Chemiluminescence-HPLC for the Assay of Guanidino Compounds

Yukio NOHARA,^{*,a} Toshihiko HANAI,^b
Toshio KINOSHITA,^c and Mitsuo WATANABE^a

Faculty of Pharmaceutical Sciences, Teikyo University,^a Sagamiko, Kanagawa 199-0195, Japan, International Institute of Technological Analysis, Health Research Foundation,^b Institute Pasteur 5F, Hyakumanben, Sakyo-ku, Kyoto 606, Japan, and School of Pharmaceutical Sciences, Kitasato University,^c 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan.

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A chemiluminescence-HPLC system for the assay of serum guanidino compounds was established. In this study, we found that lucigenin added to the reaction mixture containing guanidino compounds and alkaline-ninhydrin reagent resulted in strong chemiluminescence. The lucigenin chemiluminescence was not derived from fluorescent derivatives of guanidino compounds or ninhydrin. The interaction of guanidino compounds and ninhydrin under alkaline conditions produced reactive oxygen species. We applied this phenomenon to the microanalysis of guanidino compounds.

Key words guanidino compound; chemiluminescence; ninhydrin; lucigenin; HPLC; chronic renal failure

Guanidino compounds are uremic toxins that accumulate in the blood of chronic renal failure patients. Guanidino compounds are candidates for markers of renal dysfunction since they are more toxic than creatinine (CTN) or blood urea nitrogen (BUN).^{1,2)} We recently found that uremic patients capable of excreting urine even on hemodialysis have markedly lower serum guanidinosuccinic acid levels compared with anuric patients.^{3,4)} HPLC coupled with fluorometry has been used to determine guanidino compound. However, the detailed investigation of guanidino compounds for the elucidation of their behavior requires higher sensitivity, since serum concentrations of guanidino compounds are far lower than those of CTN or BUN.

We previously developed fluorometry of guanidino compounds using an alkaline-ninhydrin reagent.^{3–7)} In this study, we found that lucigenin added to the reaction mixture containing guanidino compounds and alkaline-ninhydrin reagent resulted in strong chemiluminescence, whereas no luminescence under alkaline conditions was detected with either guanidino compounds or ninhydrin alone. This phenomenon was also seen in the microanalysis of guanidino compounds.

Figure 1A shows the serial changes in fluorescence intensities after mixing guanidinoacetic acid (GAA) with alkaline-ninhydrin reagent. The reaction mixture contained ninhydrin 0.4 mM, GAA 4 mM, and sodium hydroxide 400 mM in a water–2-propanol mixture (94:6 v/v%). The fluorescence intensity increased gradually after 2.5 min, and plateaued after 15 min.

Figure 1B shows lucigenin chemiluminescence intensities from an aliquot of reaction mixture during reaction between GAA and alkaline-ninhydrin reagent. The reaction mixture contained ninhydrin 0.2 mM, GAA 2 mM, lucigenin 0.065 mM, and sodium hydroxide 200 mM in a water–2-propanol mixture (97:3 v/v%). The reaction mixture during reaction be-

tween GAA and alkaline-ninhydrin reagent was used in Fig. 1A, lucigenin solution was mixed in a cuvette. The lucigenin chemiluminescence was measured using a spectrofluorometer at 465 nm, and the lamp was turned off during measurement. Although the fluorescence intensity was weak at 2.5 min (Fig. 1A), the chemiluminescence intensity from the reaction mixture was maximum. These findings indicate that lucigenin chemiluminescence was mainly due to the reactive oxygen species from the interaction of the guanidino group of GAA with the diketone of *o*-carboxyphenylglyoxal changed from ninhydrin under alkaline conditions, not from fluorescent derivatives of the GAA and ninhydrin.

We applied this phenomenon to the microanalysis of guanidino compounds. Chemiluminescence analysis is a sensitive method because there is no background light source. The lucigenin has high chemiluminescence efficiency and does not require a catalyst, unlike luminol, and therefore lucigenin was used for chemiluminescence-HPLC of guanidino compounds.

The HPLC consisted of an HP 1100 liquid chromatograph (Yokogawa Analytical Systems Inc., Tokyo, Japan) with three isocratic pumps (Model G1310A Iso Pump, Yokogawa), an autosampler (Model G1313A ALS, Yokogawa), and an A/D converter (Model HP 35900E Dual Channel Interface, Yokogawa). Detection was performed with a chemiluminescence detector (Model CLD-10A, Shimadzu Seisakusho Co., Kyoto, Japan). The host computer was a Vectra XM series 3 16/90 personal computer (Yokogawa) equipped with Chem Station chromatographic software (Yokogawa).

The eluent was a mixture of sodium dihydrogenphosphate 5 mM solution (pH 4.6) containing 1-octanesulfonic acid sodium salt 5 mM and 2-propanol (85:15 v/v%). It was delivered from pump A at a flow rate of 1.0 ml/min. A 5 μ l sample was injected by the autosampler. Guanidino compounds that formed ion pairs with 1-octanesulfonic acid were separated on the column (Senshu Pak PEGASIL ODS, 150 $\times$ 4.6 mm i.d., particle size 5 μ m). The eluate from the separation column was mixed with reagent solution (ninhydrin 5 mM and lucigenin 0.098 mM in a water–2-propanol mixture, 98:2 v/v%) and sodium hydroxide 500 mM solution delivered from pumps B and C at a flow rate of 0.5 ml/min. The mixture was

Fig. 1. Serial Changes in Fluorescence Intensities after Mixing Guanidinoacetic Acid with Alkaline-Ninhydrin Reagent (A), and Lucigenin Chemiluminescence Intensities from an Aliquot of Reaction Mixture during the Reaction between Guanidinoacetic Acid and Alkaline-Ninhydrin Reagent (B)

* To whom correspondence should be addressed. e-mail: d96003@pharm.teikyo-u.ac.jp

Fig. 2. Chromatogram of a Standard Mixture of Guanidino Compounds (GAA, GPA, and MG: 50 pmol/Injection; CT, GBA, GSA, and TAU: 100 pmol/Injection; Arg and G: 200 pmol/Injection; CTN: 500 pmol/Injection) (A), Chromatograms of a CRF Patient Serum before (B) and after (C) Addition of Ninhydrin 5 mM to the Mobile Phase

reacted in a coil (5 m $\times$ 0.5 mm i.d.), placed in a detector at 25 $^{\circ}$ C, and the chemiluminescence was monitored with the chemiluminescence detector.

Figure 2A shows a chromatogram of a guanidino compound standard mixture containing arginine (Arg), creatine (CT), CTN, guanidine (G), GAA, guanidinobutyric acid (GBA), guanidinopropionic acid (GPA), guanidosuccinic acid (GSA), methylguanidine (MG), and taurocyamine (TAU). GSA, GBA, GPA, CTN, Arg, G, and MG were clearly separated.

Figures 2B and 2C show chromatograms of a chronic renal failure (CRF) patient serum before (Fig. 2B) and after (Fig. 2C) the addition of ninhydrin 5 mM to the mobile phase. The sample solution was obtained by deproteinization of serum according to the method reported by Kubo and coworkers.⁷⁾ Guanidino compounds were not detected by the chemiluminescence detector (Fig. 2B), but some unknown peaks were detected because lucigenin reacts with reductants or oxidizing agents under alkaline conditions. In our procedure, lucigenin chemiluminescence of GSA, CTN, Arg, G, and MG, which are found in serum of CRF patients, was detected following the addition of ninhydrin to the mobile phase (Fig. 2C). The detection limits (S/N=2) were as follows: GSA and GBA, 10 pmol/injection; GPA, 5 pmol/injection; CTN, 2 nmol/injection; Arg and G, 40 pmol/injection; and MG, 25 pmol/injection. This chemiluminescence-HPLC system has

sensitivity similar to HPLC coupled with fluorometry using an alkaline-ninhydrin reagent as reported by Kobayashi *et al.*⁷⁾

This method is specific for the guanidino group, not amine. On the other hand, this chemiluminescence-HPLC system is an easy and simple one, because the reaction of alkaline-ninhydrin reagent with guanidino compounds generates lucigenin chemiluminescence immediately, and the lucigenin chemiluminescence reaction proceeds at room temperature, whereas the fluorescence reaction used in the conventional detection of guanidino compounds requires heating of the reaction mixture.

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References and Notes

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