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POST-COLUMN DERIVATIZATION SYSTEM FOR THE FLUORIMETRIC DETERMINATION OF GUANIDINO COMPOUNDS WITH NINHYDRIN BY REVERSED-PHASE ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

YOSHIE KOBAYASHI*, HIROAKI KUBO and TOSHIO KINOSHITA

School of Pharmaceutical Sciences, Kitasato University, 5-9-1, Shirokane, Minato-ku, Tokyo 108 (Japan)

SUMMARY

A post-column derivatization system for the fluorimetric determination of guanidino compounds by high-performance liquid chromatography was developed. A mobile phase containing ninhydrin was used as the fluorigenic reagent. Ten guanidino compounds were separated within 25 min on a Nucleosil C_8 (5 μm) column (15 cm × 4.6 mm I.D.) by isocratic, reversed-phase ion-pair chromatography and detected as fluorophors derived from condensation with ninhydrin in an alkaline stream. In this simplified system only two pumps required to deliver the mobile phase and the alkaline solution. This method was applied to serum from patients on haemodialysis therapy.

INTRODUCTION

Guanidino compounds have been demonstrated to be accumulated in the biological fluids of hyperargininaemic[1] and uraemic[2,3] patients, and their identification and determination are therefore necessary.

In the past, various methods, including classical column chromatography, thin-layer chromatography, paper chromatography and gas chromatography, and also spectrophotometric and fluorimetric procedures, were reported for the determination of guanidino compounds in biological fluids. The disadvantages of these methods are mainly the requirement for large sample volumes and the lack of detection sensitivity.

More recently, sensitive analytical methods based on high-performance liquid chromatography (HPLC) with 9,10-phenanthraquinone[4–8], ninhydrin[9–11] or benzoin[12–14] as fluorigenic reagents have been reported for the determination of guanidino compounds. Most of these methods require gradient elution for the separation of guanidino compounds and either pre- or post-column derivatization for fluorimetric detection. Pre-column derivatization requires time-consuming sample pretreatment and post-column derivatization requires three (or more) pumps to deliver the mobile phase, fluorigenic reagent and an alkaline solution. Hence these systems are very complicated.

This paper describes a post-column derivatization system in which the mobile phase contains ninhydrin as the fluorigenic reagent, and the separation of ten guanidino compounds by isocratic reversed-phase ion-pair HPLC.

EXPERIMENTAL

Apparatus

The chromatographic system was assembled from a Model 6000A solvent delivery pump, a Model U6K injector (Waters Assoc., Milford, MA, USA) and a Soma S-3800 reaction system, equipped with a mixing tee, a reciprocating pump, a pulse-damping device and a reaction coil, consisting of a stainless-steel tube (5 m × 0.5 mm I.D.) in a heating bath (Soma Optics, Tokyo, Japan). The analytical column was a Nucleosil C_8 (5 μm) column (15 cm × 4.6 mm I.D.) (Macherey, Nagel & Co., Düren, F.R.G.). A Hitachi F-1000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) was used to detect the fluorophor with an excitation wavelength of 395 nm and an emission wavelength of 500 nm. The detection signal was recorded with a recorder (National Pen Recorder, Matsushita Communication Industry, Osaka, Japan) and an integrator (Waters Assoc. 740 Data Module).

Reagent and chemicals

L-Arginine (ARG) monohydrochloride, guanidine (G) hydrochloride, creatinine (CRN), taurocyamine (TAU) and ninhydrin were purchased from Wako (Osaka, Japan), guanidinoacetic acid (GAA), N-acetyl-L-arginine (AcARG), guanidinosuccinic acid (GSA), methylguanidine (MG) hydrochloride and β-guanidinopropionic acid (GPA) from Sigma (St. Louis, MO, U.S.A.), γ-guanidinobutyric acid (GBA) from Nutritional Biochemicals (Cleveland, O.H. U.S.A.) and sodium octanesulphonate from Aldrich (Milwaukee, WI, U.S.A.). Water, acetonitrile and methanol were of liquid chromatographic grade. All other chemicals were of analytical-reagent grade.

The mobile phase was prepared to contain 15 mM sodium octanesulphonate and 5 mM ninhydrin in water–acetonitrile–methanol (92:3:5, v/v), adjusted to pH 4.0 or 3.5 with acetic acid. The alkaline solution was 0.5 M sodium hydroxide. Serum samples from patients were obtained from Kitasato University Hospital.

Preparation of physiological fluids

A 100-μl sample of serum in a 1.5-ml tapered polypropylene centrifuge tube was vortex-mixed with 20 μl of 20% trichloroacetic acid solution for a few seconds. The mixture was centrifuged at 10 000 g for 2 min. A 60-μl aliquot of the supernatant was transferred into another tube. The supernatant was mixed with 15 μl of 0.4 M sodium hydroxide and the solution was adjusted to about pH 2.5–3.0. A 50-μl aliquot of the supernatant was injected into the chromatograph. Standard sera, supplemented with various known amounts of guanidino compounds, were prepared and analysed. Peak-height measurements were performed for construction of the calibration graph.

RESULTS

Optimization of the chromatographic system

The optimal reaction conditions for the post-column derivatization and the
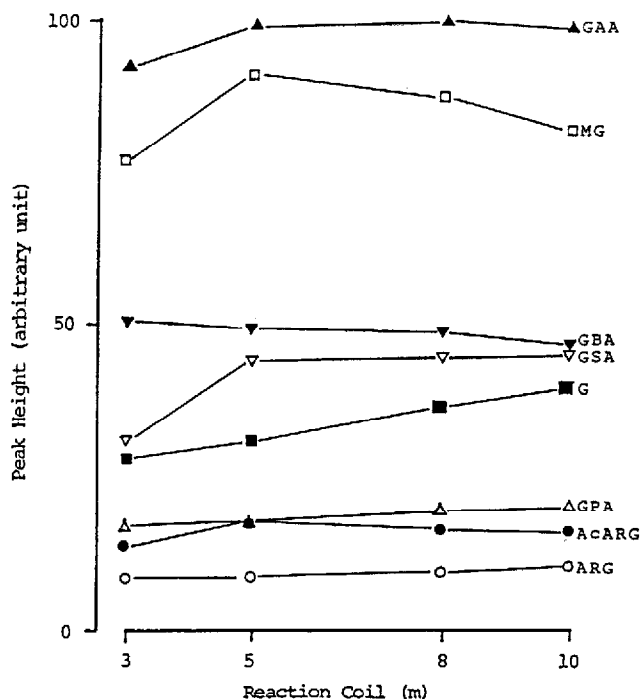
Fig. 1. Effect of reaction-coil length on the fluorescence intensity of various guanidino compounds. For abbreviations and concentrations, see text.

separation of guanidino compounds by HPLC were examined by injecting 10 μl of a standard solution of guanidino compounds (1.48 nmol of GSA, 3.24 nmol of GAA, 1.85 nmol of AcARG, 0.99 nmol of GPA, 6.8 nmol of G, 1.18 nmol of ARG, 4.56 nmol of MG and 2.06 nmol of GBA) into the chromatograph, with a mobile phase flow-rate of 1.0 ml/min and an alkaline solution flow-rate of 0.5 ml/min.

Reaction coil

Reaction coils (0.5 mm I.D.) of various lengths were tested to give the optimal fluorescence intensity for guanidino compounds. Fig. 1 shows the effect of the coil length on the fluorescence intensity. The fluorescence intensity showed a plateau with reaction coils of 5–8 m and a gradual decrease with longer reaction coils. Therefore, a 5-m reaction coil was used for the determination of guanidino compounds.

Reaction temperature

The effect of temperature on the reaction of guanidino compounds with nin-hydrin in the alkaline solution was studied in the range 50–90°C (Fig. 2). The fluo-rescence intensity increased with increasing temperature up to a maximum at *ca.* 75°C, followed by a gradual decrease at higher temperatures. Therefore, a reaction temperature of 75°C was used in subsequent work.

Alkali concentration

Fig. 3 shows the fluorescence intensity for guanidino compounds in sodium
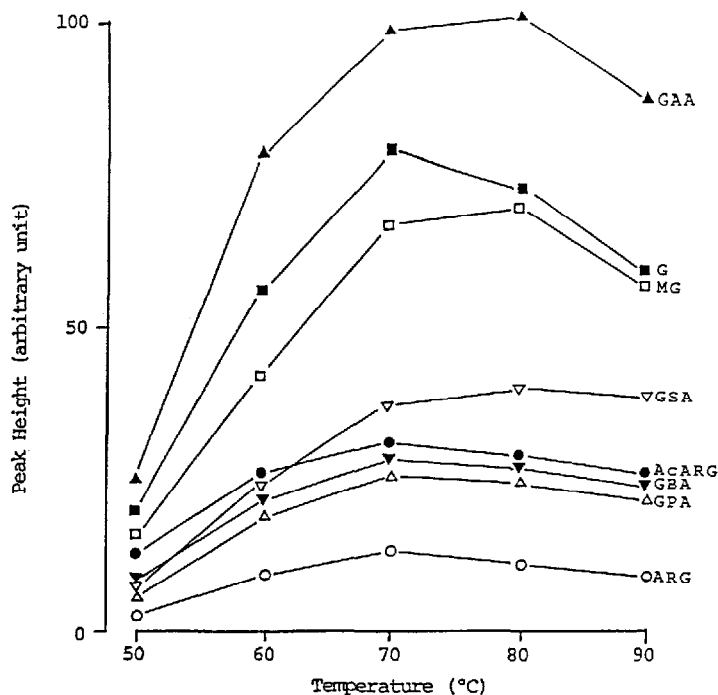
Fig. 2. Effect of reaction temperature on the fluorescence intensity of various guanidino compounds. For abbreviations and concentrations, see text.
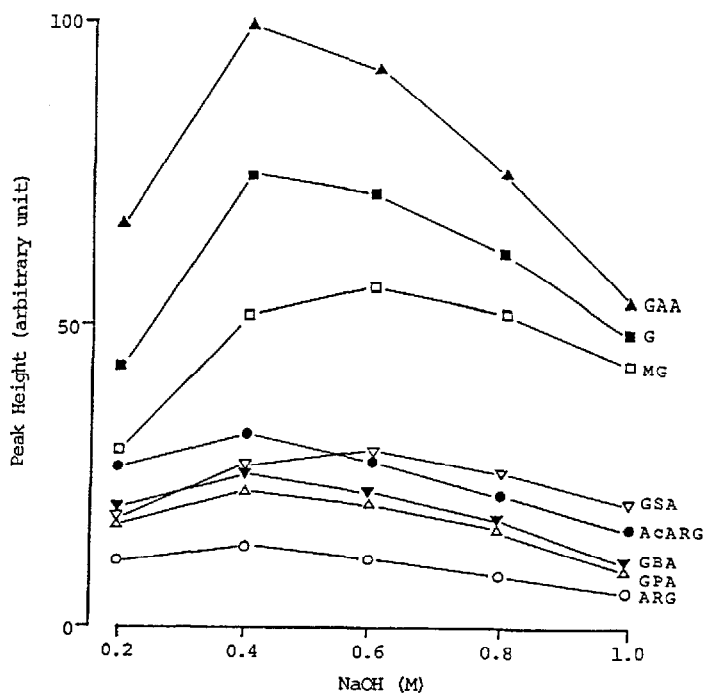

Fig. 3. Effect of alkali concentration on the fluorescence intensity of various guanidino compounds. For abbreviations and concentrations, see text.
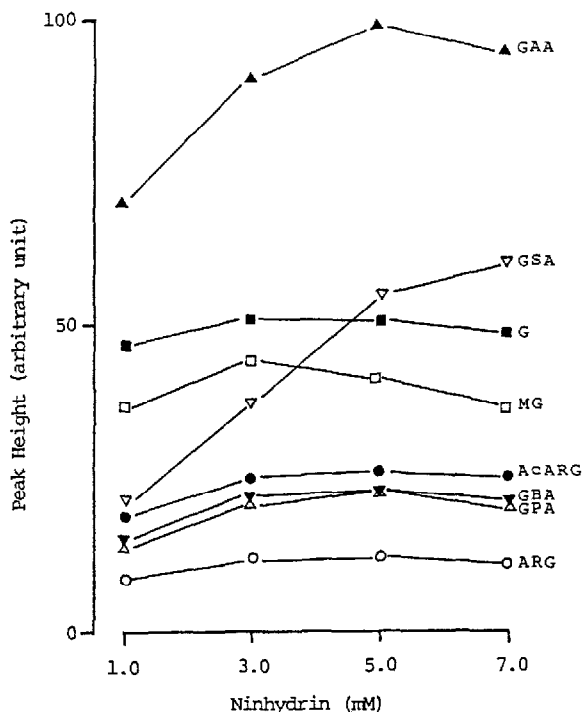
Fig. 4. Effect of ninhydrin concentration on the fluorescence intensity of various guanidino compounds. For abbreviations and concentrations, see text.
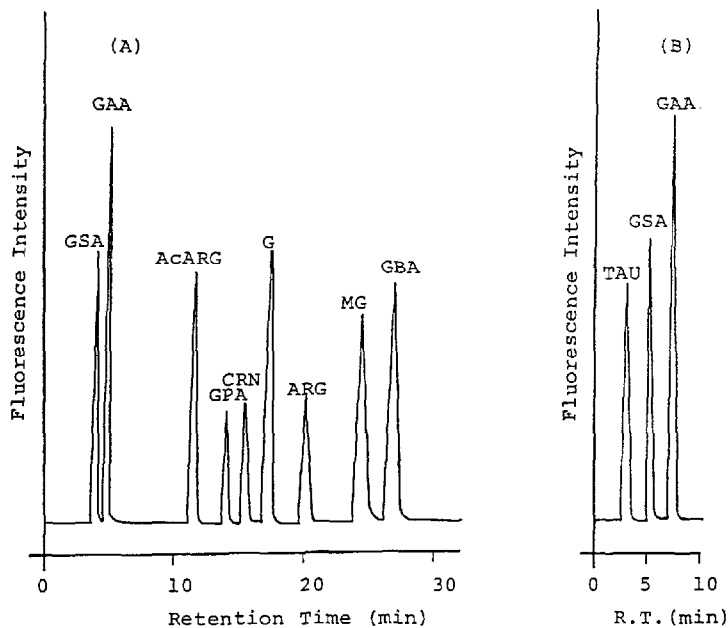

Fig. 5. Chromatograms of a standard mixture of guanidino compounds. TAU, 0.160 nmol; GSA, 0.148 nmol; GAA, 0.324 nmol; AcARG, 0.185 nmol; GPA, 0.099 nmol; CRN, 7.20 nmol; G, 0.680 nmol; ARG, 0.118 nmol; MG, 0.456 nmol; GBA, 0.206 nmol. Mobile phase pH: (A) 4.0; (B) 3.5. For abbreviations, see text.

hydroxide solution in the concentration range 0.2–1.0 M. The fluorescence intensity increased with increasing concentration up to *ca.* 0.4 M, followed by a gradual decrease. Therefore, a concentration of the alkaline solution of 0.5 M was used in subsequent work.

Ninhydrin concentration

Fig. 4 shows the effect of the ninhydrin concentration on the fluorescence intensity for each guanidino compound. The fluorescence intensity began to level off at *ca.* 3 mM, except for GAA and GSA. Therefore, the final concentration of ninhydrin in the mobile phase selected was 5 mM.

Chromatographic separation and quantitative response

Fig. 5A shows a chromatogram obtained from a standard solution of nine guanidino compounds with the mobile phase at pH 4.0, and Fig. 5B shows a chromatogram obtained from a standard mixture of three guanidino compounds (GSA, GAA, and TAU) with the mobile phase at pH 3.5. Complete separation of the compounds was achieved within 25 min.

Calibration graphs for the guanidino compounds are shown in Fig. 6. All the compounds showed a linear response in the indicated range. The correlation coefficients of all the curves were 0.997. The coefficients of variation (C.V., within-run, $n = 10$) for the assay of the compounds were tested against the standard solution and are given in Table I. The recoveries of each guanidino compound added to 100
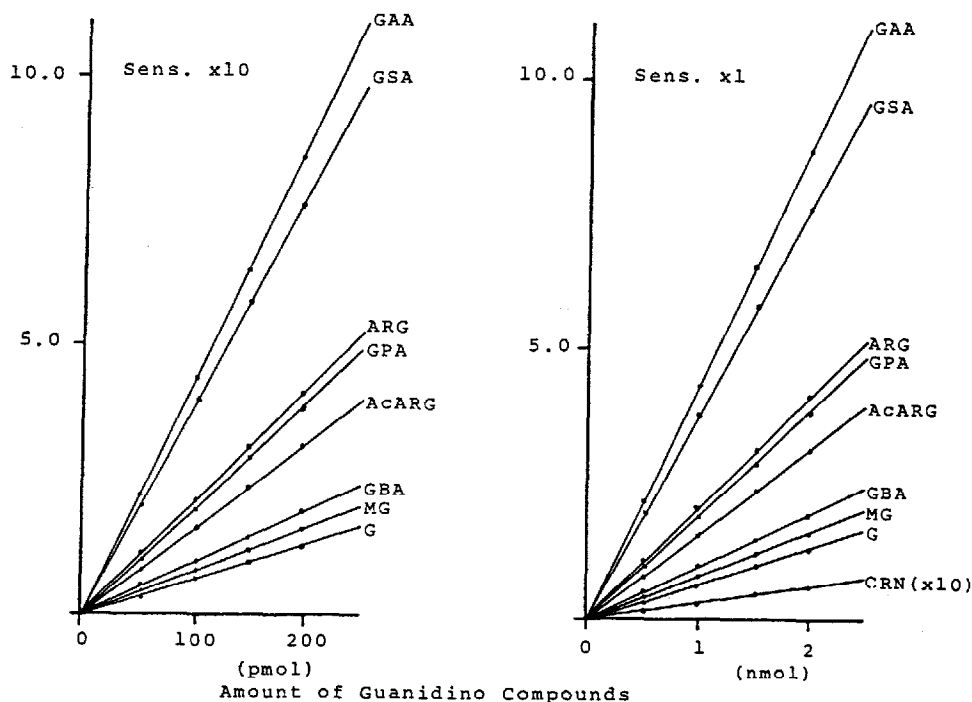

Fig. 6. Calibration graphs for guanidino compounds. For abbreviations, see text.

TABLE I

REPRODUCIBILITY OF ANALYSES AND RECOVERY OF VARIOUS GUANIDINO COMPOUNDS

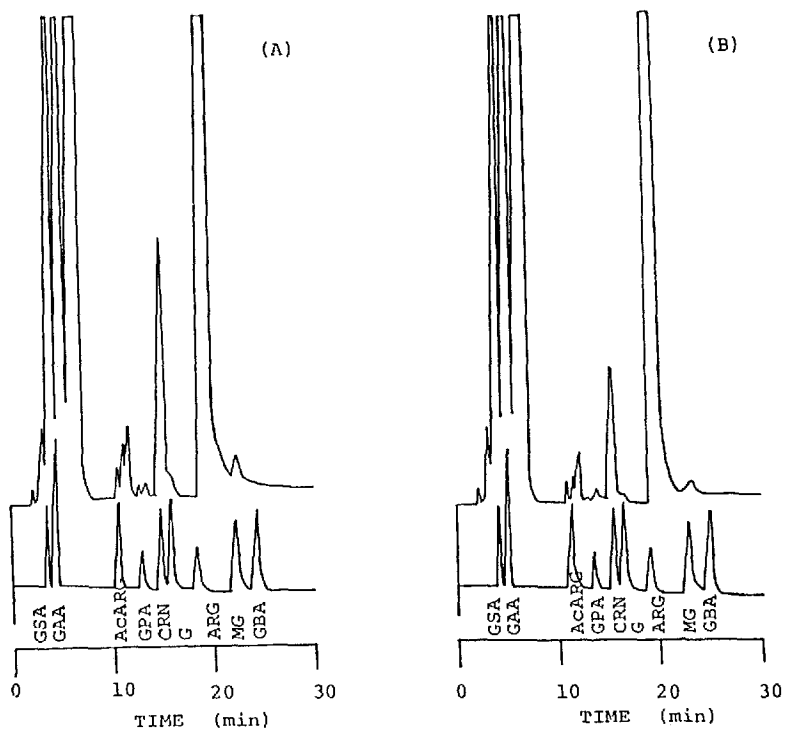| Compound*	Concentration (nmol)	Within-run C.V. (%) (n = 10)	Recovery (%)
GSA	0.146	1.92	100
GAA	0.347	2.50	98.9
AcARG	0.198	2.04	106
GPA	0.106	2.22	96.1
CRN	1.20	2.33	102
G	0.728	1.61	106
ARG	0.212	2.09	96.5
MG	0.488	3.00	107
GBA	0.221	1.59	100

* For abbreviations, see text.

Fig. 7. Chromatograms of the serum sample of a patient (A) before and (B) after haemodialysis therapy

μl of serum are also shown in Table I. The limits of detection, determined from the peak height at twice the noise level, were as follows: GAA, 4 pmol; GSA, 7 pmol; ARG and GPA, 10 pmol; AcARG, 15 pmol; GBA and MG, 20 pmol; G, 30 pmol and CRN, 0.5 nmol.

Analysis of physiological fluids

Fig. 7 shows chromatograms obtained from the serum of a patient (A) before and (B) after haemodialysis therapy. The creatinine level apparently decreased after haemodialysis.

DISCUSSION

The post-column derivatization method for the fluorimetric determination of guanidino compounds requires three (or more) pumps to deliver the mobile phase, the alkaline solution and a fluorigenic reagent, because the fluorigenic reagent is not stable in alkaline solution and some reagents are insoluble in water. Therefore, we devised a new post-column derivatization system in which the fluorigenic reagent is dissolved in the mobile phase. We have previously reported a system in which 1,2-naphthoquinone-4-sulphonate (NQS) is the fluorigenic reagent for the determination of streptomycin[15] and guanidino compounds[16]. The system is simplified because only two pumps are used to deliver the fluorigenic reagent-containing mobile phase and the alkaline solution. In this work, we used ninhydrin instead of NQS, because it is soluble in water and also far more sensitive for guanidino compounds. The excitation (395 nm) and emission (500 nm) wavelengths chosen for the fluorophors in the effluent from the detector agreed well with those reported by Conn and Davis[17].

The separation of guanidino compounds has previously been performed by HPLC on ion-exchange[4–6,8–10,12] and reversed-phase[7,11,13,14] columns. In ion-exchange and some reversed-phase column chromatographic methods, the separation requires gradient elution with several buffers. In another method of reversed-phase column chromatography, the separation requires two columns in series[7], and only five guanidino compounds are separated on one column[11]. In the NQS post-column derivatization system, we separated ten guanidino compounds on a Nucleosil C_8 column, using octanesulphonate as the counter ion, and reported the influence of the counter ion concentration and mobile phase pH on the separation. This resulted in a simplified method and a shorter analysis time (*ca.* 25 min). Ninhydrin concentrations of 1–7 mM in the mobile phase did not influence the retention time of the guanidino compounds. As shown in Fig. 7, the concentration of creatinine decreased after haemodialysis. The results were similar for three patients on haemodialysis therapy.

The advantages of this method are speed, simplicity, specificity, sensitivity and precision. Hence the method can be used for the routine determination of guanidino compounds in physiological fluids of uraemic patients. The new post-column derivatization system should be applicable to other post-column derivatization reagents and other guanidino compounds, such as drugs.

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