fluids. A high-performance cation-exchange chromatographic procedure has been developed for separating all guanidino compounds in physiologic fluids, and detecting these compounds fluorometrically by utilizing the fluorophor that is formed when guanidino compounds react with 9,10-phenanthrenequinone. 9,10-Phenanthrenequinone has been reported to form a fluorescent product with mono-substituted guanidines in alkaline media [31]. The reagent has been used for manual fluorometric assay of arginine, arginine peptides and mono-substituted guanidines and it has been demonstrated to be more sensitive than the Sakaguchi reaction.

EXPERIMENTAL

Chemicals

The standard samples of guanidinosuccinic acid, guanidinoacetic acid, guanidinopropionic acid, guanidinobutyric acid, 4-guanidinobenzoic acid, arginine, creatine, creatinine, guanidine hydrochloride and methylguanidine hydrochloride were obtained from Sigma (St. Louis, Mo., U.S.A.). α-Amino-β-guanidinopropionic acid hydrochloride was purchased from Pierce (Rockford, Ill., U.S.A.). 9,10-Phenanthrenequinone was obtained from Tokyo Chemicals (Tokyo, Japan). N,N-Dimethylformamide was purchased from Nakarai Chemicals (Kyoto, Japan).

Eluent and reagent solutions

The separation conditions for guanidino compounds were studied using buffer solutions in the pH range 3—11, and alkali solutions, which will be described later. The buffer solutions established as eluents are listed in Table I. The buffers were prepared in 4-l batches to ensure reproducibility during the analyses. The pH of each eluent was adjusted with either concentrated HCl or 1.0 *N* NaOH. 9,10-Phenanthrenequinone is unstable in alkaline solution and insoluble in water, so it is necessary to prepare it in an organic solvent such as methanol or dimethylformamide. A 2.5 m*M* solution of 9,10-phenanthrenequinone was prepared by dissolving the reagent in dimethylformamide. The

TABLE I

COMPOSITION OF BUFFERS

Reagent	Buffers			
	First	Second	Third	Fourth
pH	3.35	4.90	8.10	
Na^+ concentration (*N*)	0.20	0.20	0.20	0.50
Sodium citrate $2H_2O$ (g/l)	19.60	19.60	19.60	0
Hydrochloric acid (36%) (ml/l)	12.4	6.3	0	0
Boric acid (g/l)	0	0	0.50	0
Sodium hydroxide (g/l)	0	0	0	20.0
Pentachlorophenol (ml/l)	0.2	0.2	0.2	0

reagent solution was stored in a refrigerator at 5° to prevent degradation of the reagent. A 2.0 *N* NaOH solution was prepared in 4-l batches to ensure accuracy during the analyses.

Chromatographic system

Studies on the reaction of guanidino compounds with 9,10-phenanthrenequinone were first carried out by a manual procedure to establish optimal conditions for reaction time, alkaline concentration, concentration of reagent and other variables. These findings were used in the design of the automated 9,10-phenanthrenequinone detection system. Fig. 1 is a schematic diagram of the automatic guanidino-compound analyzer which was constructed. Three minipumps served to pump the eluent, the alkaline solution and 9,10-phenanthrenequinone solution through the system. An 8-port motor valve was inserted between the four buffer chambers and the eluent pump. The eluent selecting valve was controlled by the electric programmer of the analyzer through a relay, and allowed any one of the eluents from the buffer chambers

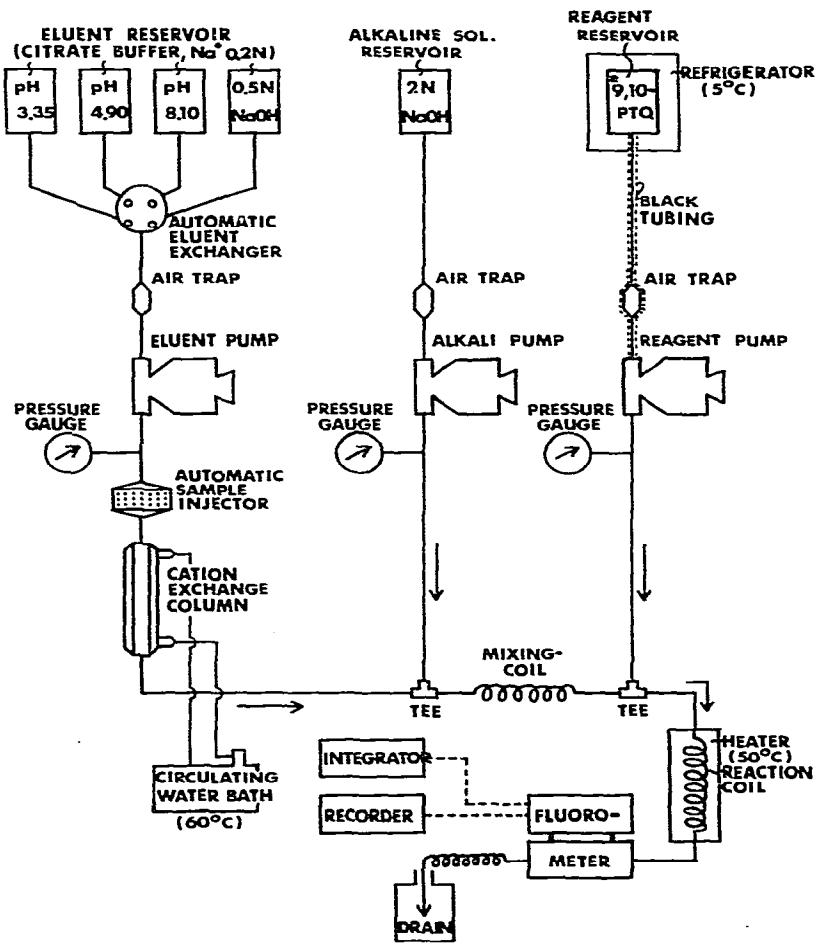

Fig. 1. Schematic diagram of automatic guanidino-compound analyzer.

to be supplied to the eluent pump. The eluent was pumped through an automated sample injection valve supplied with a 100-μl sample loop. A jacketed, stainless-steel column, 17 × 0.46 cm I.D., was used in this investigation. The column was slurry packed with CK-10-S cation-exchange resin with a mean particle size of 11.5 μm (Mitsubishi Kasei Co., Tokyo, Japan). The PTFE tubing connecting the reagent reservoir to the reagent pump was covered with black tubing to prevent exposure of the reagent to light. The column was operated at 60° using a Haacke constant-temperature circulator. The column effluent was first mixed with a stream of 2 *N* NaOH by means of a mixing coil. The alkalinated stream was then fed to another tee-junction where the 9,10-phenanthrenequinone reagent stream was introduced. The mixture flowed through a reaction coil, 800 × 0.05 cm I.D. where it was heated to 50°. At a total flow-rate of 48 ml/h the reaction coil provided approximately 2 min for dwell time.

An FP-100 fluorescence photometer (Japan Spectroscopic Co., Tokyo, Japan) was used to detect the fluorescence components eluting from the column. The excitation lamp was a high-pressure mercury lamp which emits strong 365 nm light. Visible light emitted from the light source was blocked by a primary filter; visible light above 460 nm emitted from the flow-cell passed through a secondary filter and impinged on the photosensitive elements of a photocell. The chromatogram was recorded with a strip chart recorder (Rikadenki, Tokyo, Japan). The chromatographic peak areas were determined with a SIC Model 500A digital integrator (Scientific Instruments, Tokyo, Japan) with baseline display which was connected between the fluoromonitor and the recorder.

Operation of the chromatograph for analysis

The chromatographic operation is controlled automatically and requires a minimum of operator effort. Table II describes the sequence of events for the high-performance liquid chromatographic (HPLC) analysis.

The guanidino compounds were separated on a cation-exchange resin using a stepwise pH gradient. The first buffer was pumped through the column for 11 min after the introduction of the sample, the second buffer for 32 min, the third buffer for 18 min and the fourth buffer for 33 min. Seven samples of deproteinized physiologic fluids were loaded in 100-μl sampling loops of the injection valve and automatically introduced into the analytical column at an appropriate time. The column was operated at a flow-rate of 24 ml/h and a column inlet pressure of 30—50 kg/cm^2. An alkalinating reagent was pumped into the mixing coil at a flow-rate of 12 ml/h. The 9,10-phenanthrenequinone reagent solution was pumped into the reaction coil at a flow-rate of 12 ml/h. As mentioned previously, the reaction coil was heated up to 50°, the optimum temperature for forming the fluorescent 9,10-phenanthrenequinone derivatives of the guanidino compounds for fluorescence measurement (see later).

Preparation of physiologic fluid samples

Serum and cerebrospinal fluid samples from normal controls and from uremic patients were used for the analysis of guanidino compounds. A 1-ml aliquot from each serum sample was centrifuged at 170 *g* for 2 h in a CF-

TABLE II

SEQUENCE OF EVENTS IN OPERATION OF AUTOMATIC GUANIDINO-COMPOUND ANALYZER

Time (min)	Events
	The main switch is turned on.
	Seven samples are loaded in the sample loops.
	The eluent pump, the alkaline pump and the reagent pump are started.
	The first eluent is pumped through the column.
000	The automatic programmer is started.
001	The repeat program is turned on. The first eluent continues.
002	The sample is introduced into the column. The integrator is started.
013	The eluent-selecting valve is switched to the second eluent.
045	The eluent-selecting valve is switched to the third eluent.
063	The eluent-selecting valve is switched to the fourth eluent.
100	The eluent-selecting valve is switched to the first eluent.
110	The integrator prints out the retention times, peak heights, peak areas and so on. The first eluent continues.
140	The programmer is automatically recycled, or the repeat programmer is turned off.
142	The alkaline flow-line is washed with water and the reagent flow-line washed with dimethylformamide.
200	The main power is turned off.

25 Centriflo® membrane [32] (Amicon, Lexington, Mass., U.S.A.), yielding approximately 0.5 ml of ultrafiltrate. A 0.1-ml aliquot of the deproteinized ultrafiltrate was applied to the column of the chromatographic system. The cerebrospinal fluid sample was used directly for analysis without any deproteinization treatment.

RESULTS

Effect of pH on the capacity factor of guanidino compounds

The effect of the pH of a 0.2 *N* sodium citrate buffer on the capacity factors of different guanidino compounds is shown in Fig. 2. For the guanidino compounds that have their pK_a values within the studied pH range, a significant drop of retention is expected with increasing pH as these solutes become less cationic. The capacity factors of guanidinosuccinic acid and guanidinoacetic acid decreased markedly around pH 3.0—3.5 and were constant above pH 5.0. The values of k for guanidinopropionic acid, creatinine and guanidinobutyric acid showed a steep decrease around pH 5.0—6.0 and were constant above pH 7.0. For guanidino amino acids such as α-amino-β-guanidinopropionic acid and arginine, a significant decrease in k was observed at a higher pH of 8.0 and 10.0, respectively. Methylguanidine and guanidine are strongly basic, and so are strongly adsorbed by the column and not eluted with the buffers studied. With a strong alkaline solution, such as 0.4 *N* or stronger NaOH, they are eluted in a reasonable time.

From these data, it was determined that a stepwise pH gradient should be adopted for the separation of a series of these guanidino compounds in a single run. Furthermore, the pH values of the buffer eluents for the gradient elution were determined using these data.

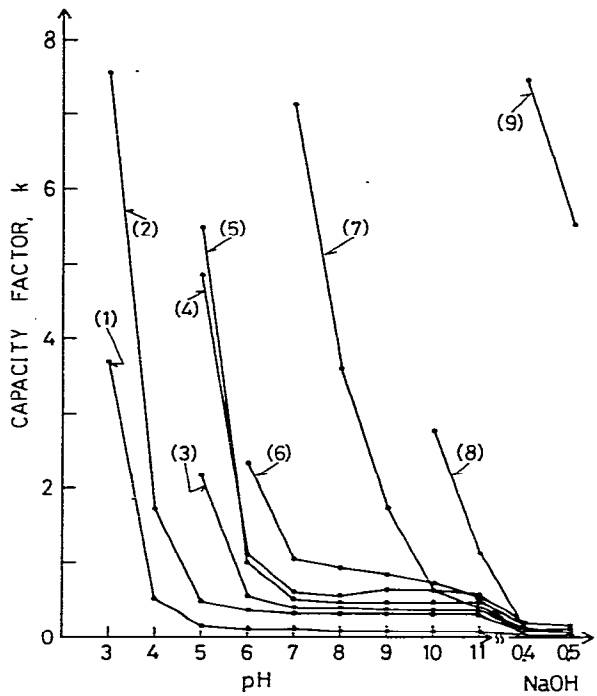

Fig. 2. Dependence of capacity factor k upon pH and alkaline concentration of eluent. (1) GSA; (2) GAA; (3) GPA; (4) CRN; (5) GBA; (6) 4-GBA; (7) α-A-β-GPA; (8) ARG; (9) MG.

Fluorescence properties of 9,10-phenanthrenequinone derivatives of guanidino compounds

9,10-Phenanthrenequinone derivatives of guanidino compounds show a fluorescence maximum around 495—525 nm and an excitation maximum around 370—386 nm. The effect of temperature on the reaction of guanidino compounds with 9,10-phenanthrenequinone was studied in the range 20—90° using a chromatographic system. Fig. 3 shows a plot of the fluorescence intensity as a function of reaction-coil temperature after passing through the coil for 2 min. The degree of fluorescence conversion of all guanidino compounds increased with increase in reaction-coil temperature up to a maximum, and then decreased at higher temperatures with increase in reaction temperature. This decrease is assumed to result from a partial degradation of the reagent or products at higher temperatures. The optimum temperature for fluorescence conversion of guanidinoacetic acid and methylguanidine is 50°, whereas that for α-amino-β-guanidinopropionic acid and guanidinosuccinic acid is 60° and for creatinine, 4-guanidinobenzoic acid, arginine and guanidinobutyric acid 70°

From these data it appeared that a temperature of 50—60° should be chosen for coil operation as a compromise between speed of fluorescence conversion, sensitivity and stability of the fluorescent derivatives formed. A temperature of 50° was tentatively chosen for coil operation for the rest of the analysis.

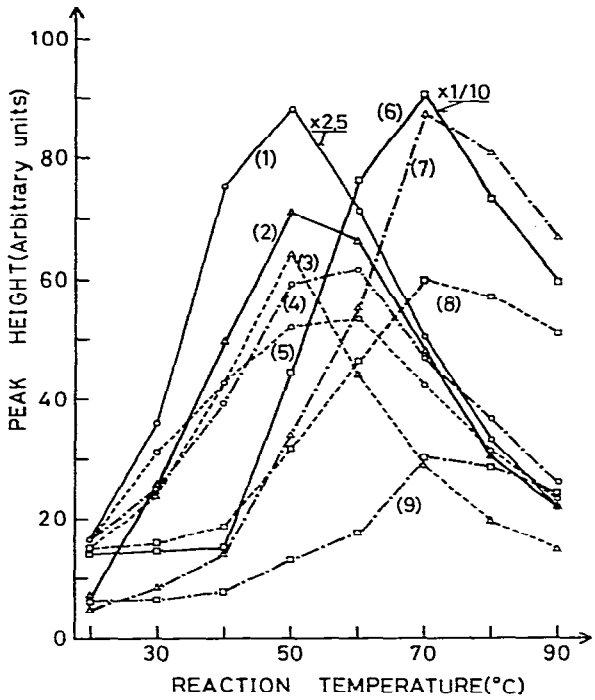

Fig. 3. Effect of reaction temperature on fluorescence conversion of guanidino compounds. (1) GAA; (2) MG; (3) α-A-β-GPA; (4) GPA; (5) GSA; (6) CRN; (7) 4-GBA; (8) ARG; (9) GBA.

Chromatographic separation and quantitative response

Fig. 4 shows a typical separation of a standard mixture of guanidino compounds, each at a concentration of 2.5 nmole/100 μl (creatinine 25 nmole/100 μl). Guanidinosuccinic acid (GSA), guanidinoacetic acid (GAA), guanidinopropionic acid (CPA), creatinine (CRN), guanidinobutyric acid (GBA), 4-guanidinobenzoic acid (4-GBA), α-amino-β-guanidinopropionic acid (α-A-β-GPA); arginine (ARG), guanidine (G) and methylguanidine (MG) were all completely resolved, except for guanidine—methylguanidine pairs. For such a chromatogram as guanidine—methylguanidine pairs, which have an R_S value of about 0.9 and a peakheight ratio (MG:G) of 23:1, the guanidine area will be underestimated [33]; this is confirmed in Table III. Therefore, quantitative analysis of guanidine was performed by using peak heights in the rest of the analysis for physiologic fluid samples. A more sensitive and rapid HPLC method has been developed for the quantitative analysis of guanidine in physiological fluids; this has been presented in a separate paper [34]. Creatine, which was eluted between the peaks of guanidinosuccinic acid and guanidinoacetic acid, could not be detected in this analytical system. The entire analysis can be completed in less than 100 min under the conditions described above. The retention times, peak heights and peak areas of equimolar quantities of the guanidino compounds are presented in Table III. The reproducibility of the

TABLE III

RETENTION TIMES, PEAK HEIGHTS AND PEAK AREAS OF A STANDARD MIXTURE OF GUANIDINO COMPOUNDS

Numerals shown in the Table are the mean values (n = 20) obtained with a digital integrator.

Compound	Retention time (min)	Peak height	Peak area
GSA	16.29	385	197086
GAA	25.02	1778	633102
GPA	37.94	632	331372
CRN	48.16	339	214222
GBA	56.82	130	48939
4-GBA	61.66	122	51527
α-A-β-GPA	65.95	1011	477322
ARG	72.76	227	103036
G	91.36	32	7636
MG	94.76	903	581403

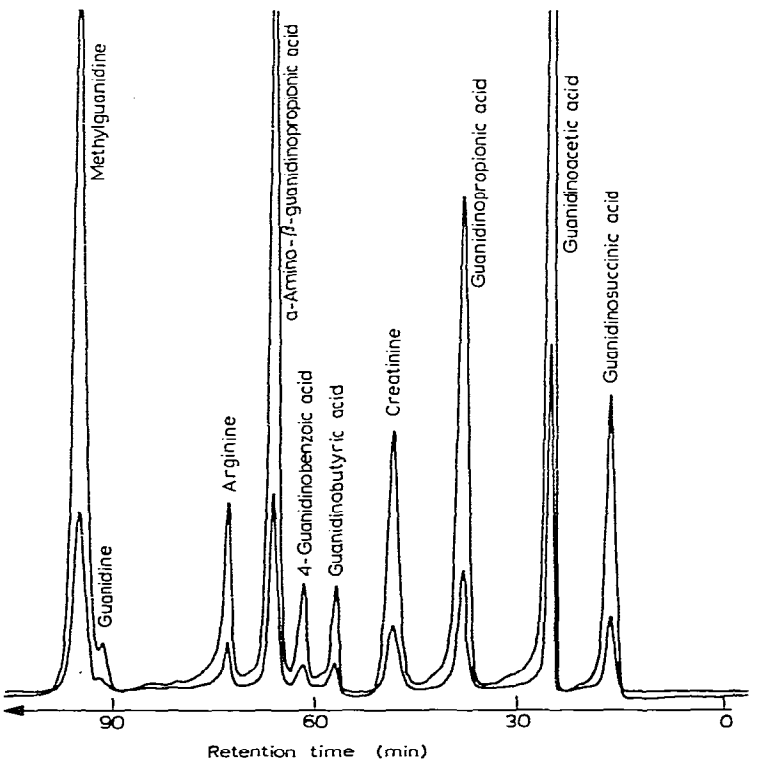

Fig. 4. Chromatogram of a standard mixture of guanidino compounds, each at a concentration of 2.5 nmoles/100 μl (creatinine 25 nmoles/100 μl).

analytical system was evaluated with twenty chromatograms obtained from the standard samples. Retention times of all guanidino compounds showed a relative standard deviation of ≤1.5%. The standard deviations for peak heights and peak areas were within 4.0% and 5.4%, respectively, for all guanidino compounds.

The fluorescence response was linear for all guanidino compounds up to at least 2.5 nmoles. As examples, guanidinosuccinic acid, guanidinoacetic acid, guanidinopropionic acid and methylguanidine are shown in Fig. 5, plotted for amounts of 0.025—2.50 nmoles. The amount of each guanidino compound in an aliquot of physiologic fluid taken for the analysis was generally less than 2 nmoles, except for arginine and creatinine. For arginine and creatinine, the linear range was tested up to 25 nmoles and 100 nmoles, respectively. The linearities of these compounds were obtained using peak areas. The minimum detectable quantity for each guanidino compound is listed in Table IV. The limit of detection was determined by the peak height at twice the noise level.

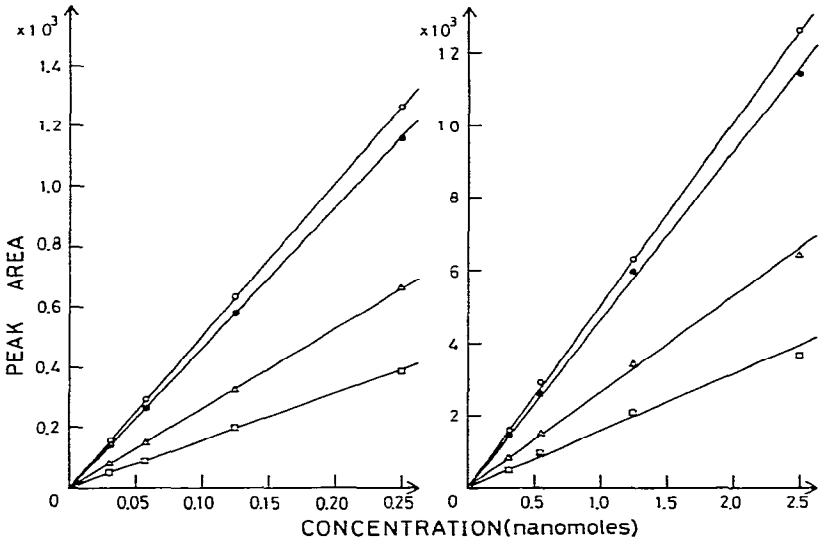

Fig. 5. Calibration curves for some guanidino compounds. GAA (○); MG (●); GPA (△); GSA (□).

TABLE IV

MINIMUM DETECTABLE AMOUNT AND CONCENTRATION OF GUANIDINO COMPOUNDS IN PHYSIOLOGIC FLUIDS

	GSA	GAA	GPA	CRN	GBA	α-A-β-GPA	ARG	G	MG
pmoles	6.5	1.4	3.9	73.0	19.0	2.5	11.0	78.0	2.7
μg/dl	0.11	0.19	0.51	8.26	2.76	0.37	1.92	4.61	0.20

Analysis of physiologic fluid samples

A typical example of a serum sample from a uremic patient is shown in Fig. 6. The labeled peaks were identified from the retention times compared to those of standard mixtures. Recently we obtained an authentic sample of taurocyamine and could identify the first peak (unlabeled) as taurocyamine. A significant peak, which is unidentified, is observed between the peaks of taurocyamine and guanidinosuccinic acid. Smaller unknown peaks also appear at 28 min, 32 min and 63 min. The amounts of the labeled peaks were estimated to be 1.96 nmoles for GSA, 0.38 nmoles for GAA, 0.26 nmoles for GPA, 63.6 nmoles for CRN, 0.41 nmoles for GBA, 0.27 nmoles for α-A-β-GPA, 0.675 nmoles for ARG, 0.31 nmoles for G and 0.68 nmoles for MG. When calculated in terms of serum levels, they represent a concentration of 343.4 μg/dl for GSA, 44.5 μg/dl for GAA, 34.1 μg/dl for GPA 7.20 mg/dl for CRN, 59.5 μg/dl for GBA, 39.5 μg/dl for α-A-β-GPA 1.18 mg/dl for ARG, 18.5 μg/dl for G and 49.7 μg/dl for MG.

Fig. 7 represents a typical example of a serum sample from a person in normal health. The first peak (unlabeled) was identified as taurocyamine by comparison of its retention time. A smaller unknown peak at 28 min, which is present in uremic serum, was also observed in normal serum. The amounts of

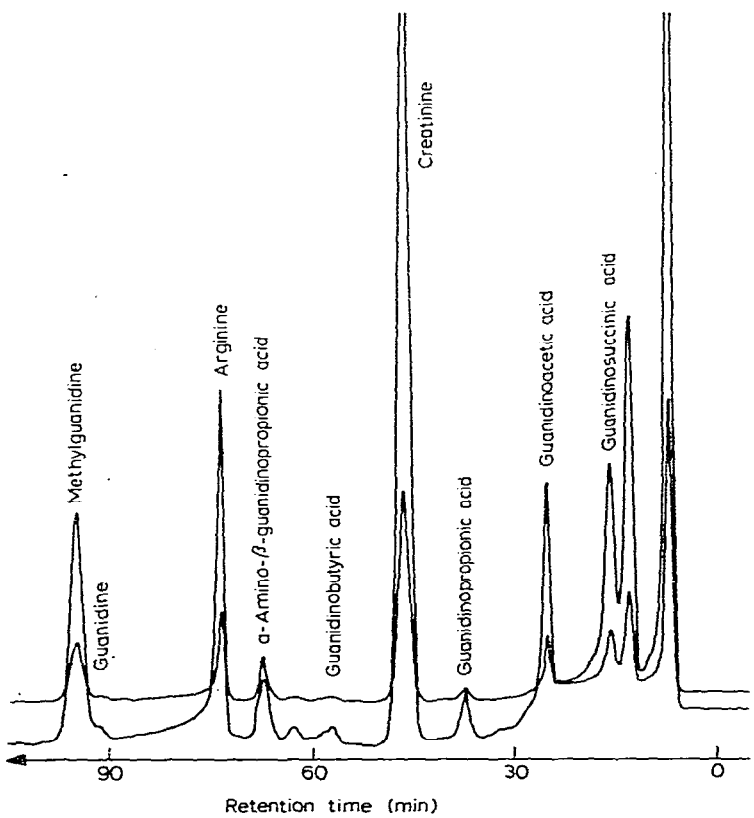

Fig. 6. Analysis of a serum sample from a uremic patient.

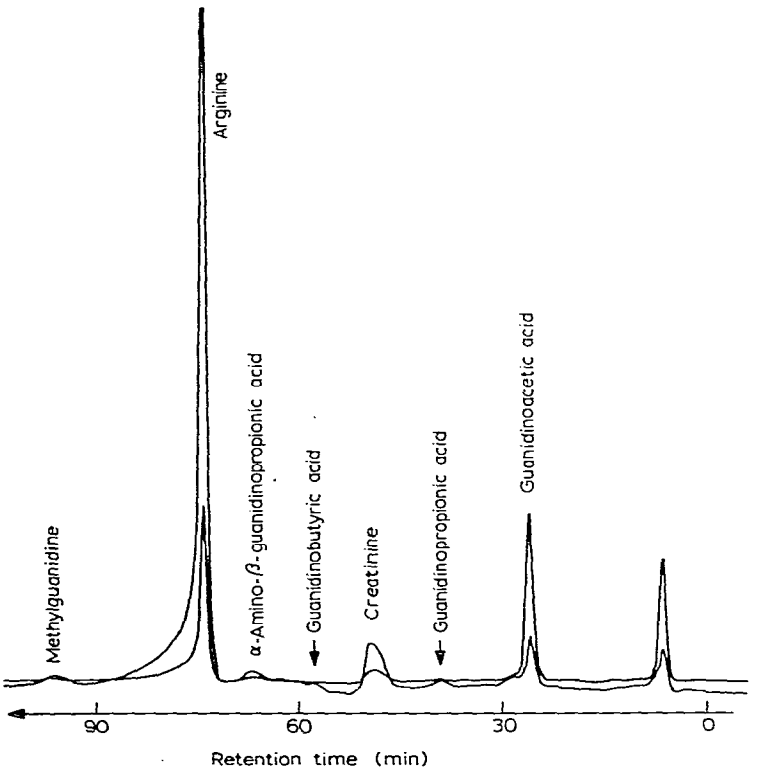

Fig. 7. Analysis of a serum sample from a normal control.

the labeled peaks were determined to be 0.303 mmoles for GAA, 0.035 nmoles for GPA, 6.17 nmoles for CRN, 0.078 nmoles for GBA, 0.030 nmoles for α-A-β-GPA, 9.46 nmoles for ARG and 0.01 nmoles for MG. In terms of serum concentration there are 35.5 μg/dl for GAA, 4.59 μg/dl for GPA, 0.698 mg/dl for CRN, 11.3 μg/dl for GBA, 4.38 μg/dl for α-A-β-GBA, 1.65 mg/dl for ARG and 0.73 μg/dl for MG.

Fig. 8 shows a chromatogram of a cerebrospinal fluid sample from a uremic patient. In the analysis of cerebrospinal fluid, the baseline was irregular because deproteinization treatment of the sample was omitted; when the filtration membrane was used the baseline became stabilized. The first peak at 5.09 min was identified as taurocyamine. An unidentified peak at 10.55 min, which also appeared in the uremic serum sample, was also observed with a significant peak. The quantities of the labeled peaks were estimated as 78.30 μg/dl for GSA, 2.01 μg/dl for GAA, 6.87 mg/dl for CRN, 3.79 mg/dl for ARG and 19.25 μg/dl for MG.

DISCUSSION

All of the analytical techniques used in the past have been directed toward determination of some specific guanidino compounds [1—8] such as guanidinosuccinic acid [3, 6—8] and methylguanidine [1—5]. The determination of

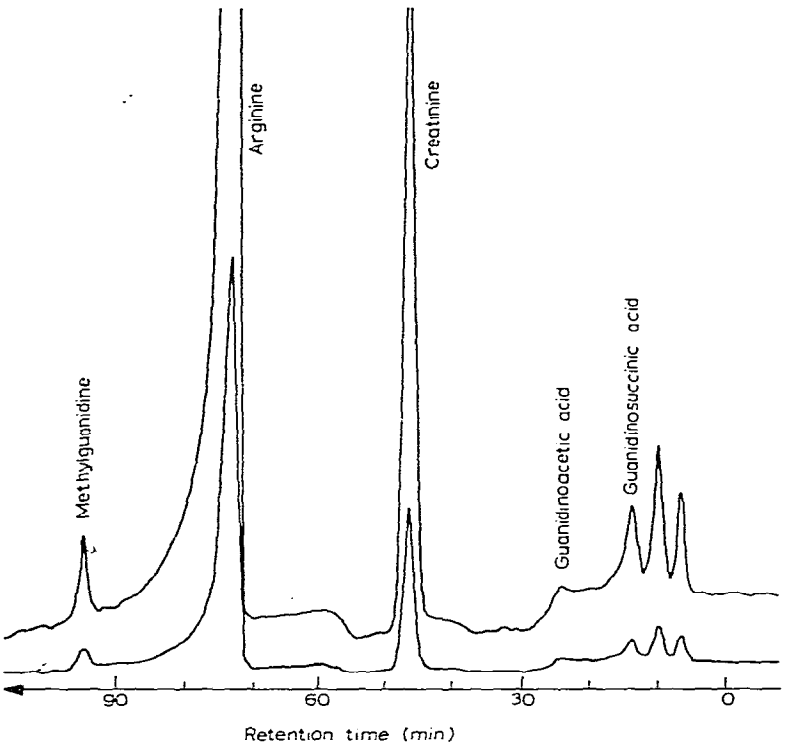

Fig. 8. Analysis of a cerebrospinal fluid sample from a uremic patient.

these guanidino compounds was mainly carried out with cation-exchange column chromatography followed by manual assay using the Sakaguchi reaction. Such methods require a large amount of time and effort, and specialized analysts. The recently described analytical method using a modified automatic amino-acid analyzer [27—30] enabled the separation of most of the guanidino compounds in biologic fluids. The entire analysis requires 4 h or more, which is still time-consuming. In this method, automatic quantitative determination was performed with biacetyl-α-naphthol, a specific reagent for guanidino compounds. The color development time required was 16—20 min [29], which made it difficult to shorten the analysis time. We have developed a high-performance cation-exchange chromatographic method for the analysis of guanidino compounds using 9,10-phenanthrenequinone reagent. Experiments were carried out to determine the optimum separation conditions for shortening the analysis time. A series of guanidino compounds were efficiently separated using a stepwise pH gradient in which a considerably stronger alkaline solution is used for the elution of stronger basic compounds. Furthermore, our efforts were focused on methods for reducing the volume of the reaction system and the fluorescence conversion time to a minimum. The present study has shown that fluorescence conversion is achieved in 2 min when the reaction is carried out at temperature above 50°. Shortening the reaction time made it

possible to give a baseline separation and to complete the analysis in less than 100 min.

The conventional analytical method based on the colorimetric assay requires a large amount of sample (5—20 ml) and concentration of the samples [4]. A recent analytical method using an amino-acid analyzer also requires concentration techniques such as lyophilization prior to introduction into the chromatographic column [29]. Nevertheless, some of the guanidino compounds containing methylguanidine were not quantitatively determined, even for the uremic serum [29, 30], because of the low sensitivity of the colorimetric method used. The limiting factor in sensitivity for the conventional analytical method for guanidino compounds is the reagent used for detection. 9,10-Phenanthrenequinone has been reported to form a highly fluorescent product with mono-substituted guanidines and has been demonstrated to be more sensitive than the Sakaguchi reaction for the detection of arginine [31]. We adopted the 9,10-phenanthrenequinone reagent for the determination of the guanidino compounds eluted from the chromatographic column. The fluorometric detection method used in our analytical system is found to be two orders of magnitude more sensitive than the conventional colorimetric detection method. Therefore, no such concentration treatment as lyophilization or evaporation is required for our analytical method. As little as 0.3 ml of physiologic fluid sample is sufficient for the analysis, since our analytical method requires a net volume of only 0.1 ml of sample. In the analysis of serum, as little as 1 ml of serum is deproteinized with the filtration membrane and then approximately 0.3 ml of the filtrate is loaded onto the analytical system. With our analytical method, all of the physiologic fluid guanidino compounds were quantitatively determined with ease in serum and cerebrospinal fluid samples from normal subjects as well as from uremic patients. The sensitivity of the method was sufficiently high for routine clinical laboratory use. Furthermore, the sensitivity of the method has proved advantageous where only samples of limited volume are available, for example cerebrospinal fluid or sera of small animals. An instrument based on the present report is now available from Japan Spectroscopic Co.

A large number of serum and cerebrospinal fluid samples from normal subjects and uremic patients are currently being analyzed and the results obtained with our analytical method will be the subject of a later report.

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