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New Method for the Fluorimetric Determination of Guanidino Compounds with Benzoin

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A new fluorimetric method for the precise and rapid determination of guanidino compounds with benzoin is described. This is based on their reaction in an aqueous potassium hydroxide solution with benzoin (dissolved in methylcellosolve), in the presence of β-mercaptoethanol and sodium sulfite, while the pH of the reaction mixture is maintained at 9.2 after the reaction. This method requires heating at 100° for only 10 min. The excitation and emission maximum wavelengths of the fluorescence from these compounds are at around 325 and 435 nm, respectively. The method is simple, selective for guanidine and monosubstituted guanidino compounds (including peptides with one or two arginyl residues) and sensitive; almost all the compounds can be determined at concentrations as low as 40—170 pmol/ml.

Keywords——guanidine; monosubstituted guanidino compounds; peptide with arginyl residue; fluorimetry; benzoin; β-mercaptoethanol; sodium sulfite; micro analysis; rapid method

Colorimetric methods for the determination of guanidino compounds and peptides containing an arginyl residue,[1–5] based on the Sakaguchi or Voges–Proskauer reaction, are not sensitive. However, fluorimetric methods based on reactions with ninhydrin[6] and 9,10-phenanthraquinone[7,8] are sensitive enough to determine the guanidino compounds which are implicated as uremic toxins responsible for uremic syndrome in small amounts of mammalian body fluids.[9–11]

We have reported a fluorimetric method for the selective determination of the guanidino compounds, based on their reactions with benzoin-dimethylformamide reagent in an aqueous potassium hydroxide solution.[12] This method is more sensitive than the other fluorimetric methods, but requires prolonged heating at 100° (45 min) to give maximum constant fluorescence. Recently, it was shown that the fluorescent products formed from the guanidino compounds in this method were the corresponding 2-substituted amino-4,5-diphenylimidazoles; they fluoresced most intensely in a weakly alkaline medium (pH 9—10) and their fluorescence was stabilized by β-mercaptoethanol.[13] This suggests that dimethylformamide is not incorporated into the fluorescent products but is decomposed in the hot, strongly alkaline solution to formic acid, which serves to make the resulting reaction mixture weakly alkaline. Therefore, the reaction of the guanidino compounds with benzoin proceeds in a strongly alkaline medium but a change to weakly alkaline conditions must then be made to obtain maximum fluorescence.

More recently, we found that the maximum fluorescence could be obtained when the fluorescence reaction at 100° was carried out for only a few minutes, that the reaction occurred even in an aqueous solution of a water-miscible solvent other than dimethyl formamide, and that β-mercaptoethanol added to the reaction mixture before heating acted as a stabilizer of the fluorescent products even under the strongly alkaline conditions of the reaction.

This paper describes a new method for the rapid and sensitive determination of the guanidino compounds with benzoin, based on the above observations. Arginine, methylguanidine, guanidinoacetic acid, guanidinosuccinic acid and phenylguanidine were employed

as model compounds to establish suitable reaction conditions for a more general analytical method.

Experimental

Reagents and Solutions——Taurocyamine was kindly supplied by Prof. A. Mori (Institute for Neurobiology, Okayama University Medical School, Okayama, Japan). Argininosuccinic acid (Ba salt, 90% purity) was purchased from Sigma (St. Louis, Mo., U.S.A.). Leupeptin was a gift from Nippon Kayaku Co. (Tokyo, Japan). Trishydroxymethylaminomethane (Tris) (Wako, Osaka, Japan) was recrystallized from aqueous 60% methanol to remove fluorescent impurities. All other chemicals were of reagent grade. Double-distilled water was used.

Benzoin Solution (4.0 mM): Dissolve 85 mg of benzoin in 100 ml of methylcellosolve. The solution is stable for at least a month when stored in a refrigerator.

β-Mercaptoethanol (0.1 M)–Na_2SO_3 (0.2 M) solution: Dissolve 0.78 g of β-mercaptoethanol and 2.52 g of Na_2SO_3 in 80 ml of H_2O and dilute with H_2O to 100 ml. The solution is usable for at least 2 weeks when stored in a refrigerator.

HCl (2 M)–Tris Buffer (0.5 M, pH 9.2) Mixture: Mix equal volumes of 4 M HCl and 1 M Tris buffer (prepared by dissolving 12.11 g of Tris in 80 ml of water, adjusting the pH to 9.2 with concentrated HCl and diluting the solution to 100 ml with H_2O). The solution is usable for at least a month when stored at room temperature.

Apparatus——Fluorescence spectra and intensities were measured with a Hitachi MPF-4 spectrofluorimeter in 10×10 mm quartz cells: spectral bandwidths of 10 nm were used in both the excitation and emission monochrometers. The fluorescence spectra, and excitation and emission maxima are uncorrected.

Procedure——Place 1.0 ml of aqueous test solution in a test tube, add 0.5 ml of benzoin solution, 0.5 ml of β-mercaptoethanol-Na_2SO_3 solution and 1.0 ml of aqueous 2 M KOH with cooling in ice-water. Heat the mixture in a boiling water-bath for 10 min. Cool in ice-water for approximately 2 min and add 1.0 ml of HCl–Tris buffer mixture. Prepare a reagent blank by treating 1.0 ml of H_2O in the same manner. Measure the fluorescence intensities of the test solution and blank at the emission maximum wavelength with irradiation at the excitation maximum (see Table I). Read the concentration of guanidino compound from a calibration curve prepared in the usual manner.

Results and Discussion

With guanidino compounds, benzoin gives an intense fluorescence in aqueous potassium hydroxide solutions of organic solvents tested (*e.g.* methylcellosolve, methanol, ethyleneglycol, tetrahydrofuran, ethanol, dioxane, acetonitrile and dimethylsulfoxide). Methylcellosolve was used in the standard procedure as a solvent for benzoin, because of its low volatility below 100° and easiness in handling. Methylcellosolve does not affect the fluorescence development over the concentration range of 10—30% (v/v) in the reaction mixture; 16.7% (v/v, 0.5 ml as benzoin solution in 3.0 ml of the reaction mixture) was used.

Concentrations of benzoin higher than 3.0 mM in the reagent solution give a maximum and constant fluorescence for all the model guanidino compounds; 4.0 mM was selected as the standard concentration. Potassium hydroxide concentrations ranging from 1.0 to 3.0 M in the alkaline solution give maximum intensity (Fig. 1); 2.0 M was selected as the standard.

The fluorescent products formed during the reaction are unstable in the absence of β-mercaptoethanol. For example, the fluorescence produced from arginine is less than 85% of that produced in its presence, and decreased at the rate of approximately 65% per hr in daylight at room temperature. Therefore, β-mercaptoethanol should be added to the reaction mixture before heating. The presence of β-mercaptoethanol prevents the decrease of fluorescence formed from model guanidino compounds at concentrations higher than 0.02 M in the reagent solution; 0.1 M solution was used in the standard procedure. The fluorescence thus obtained is stable for more than 4 hr in daylight at room temperature when the pH of the heated reaction mixture is adjusted to 9.2. The mechanism of the stabilization by β-mercaptoethanol remained unknown, but it could be ascribed to its reducing properties, since other reducing agents (*e.g.* sodium thiosulfate, sodium sulfite, sodium borohydride and sodium formate) also stabilized the fluorescence fairly effectively.

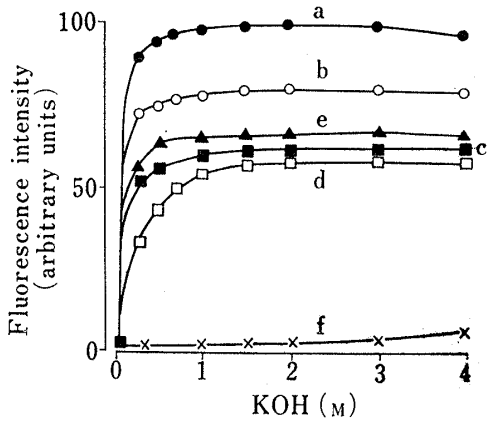

Fig. 1. Effect of the Concentration of Potassium Hydroxide on the Fluorescence Development

Portions (1.0 ml) of solutions of guanidino compounds were treated according to the standard procedure but with various concentrations of potassium hydroxide and the final reaction mixtures were adjusted to pH 9.2 with mixtures of hydrochloric acid (0—8 M) and Tris buffer (1 M, pH 9.2) (1:1, v/v). a: arginine; b: methylguanidine; c: guanidinoacetic acid; d: guanidinosuccinic acid; e: phenylguanidine; f: reagent blank. a—d: 10 nmol/ml; e: 2.5 nmol/ml.

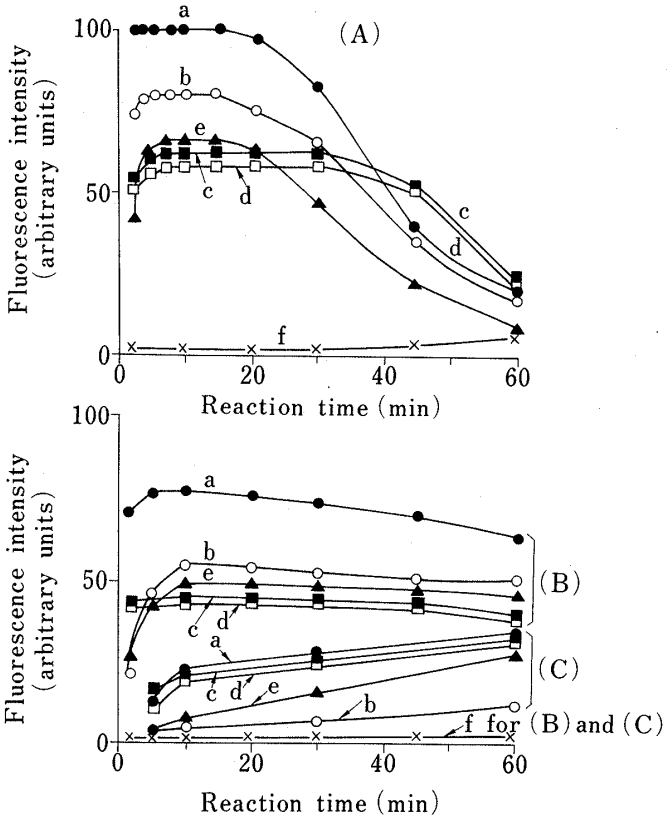

Fig. 2. Effects of Reaction Temperature and Time on the Fluorescence Development

Portions (1.0 ml) of solutions of guanidino compounds were treated according to the standard procedure but for various reaction times at different reaction temperatures.
(A): 100°; (B): 68°; (C): 24°.
a: arginine; b: methylguanidine; c: guanidinoacetic acid; d: guanidinosuccinic acid; e: phenylguanidine; f: reagent blank. a—d: 10 nmol/ml; e: 2.5 nmol/ml.

Although the fluorescence of the reagent blank obtained in both the presence and absence of β-mercaptoethanol is very weak (the intensity is 2% of that given by 10 nmol/ml solution of arginine), it doubles per hr in daylight, but not in the dark. This increase can be halted by the addition of sodium sulfite solution to the reaction mixture before heating, at a concentration higher than that of β-mercaptoethanol used: 0.2 M solution was used in the standard procedure.

Temperatures higher than approximately 60° develop the fluorescence more rapidly and more intensely (Fig. 2, (A) and (B)). At 100°, which was selected for convenience, the fluorescence intensity reaches a stable maximum after heating for 7—15 min; heating for 10 min gave repoducible results.

The fluorescence reactions apparently occur even at room temperature (Fig. 2, (C)). However, when the reaction mixtures of guanidino compounds containing a carboxyl group (arginine, guanidinoacetic acid and guanidinosuccinic acid) are left to stand at room temperature (24°) for 60 min before being heated, and are then heated at 100° as recommended, the fluorescence is about 20% lower than that obtained without standing before heating. This is probably due to partial decomposition of the guanidino compounds in the strongly alkaline medium to form non-reacting species. The decrease in the fluorescence intensity is not observed when the reaction mixture is heated after standing for even longer than 1 hr under cooling in ice-water. Therefore, the reactant solution should be cooled while being mixed to obtain reproducible results.

The heated reaction mixture is made weakly alkaline by adding a mixture of hydrochloric acid to neutralize the potassium hydroxide used in the reaction and the Tris buffer. Although the pH of the resulting mixture does not greatly affect the fluorescence intensity in the range of 8.5—10, pH 9.2 gives the maximum intensity (Fig. 3); 2 M hydrochloric acid and 0.5 M Tris buffer of pH 9.2 were used in the standard procedure. When 0.2 M borate buffer was used in place of the Tris buffer, the blank fluorescence increased 4 times compared with that obtained with the Tris buffer, probably due to the formation of fluorescent benzoin-borate complex.

The excitation and emission maxima of the fluorescence from arginine occur at 325 and 435 nm, respectively (Fig. 4). The fluorescence spectra of the other model compounds are very similar in shape and maxima to that of arginine.

Guanidine and monosubstituted guanidino compounds other than the model compounds, including peptides with an arginyl residue, fluoresce under the conditions recommended. The excitation and emission maxima of the fluorescences from various compounds (including the model compounds), their relative fluorescence intensities and the minimum detectable amounts are shown in Table I. The excitation and emission maximum wavelengths are not characteristic of individual compounds. Compounds with one guanidino group in the molecule show 57—264% of the fluorescence intensity given by arginine at equimolar concentration. The intensities from peptides with two arginyl residues are approximately twice that from arginine.

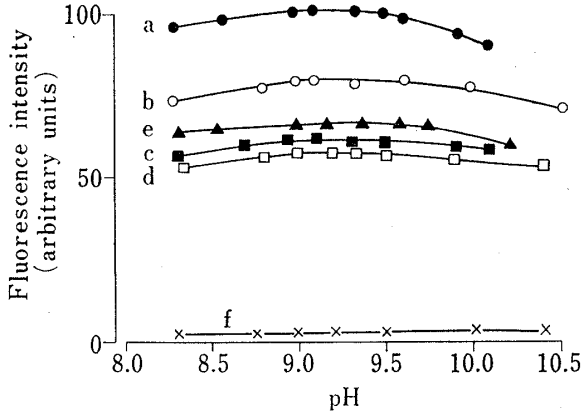

Fig. 3. Effect of pH on the Fluorescence Developed

Portions (1.0 ml) of solutions of guanidino compounds were treated according to the standard procedure, but the final reaction mixtures were adjusted to various pHs with mixtures of hydrochloric acid (4 M) and Tris buffer (1 M, various pHs) (1: 1, v/v).
a: arginine; b: methylguanidine; c: guanidinoacetic acid; d: guanidinosuccinic acid; e: phenylguanidine; f: reagent blank. a—d: 10 nmol/ml; e: 2.5 nmol/ml.

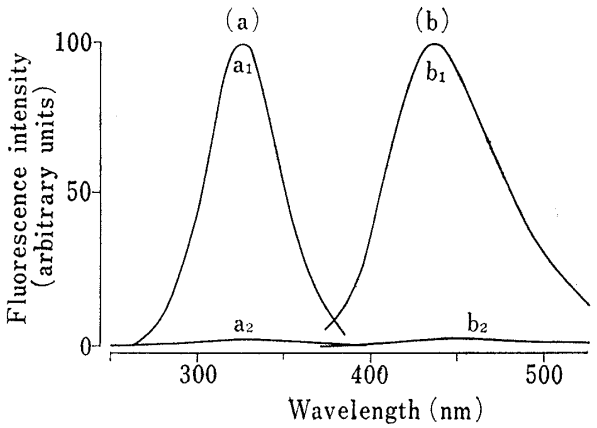

Fig. 4. Excitation and Emission Spectra of the Final Reaction Mixture of Arginine

a: excitation spectra; b: emission spectra.
a_1 and b_1: a portion (1.0 ml) of 10 nmol/ml arginine solution was treated by the standard procedure.
a_2 and b_2: reagent blank corresponding to a_1 and b_1.

The fluorescence intensity from leupeptin is only 10% of that given by an equimolar concentration of arginine. This is ascribable to Schiff base formation of the amino group of the guanidino moiety with the formyl group in the molecule. Leupeptin acid does not form such a Schiff base and therefore fluoresces as strongly as arginine.

The fluorescences from creatine, creatinine and argininosuccinic acid are 1/400—1/500 times that from arginine at equimolar concentration. This indicates that disubstituted guanidino compounds effectively do not react with benzoin under the conditions recommended.

Various other biologically important substances do not fluoresce and also do not affect the fluorescence development from arginine (5.0 nmol/ml), even at concentrations of 0.5 μmol/ml, *e.g.* urea, uric acid, allantoin, putrescine, spermine, glycine, 16 different L-α-amino acids

Table I. Fluorescence Excitation and Emission Maxima from Guanidino Compounds, and Their Relative Fluorescence Intensities and Lower Limits of Determination

	Excitation maximum[a] (nm)	Emission maximum[a] (nm)	Relative fluorescence intensity[a,b]	Lower limit of determination[c] (pmol/ml)
Arginine	325	435	100	100
Agmatine	325	435	66	160
n-α-Acetylarginine	325	435	98	100
Methylguanidine	327	437	79	130
Guanidinoacetic acid	325	435	61	160
Guanidinopropionic acid	325	435	85	120
Guanidinobutyric acid	325	435	122	80
Guanidinosuccinic acid	325	435	57	170
Taurocyamine	325	432	97	100
Homoarginine	325	435	85	120
Canavanine	325	435	113	90
Phenylguanidine	323	428	264	40
Guanidine	322	432	57	170
Streptomycin	325	433	276	40
Arg–Val–Tyr–Ile–His–Pro–Phe (Angiotensin III)	325	437	101	100
Asp–Arg–Val–Tyr–Ile–His–Pro–Phe (Angiotensin II)	327	437	126	80
Thr–Lys–Pro–Arg (Tuftsin)	325	435	109	90
Pyr–His–Trp–Ser- Tyr–Gly–Leu–Arg–Pro–Gly–NH_2 (LH–Releasing hormone)	328	438	127	80
Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg (Bradykinin)	327	435	209	50
Pyr–Leu–Tyr–Glu–Asn–Lys–Pro–Arg–Arg–Pro–Thr–Ile–Leu (Neurotensin)	328	435	237	40
Acetyl–Leu–-LeuArg (Leupeptin acid)	325	435	110	90
Acetyl–Leu–Leu–Arginal (Leupeptin)	325	435	10	1000

a) Portions (1.0 ml) of 10 nmol/ml solutions of the corrpounds were treated according to the standard procedure.
b) The fluorescence intensity from arginine was taken as 100.
c) Defined as the concentration which gives a fluorescence intensity 1.5 times the blank fluorescence.

other than arginine, glutamine, asparagine, glutathione, adenine, formaldehyde, acetaldehyde, pyruvic acid, 2-oxoglutaric acid, phenylpyruvic acid, oxalic acid, fumaric acid, D-glucose, D-fructose, D-glucuronolactone, D-glucosamine, L-ascorbic acid, cortisone and epiandrosterone. Heparin (10 μg/ml), sodium citrate (5 mg/ml) and EDTA-2Na (1 mg/ml), which are used as anticoagulants for blood, do not interfere with the fluorescence development from arginine (5.0 nmol/ml). Perchloric acid and acetic acid (both 0.5 mmol/ml as their sodium salts) have no effect on the fluorescence development from arginine (5.0 nmol/ml). Trichloroacetic acid (0.5 mmol/ml as its potassium salt) interferes with the fluorescence development and reduces the fluorescence from arginine (5.0 nmol/ml) to 39% of that in its absence. Therefore, this acid cannot be used for deproteinization of biological samples. These observations suggest that the proposed method is selective for guanidine and monosubstituted guanidino compounds.

The sensitivity of the proposed method is more than 1.5 times that of the benzoin-dimethyl formamide method, 2—7 times that of the 9,10-phenanthraquinone method and 5—25 times that of the ninhydrin method, depending on the monosubstituted guanidino compound in question.

The calibration curves for all the guanidino compounds tested (Table I) are linear up to at least 500 nmol/ml and pass through the origin.

The precision was examined by performing 30 analyses on 0.5 and 10 nmol/ml solutions

of arginine. The coefficients of variation were 2.4 and 1.6%, respectively.

The proposed method is the most sensitive and rapid among fluorimetric methods so far reported for the determination of guanidino compounds. This method should be useful for both pre- and post-column derivatization in the high-performance liquid chromatographic determination of uremic toxins and peptides with an arginyl residue, whereas the 9,10-phenanthraquinone method can be used only for post-column derivatization because the method gives a single fluorescent product, 2-amino-1H-phenanthro[9,10-*d*]-imidazole, from all guanidino compounds.[14–16]

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References and Notes

1) S. Sakaguchi, *J. Biochem.*, **5**, 13 (1925).
2) C.J. Weber, *J. Biol. Chem.*, **86**, 217 (1930).
3) J.F. Van Pilsum, R.P. Martin, E. Kito, and J. Hess, *J. Biol. Chem.*, **222**, 225 (1956).
4) H. Rosinberg, A.H. Ennor, and J.F. Morrison, *Biochem. J.*, **63**, 153 (1956).
5) M.J. Micklus and L.M. Stein, *Anal. Biochem.*, **54**, 545 (1973).
6) R.B. Conn and R.B. Davis, *Nature* (London), **183**, 1053 (1959).
7) S. Yamada and H.A. Itano, *Biochim. Biophys. Acta*, **130**, 538 (1966).
8) T. Sakaguchi, S. Tanabe, H. Yagi, T. Miyawaki (née Iijima), and A. Saito, *Yakugaku Zasshi*, **97**, 89 (1979).
9) I.M. Stein, G. Perez, R. Johnson, and N.B. Cummings, *J. Lab. Clin. Med.*, **77**, 1020 (1971).
10) S. Giovannetti and G. Barsotti, *Nephron*, **14**, 123 (1975).
11) M. Matsumoto, H. Kishikawa, and A. Mori, *Biochem. Med.*, **16**, 1 (1976).
12) Y. Ohkura and M. Kai, *Anal. Chim. Acta*, **106**, 89 (1979).
13) M. Kai, M. Yamaguchi, and Y. Ohkura, *Anal. Chim. Acta*, **120**, 411 (1980).
14) H.A. Itano and S. Yamada, *Anal. Biochem.*, **48**, 483 (1972).
15) K. Yasuda and W.S. Chilton, *Anal. Biochem.*, **74**, 609 (1976).
16) H.A. Itano, K. Hirota, I. Kawasaki, and S. Yamada, *Anal. Biochem.*, **76**, 134 (1976).