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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSER FOR GUANIDINO COMPOUNDS USING BENZOIN AS A FLUOROGENIC REAGENT

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SUMMARY

An automatic analyser based on high-performance liquid chromatography has been developed for the quantification of biogenic guanidino compounds in human physiological fluids. Fourteen guanidino compounds are mutually separated within 35 min on a cation-exchange column with a stepwise gradient elution of pH and/or ionic strength in the mobile phase and then converted automatically to their fluorescent derivatives with benzoin. The method in this system is simple, rapid and sensitive; the lower limits of detection are 5—50 pmol for monosubstituted guanidino compounds, 1 nmol for creatine and 20 nmol for creatinine in 100 μl of injection volume.

INTRODUCTION

Guanidino compounds such as methylguanidine and guanidinosuccinic acid have been implicated as uremic toxins in uremic syndrome because these compounds accumulate in body fluids of uremic patients [1—3] and give rise to a symptom complex similar to uremia [4, 5].

Among the current analytical methods for guanidino compounds, ion-exchange chromatography coupled with automatic colorimetric or fluorimetric detection, including high-performance liquid chromatography (HPLC), is the most popular because of its simplicity in operation, though other chromatographic methods based on paper [6, 7], thin-layer [8—10] and gas chromatography [11—13] have been reported. However, colorimetric detection of the compounds by means of the Sakaguchi or Voges-Proskauer reaction in ion-exchange chromatography has a limited sensitivity and thus necessitates a large amount of sample [14—16]. On the other hand, fluorimetric detection based

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on post-column derivatization after HPLC, using ninhydrin or 9,10-phenanthraquinone as fluorogenic reagent can offer a method sensitive enough to measure the compounds at the picomole level [17—19].

Recently we have developed a manual fluorimetric method for the selective determination of guanidino compounds using benzoin [20, 21]. This reagent is a non-fluorescent material, but reacts with the guanidino moiety of the compounds in an alkaline medium and gives highly fluorescent derivatives — 2-substituted amino-4,5-diphenylimidazoles [22]. Therefore this reaction is applicable to the fluorimetric detection of guanidino compounds as both pre- and post-column derivatives in HPLC. The application of the reaction to pre-column derivatization of the compounds in HPLC has been described in a separate paper [23]; this method is most sensitive and useful for the determination of guanidino compounds at the femtomole level; however, the limited separation of the fluorescent derivatives did not permit the assay of biogenic guanidino compounds without a clean-up procedure for removal of oligopeptides in biological samples.

In this paper we describe the HPLC conditions for the rapid simultaneous separation of guanidino compounds on a cation-exchange column and the application of the benzoin reaction to the post-column fluorescence derivatization of the compounds in order to assemble an automatic analyser for routine assay of biogenic guanidino compounds in human urine and serum from normal subjects or uremic patients. The following fourteen guanidino compounds were used as representative compounds for the investigations; taurocyamine, TC; guanidinosuccinic acid, GSA; creatine, CR; guanidinoacetic acid, GAA; N^{α}-acetylarginine, AcARG; argininic acid, ARA; guanidinopropionic acid, GPA; creatinine, CRN; guanidinobutyric acid, GBA; arginine, ARG; phenylguanidine, PG; guanidine, G; methylguanidine, MG; agmatine, AGM.

EXPERIMENTAL

Chemicals and solutions

All chemicals were of analytical reagent grade, unless otherwise noted. Distilled water was used. Benzoin (Wako, Osaka, Japan) was recrystallized from absolute methanol. Tris(hydroxymethyl)aminomethane (Tris) (Wako, Osaka, Japan) was recrystallised from 60% (v/v) aqueous methanol to remove fluorescent impurities. Standard solutions of the guanidino compounds were prepared in 0.05 *M* hydrochloric acid.

Mobile phase for HPLC. Three aqueous eluents are required for separation of the guanidino compounds. Buffer A (pH 3.5): dissolve 5.25 g of trisodium citrate dihydrate, 2.7 g of sodium chloride, 8.1 g of citric acid and 7.2 mg of sodium pentachlorophenol as a preservative in approximately 400 ml of water and dilute with water to 500 ml (the final concentration of each component is 36, 92, 77 and 0.05 m*M*, respectively). Buffer B (pH 5.0): dissolve 13.35 g of trisodium citrate dihydrate, 20 g of sodium chloride, 3.05 g of citric acid and 7.2 mg of sodium pentachlorophenol in approximately 400 ml of water, and dilute with water to 500 ml (the final concentration of each component is 91, 684, 29 and 0.05 m*M*, respectively). Potassium hydroxide solution (1.0 *M*):

dissolve 28.05 g of potassium hydroxide in about 400 ml of water and dilute with water to 500 ml. The solutions were thoroughly degassed in the usual manner before use.

Reagents for post-column derivatization. Benzoin solution (4.0 m*M*): dissolve 0.425 g of benzoin in 500 ml of a mixture of methylcellosolve and water (6:4, v/v). Potassium hydroxide solution (4.0 *M*): dissolve 122.2 g of potassium hydroxide in about 400 ml of water and dilute with water to 500 ml. Sodium dihydrogen phosphate (1.6 *M*)—Tris (1.0 *M*) mixture: dissolve 110.5 g of sodium dihydrogen phosphate and 60.55 g of Tris in about 400 ml of water and dilute with water to 500 ml. The solutions were degassed before use.

Chromatographic system and its operation

Fig. 1 is a schematic diagram of the HPLC analyser constructed for analysis of the guanidino compounds.

A cation-exchange column (80 mm × 4 mm I.D.; packing material Hitachi 2619 resin; particle size 5 μm) was used in the HPLC with a stepwise gradient elution of pH and/or ion strength by using buffers A and B, and 1.0 *M* potassium hydroxide. The temperature of the column with a water-jacket was kept at 80°C by an Hitachi constant-temperature circulator. The mobile phase (buffers A and B, and 1.0 *M* potassium hydroxide) was pumped at a flow-rate of 0.5 ml/min by an Hitachi 638-30 high-performance liquid chromatograph which had a programming controller of the electronic valves placed prior to

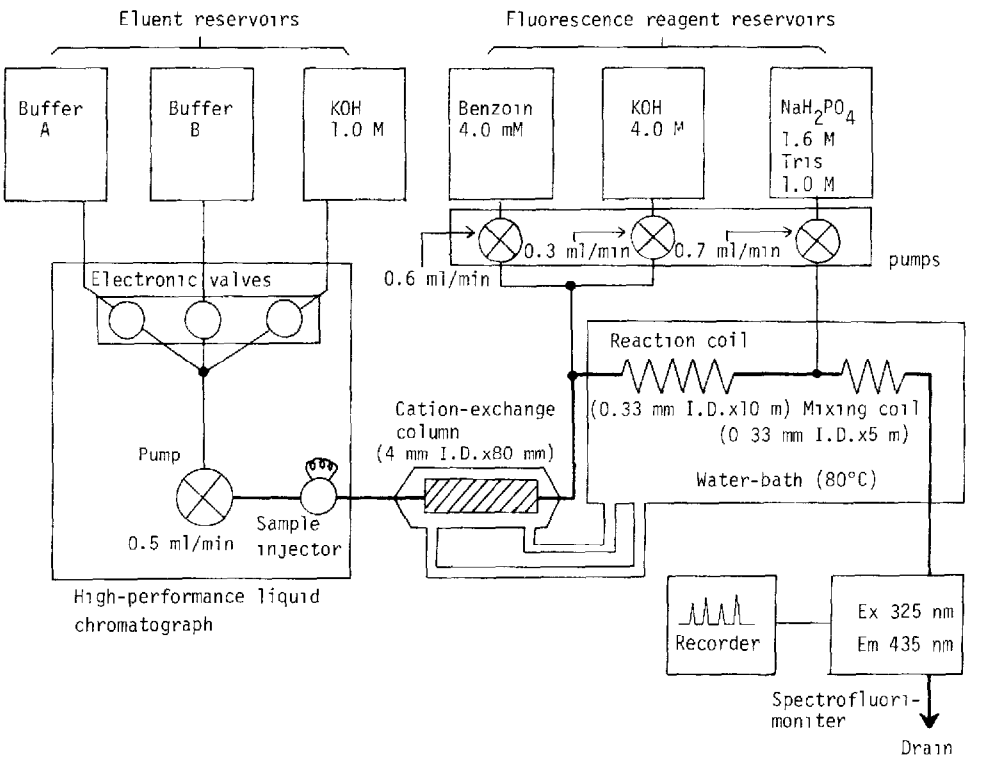

Fig. 1. Schematic diagram of HPLC analyser for guanidino compounds.

the pump inlet for various gradient elutions. Buffer A was first run into the column for 2 min, then a mixture of buffers A and B (1:1, v/v) for 2 min, then buffer B for 11 min and 1.0 *M* potassium hydroxide for 10 min to separate the guanidino compounds; then successively the column was equilibrated with buffer A for 20 min before the start of the next sample (Fig. 2). The above change of eluents was automatically controlled with the electronic programmer of the chromatograph.

The eluate from the column was conducted to the fluorescence reactor system through a PTFE tube (20 cm × 0.33 mm I.D.). All the coils in the reaction system are made of PTFE. Benzoin (4.0 m*M*) and potassium hydroxide (4.0 *M*) solutions were first added to the eluate stream at a tee-connector by an Hitachi reagent-delivery pump for amino acid analyser and an Hitachi 663-C chemical pump at flow-rates of 0.6 and 0.3 ml/min, respectively, and then the mixture was passed through a reaction coil (10 m × 0.33 mm I.D.) immersed in an 80°C water-bath. After the fluorescence reaction, sodium dihydrogen phosphate (1.6 *M*)—Tris (1.0 *M*) mixture was added to the reaction mixture at a flow-rate of 0.7 ml/min by an Hitachi reagent-delivery pump for amino acid analyser and the mixture was passed through a mixing coil (5 m × 0.33 mm I.D.). The fluorescence intensity from each guanidino compound in the last eluate was monitored at 435 nm emission against 325 nm excitation (both slit-widths 5 nm) by an Hitachi 650-10LC spectrofluorimeter equipped with a flow cell (18 μl) and a xenon lamp.

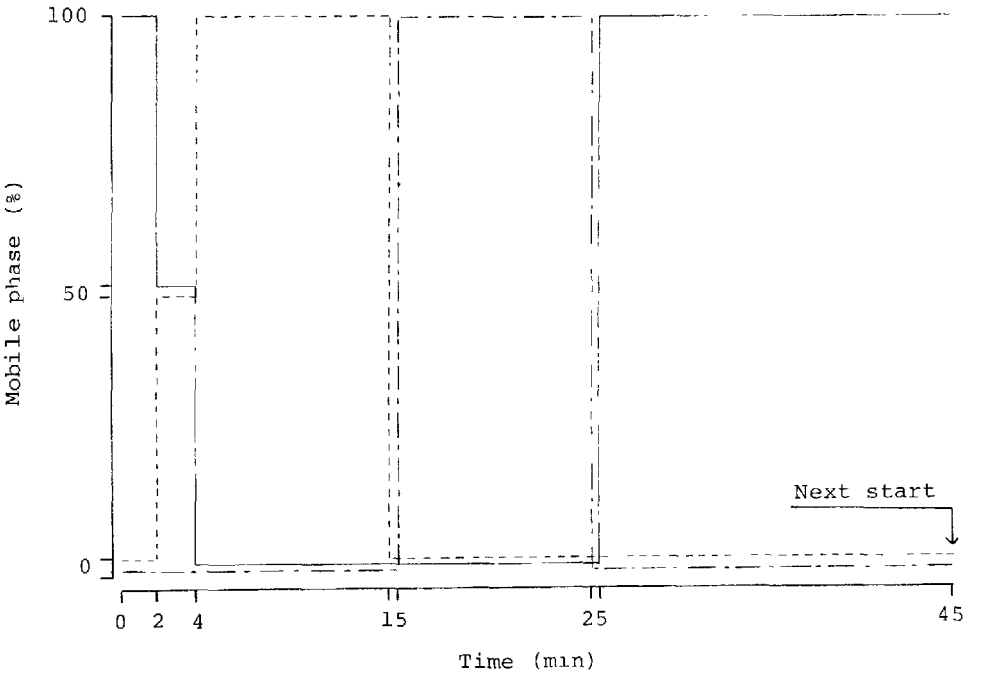

Fig. 2. Elution mode of the stepwise gradient. (——), buffer A; (- · -), buffer B; (- — -), potassium hydroxide.

Preparation of physiological fluids

Urine and serum specimens were obtained from healthy volunteers in our laboratory and from patients with chronic renal failure being hemodialysed at Japan Red Cross Fukuoka Hospital (Fukuoka, Japan).

Urine samples. To 100 μl of urine centrifuged at approximately 1000 *g* for 5 min, were added 800 μl of 7.5 nmol/ml phenylguanidine. An aliquot (100 μl) of the mixture was applied to the HPLC analyser.

Serum samples. A 100-μl aliquot of serum was mixed with 50 μl of 1.8 *M* perchloric acid and 25 μl of 25 nmol/ml phenylguanidine. The mixture was centrifuged at 1500 *g* for 10 min. A 100-μl aliquot of supernatant was neutralized by adding 50 μl of 0.6 *M* potassium carbonate and then the potassium perchlorate formed was removed to avoid the precipitation of the salt in the lines of the HPLC analyser. The pH of the resultant supernatant was adjusted to approximately 1.7 with about 20 μl of 0.7 *M* hydrochloric acid. An aliquot (100 μl) of the final mixture was applied to the HPLC analyser.

RESULTS AND DISCUSSION

Separation of guanidino compounds

The conventional separation of guanidino compounds has been performed by chromatography using a strong cation-exchange resin, based on the separation technique for basic amino acids [24]. A resin of this type was used in our investigations. The resin particles were small and arranged to be of the same diameter (5 μm) since well-regulated small particles of resin provide an

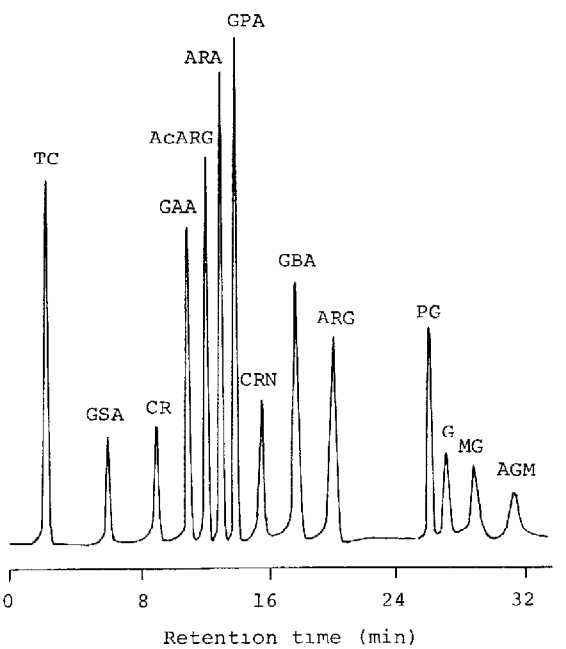

Fig. 3. Chromatogram of a standard mixture of guanidino compounds, each at the amount of 800 pmol, subjected to HPLC (CR, 40 nmol; CRN, 0.8 μmol). For the separation and detection conditions of the HPLC analyser, see text.

increased theoretical plate number [25]. Thus the resin packed in a short column (80 mm × 4 mm I.D.) gave a high resolution of the guanidino compounds in HPLC. Fig. 3 shows a chromatogram of a standard mixture of fourteen guanidino compounds obtained with the HPLC analyser. Complete separation of the compounds on the column is achieved within 35 min in a single run with a stepwise gradient elution (the operational procedure is described in Experimental).

The guanidino compounds tested, except for the strongly basic compounds such as PG, G, MG and AGM, were retained on the column and resolved using a sodium citrate buffer (0.1 *M*, pH 4.0) with isocratic elution; their retention times were affected by the pH and ionic strength in the buffer. The ionic strength was controlled by the addition of sodium chloride to the buffer. The rise of pH and/or ionic strength in the buffer resulted in an early elution of the guanidino compounds. On the other hand, G, MG, PG and AGM were strongly retained on the column and not eluted with the acidic buffer. However, with an alkaline solution such as 1.0 *M* potassium hydroxide, they were resolved with reasonable retention times. From the above preliminary studies on the separation of the guanidino compounds, the combination of three eluents — buffer A (pH 3.5), buffer B (pH 5.0) and 1.0 *M* potassium hydroxide — as mobile phase was employed for HPLC (the constituent of each eluent and the elution mode for the stepwise gradient are described in Experimental). Buffer A is mainly used for the separation of TC and GSA, buffer B for CR, GAA, AcARG, ARA, GPA, CRN, GBA and ARG, and 1.0 *M* potassium hydroxide for PG, G, MG and AGM.

The retention times of the guanidino compounds are also influenced by the column temperature. Increased temperature facilitates early elution of the guanidino compounds, especially late-eluting compounds such as G, MG and AGM, without deterioration of resolution and peak shape. For example, when the column was operated at ambient temperature (23°C), MG eluted late at about 44 min, but at 80°C this compound was eluted at 29.2 min. Consequently the column was operated at 80°C not only to shorten the analytical time but also to obtain a definite retention time of each guanidino compound. Good durability of the column was also observed; the column can be used for more than 2000 analyses.

Fluorescence reactor and detection

In the manual method [21], the fluorescence reaction of the guanidino compounds with benzoin needs a strongly alkaline medium and heating conditions to minimize the reaction time; also the fluorescent derivatives produced fluoresce most intensely in a weakly alkaline solution (pH 8.5—10.5). Thus the eluate from the column was first mixed with benzoin and potassium hydroxide, and then the mixture was heated at 80°C through a reaction coil for approximately 45 sec and made weakly alkaline by adding a mixture of sodium dihydrogen phosphate and Tris. The excitation and emission maxima of the fluorescence from all the guanidino compounds tested, obtained with this detection system, were around 325 and 435 nm, respectively. These data agreed with those obtained with the manual method previously described [21].

Reaction conditions of the post-column fluorescence derivatization were

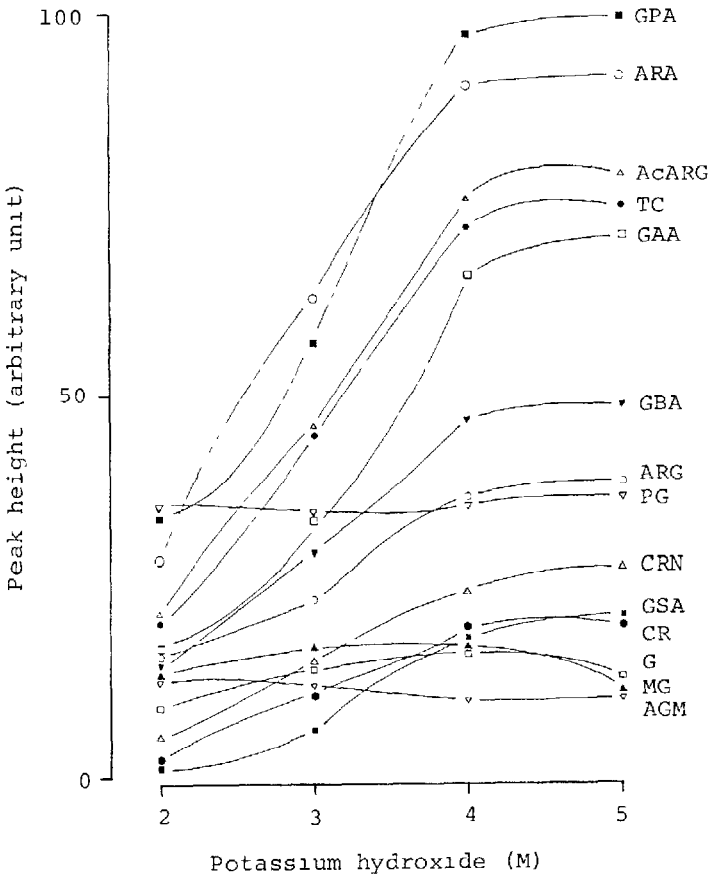

Fig. 4. Effect of potassium hydroxide concentration on the fluorescence development of guanidino compounds. The separation conditions and the amounts of the guanidino compounds are the same as in Fig. 3

examined to construct the fluorescence reactor system. A potassium hydroxide solution in the concentration range 4.0—5.0 *M* is required to obtain a maximum fluorescence intensity, corresponding to the peak height of each guanidino compound, as shown in Fig. 4; 4.0 *M* potassium hydroxide was used in the reactor system. The benzoin concentration also influences development of the fluorescence from each guanidino compound. With increasing concentration of benzoin in the range 2.0—8.0 m*M*, the fluorescence intensities of the compounds other than CR and CRN decrease slightly, but the intensities of both CR and CRN increase (Fig. 5). In the system, 4.0 m*M* benzoin was selected to obtain fairly large fluorescence intensities from the guanidino compounds except for CR and CRN, because concentrations of CR and CRN in human urine and serum are much higher than those of the other guanidino compounds. An elevated reaction temperature is required for development of the fluorescence from all the guanidino compounds tested, as shown in Fig. 6. When the reaction temperature was higher than 80°C, an irregular baseline frequently occurred on the chromatogram; this may be caused by air bubbles generated in the reaction coil. Thus a compromise temperature of 80°C was

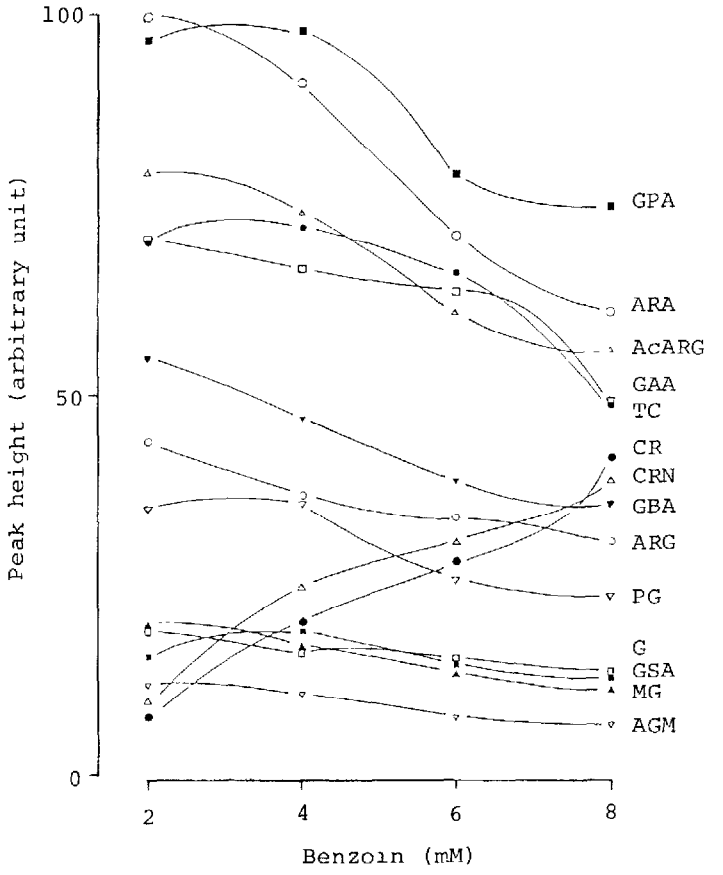

Fig. 5. Effect of benzoin concentration on the fluorescence development of guanidino compounds. The separation conditions and the amounts of the guanidino compounds are the same as in Fig 3.

used for the reaction in the reactor system, and this temperature was maintained by using the same water-bath as that used for the column. In order to attain intense fluorescence of the benzoin derivatives, it was necessary to adjust the pH of the mixture after the benzoin reaction to a weakly alkaline pH by adding a pertinent acidic salt. When a mixture of sodium dihydrogen phosphate (1.6 *M*) and Tris (1.0 *M*) was added to the reaction mixture, the pH of the final eluate was sufficiently lowered and ranged from 8.5 to 10.7 during the operation of the HPLC analyser, even with stepwise gradient elution of the pH in the mobile phase. At these pH values the derivatives of all the guanidino compounds tested fluoresce at least five times as strongly as those without adjustment of the pH.

A linear relationship was observed between the peak height and the amount of each guanidino compound in the injection volume (100 μl) up to at least 8 nmol for monosubstituted guanidino compounds, 100 nmol for CR and 8 μmol for CRN under the established conditions of the fluorescence reactor system. The lower limits of detection are 5 pmol for ARA and GPA, 8 pmol for TC, GAA and AcARG, 12 pmol for GBA, ARG and PG, 30 pmol for GSA,

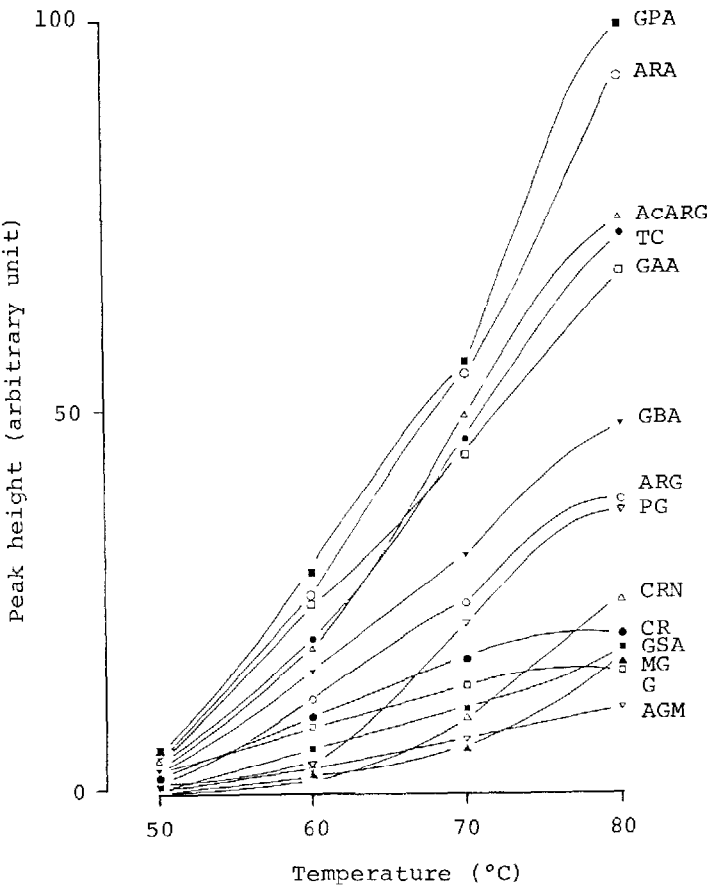

Fig. 6. Effect of reaction temperature on the fluorescence development of guanidino compounds. The separation conditions and the amounts of the guanidino compounds are the same as in Fig. 3.

G and MG, 50 pmol for AGM, 1 nmol for CR, and 20 nmol for CRN. The limit is defined as the amount in 100 μl of injection volume giving a peak height of twice the noise level.

Analysis of biogenic guanidino compounds in human urine and serum

Typical chromatograms obtained by the HPLC analyser are shown in Fig. 7 for a normal urine and in Fig. 8 for serum from a normal subject and from a patient with chronic renal failure. The guanidino compounds in the samples were identified on the basis of their retention times in comparison with standard compounds and also by co-chromatography of the standards and samples with different elution of the mobile phase, i.e. using a lower pH and/or ionic strength than those used for the recommended procedure. This elution provided better separation of the guanidino compounds but their elution was delayed. In addition, their retention times were not affected by the biological matrix of the samples. Unidentified peaks were also observed in the chromatograms, though the benzoin reaction works only on compounds with a guanidino moiety [20, 21]. The peaks are probably from native fluorescent

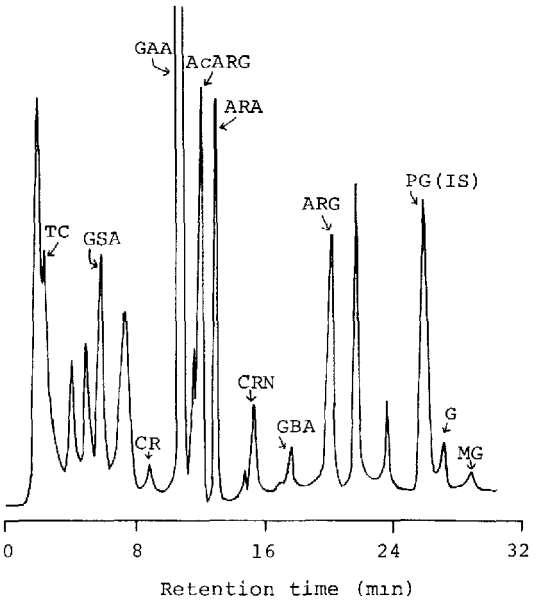

Fig. 7. Chromatogram of guanidino compounds in urine from a healthy man. Phenyl guanidine (6 nmol) was spiked in 100 μl of urine as an internal standard and the sample was treated as described in Experimental.

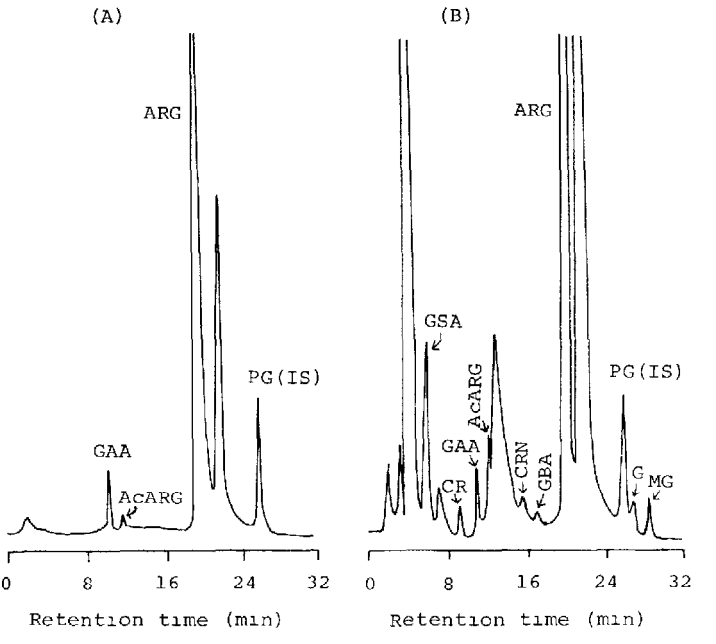

Fig. 8. Chromatograms of guanidino compounds in sera from (A) a healthy man, and (B) a uremic patient. Phenylguanidine (625 pmol) was spiked in 100 μl of each serum and the samples were treated as described in Experimental.

substances (including drugs administered to the patient during therapy), and/or unknown guanidino compounds and/or peptides with an arginyl residue which were not removed by deproteinization. Oligopeptides with one or two arginyl residues such as tuftsin, angiotensins I, II and III, kallidin, bradykinin, luteinizing-hormone releasing hormone, substance P and neurotensin, were examined for their retention times in HPLC. These peptides were co-eluted at a retention time of approximately 22 min next to the elution of ARG. Therefore, in the chromatogram of the patient serum, one of the unkown big peaks around 22 min may be ascribed to some peptides characteristic of uremia since several peptides such as middle molecule substances also have been suspected of contributing to the toxic manifestation in uremic syndrome [26, 27]. Other unidentified peaks in the chromatograms were not studied.

For a precise and facile quantification of the guanidino compounds, phenylguanidine was used as an internal standard. The calibration curves for both urine and serum, which were made by plotting the ratios of net peak heights of the spiked guanidino compounds against the peak height of the internal standard, were linear in the relationship between the ratios and amounts of the guanidino compounds added to urine or serum. The calibration curves are

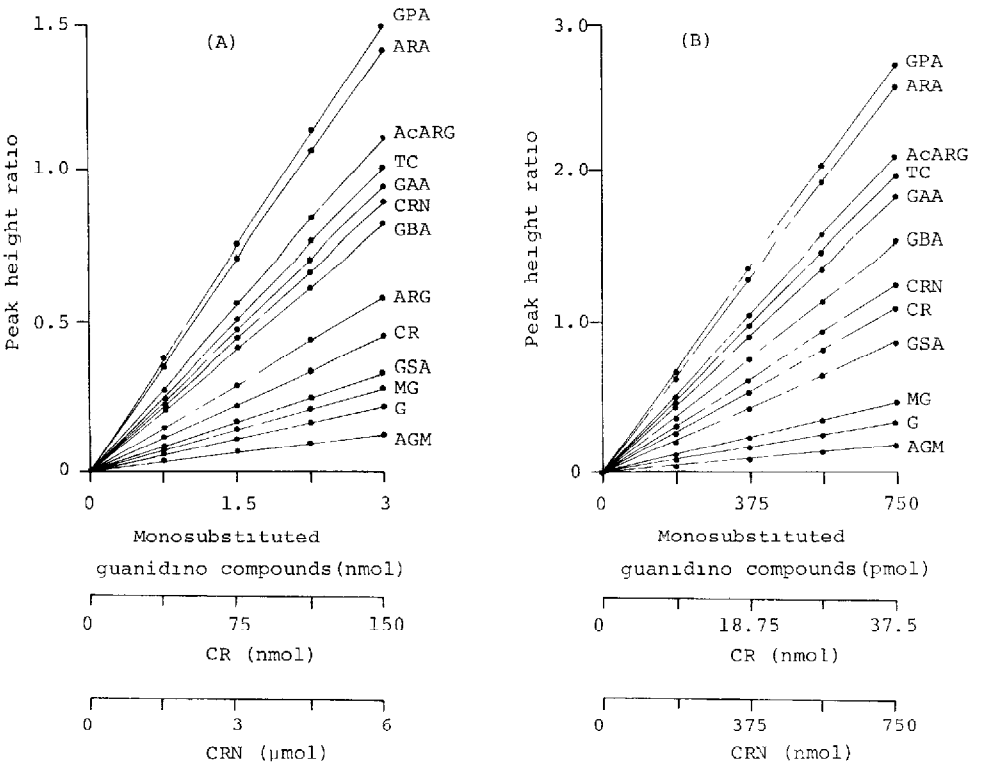

Fig. 9. Calibration curves for guanidino compounds in (A) urine, and (B) serum. (A) To 100-μl portions of a pooled urine, 0—3 nmol of the monosubstituted guanidino compounds, 0—150 nmol of CR, 0—6 μmol of CRN and 6 nmol of phenylguanidine (IS) were added. (B) To 100-μl portions of a pooled serum, 0—750 pmol of the monosubstituted guanidino compounds, 0—37.5 nmol of CR, 0—750 nmol of CRN and 625 pmol of phenylguanidine (IS) were added. The curve for ARG was not constructed because the peak of ARG in serum was too high compared with that of the internal standard.

TABLE I

CONCENTRATIONS OF GUANIDINO COMPOUNDS* IN URINE FROM HEALTHY MEN

Age	Guanidino compound (μmol/day)										
	TC	GSA	CR	GAA	AcARG	ARA	CRN	GBA	ARG	G	MG
26	11 34	24 94	N D.**	323 00	17.51	8 50	11333	7 36	13.04	11.34	N.D
25	6 06	28 34	250 00	516.25	15 00	15 84	15834	17 50	30 84	18 34	10 00
23	N D	29.41	N D	340 00	25 50	7 94	17000	14 74	17 00	18 14	6 80
23	16 15	22 80	N.D	544.67	10.45	6 65	13300	8 55	17 10	15 20	5 70
24	N D	22.40	210 00	583.00	26.60	12 60	13066	7 94	27 02	15 82	6.54
28	N D.	16 44	316.60	437.00	11 40	5 39	8866	4 75	16 15	8 24	3 80
34	N.D.	42 54	513 34	366.63	21 26	23 46	24934	5 14	28.60	16 14	8 80
28	N.D.	38 00	570 00	709.30	25 33	11 40	17732	6 33	30.40	17 73	7 60
29	N.D	9 37	N D	405 30	3 87	8 70	11600	5 80	7.73	17 40	9.67
Mean	11 38	26 06	371 98	477 48	17 42	11.16	14851	8 67	20.87	15.37	7 36
± S D	+4 75	+10 10	±160.76	±135 00	±7.90	±5.61	+4742	±4.45	±8 46	±3 42	±2 09

*Other guanidino compounds could not be successfully determined because of their minute amounts
**N D = not detected.

TABLE II

CONCENTRATIONS OF GUANIDINO COMPOUNDS* IN SERA FROM HEALTHY MEN

Age	Guanidino compounds (nmol/ml)				
	CR	GAA	AcARG	G	MG
26	11.22	1.61	0.92	N.D.**	N.D.
29	23.21	2.18	1.02	N.D.	N.D.
34	18 03	2.45	0.80	N.D.	N.D.
22	32.86	2.50	0.92	N.D.	0.46
24	16 43	3.24	1.14	N.D.	N D.
25	8.19	3.17	0.72	0.69	0.46
24	19.18	2.73	0.98	N.D.	0.38
26	9.63	2.51	0.79	0.31	0.77
Mean	17.34	2.55	0.91	0.50	0.52
±S.D.	±8 11	±0.52	±0.14	±0 27	±0.17

*Other guanidino compounds could not be successfully determined because of their minute amounts, except for ARG.
**N.D. = not detected

shown in Fig. 9. The correlation coefficients (r) of all the curves were more than 0.997 and no change of the slopes in the graphs was observed depending on the urine or serum used.

The recovery of each guanidino compound added to 100 μl of urine in amounts of 3—6 nmol for monosubstituted guanidino compounds, 300 nmol for CR, and 6 μmol for CRN was in the range 95—105% (C.V. 6.2%); when the compounds were added to 100 μl of serum in the amounts of 0.75 nmol for monosubstituted guanidino compounds, 37.5 nmol for CR and 750 nmol for CRN, the recoveries were in the range 97—104% (C.V. 6.3%). The values are the average of ten independent analyses.

TABLE III

CONCENTRATIONS OF GUANIDINO COMPOUNDS* IN SERA FROM PATIENTS WITH CHRONIC RENAL FAILURE

	Guanidino compounds (nmol/ml)							
	GSA	CR	GAA	AcARG	CRN	GBA	G	MG
1	4.64	40.50	3.05	2.56	325.87	0.19	2.25	2.82
2	13.90	31.25	2.07	1 71	506.75	N.D.**	2.25	5.19
3	22.21	53.47	1.92	1.65	451.34	N D	2.46	6.66
4	6.95	121.42	1.97	1.57	377.45	N.D.	1.97	3.03
5	15.53	80.21	1.60	1.75	599.00	N.D.	2.66	7.21
6	4.13	104.25	1.06	2.27	585.03	N.D.	2.41	2.89
7	25.20	182.03	2.12	N.D.	677.22	N.D.	2.62	3.06
8	3.39	64.21	2.18	0.57	200.00	0.40	2.07	2.18
9	25.51	64.67	2.39	1.80	646.67	0.30	3.03	5.17
10	20.05	86.02	2.58	2.34	860.21	0.41	2.21	3.69
Mean	14.15	82.80	2.09	1.80	522.95	0.33	2.39	4.19
±S.D.	±8.89	±44.46	±0.54	±0.58	±192.12	±0.10	±0.31	±1.75

*Other guanidino compounds could not be successfully determined because of their minute amounts, except for ARG.
**N.D. = not detected.

The precision of the method for the determination of the biogenic guanidino compounds was also examined by performing five analyses separately on pooled urine and serum. The coefficients of variation for the asay of the compounds [the compound and concentration (nmol/ml) in parentheses] in urine were 5.3% (GSA, 15.6), 6.0% (CR, 451.0), 8.1% (GAA, 312.0), 3.1% (AcARG, 10.9), 4.6% (ARA, 6.6), 9.1% (CRN, 6393.2), 1.1% (GBA, 5.4), 3.1% (ARG, 18.5), 3.1% (G, 10.2) and 3.5% (MG, 3.1), and for the assay of the compounds in serum were 3.9% (GSA, 7.0), 5.8% (CR, 121.4), 8.4% (GAA, 2.0), 9.6% (AcARG, 1.6), 3.2% (CRN, 377.5), 3.6% (G, 2.0) and 3.8% (MG, 3.0).

The concentrations of the guanidino compounds in urine and serum samples from healthy men, and sera from patients with chronic renal failure in maintenance with hemodialysis, were determined by this method (Tables I—III). Relatively many guanidino compounds were identified in the urine compared with those in sera from healthy men. Higher concentrations of GSA, CR, CRN, G and MG than those in the normal sera were observed in the patient sera. GAA and AcARG were present in the normal sera but at levels not significantly different from those in the patient sera. The mean values for the individual guanidino compounds are in good agreement with published data [17, 19].

The present method for the automatic determination of guanidino compounds is rapid and gives satisfactory sensitivity in the analysis of physiological fluids; the sensitivity permits use of less than 100 μl of urine and serum from normal subjects or uremic patients, and this HPLC analyser will be an invaluable tool in clinical studies of guanidino compounds in uremic syndrome.

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