Aromatic Amidines as Fluorogenic Reagents for Reducing Carbohydrates

Masaaki KAI*, Kazuhiko TAMURA* Masatoshi YAMAGUCHI** and Yosuke OHKURA*

*Faculty of Pharmaceutical Sciences, Kyushu University,
Maidashi, Fukuoka 812

**Faculty of Pharmaceutical Sciences, Fukuoka University,
Nanakuma, Fukuoka 814-01

Aromatic amidines such as 4-methoxybenzamidine, benzamidine, 3,4-dimethoxybenzamidine, 2-amidinonaphthalene and 4-amidinopyridine give fluorescences when reducing carbonhydrates are heated in an alkaline medium with these amidines for a few minutes. Of the amidines tested, 4-methoxybenzamidine and benzamidine are favorable fluorogenic reagents for reducing carbohydrates. Both the aromatic amidines permit the fluorometric determination of reducing carbohydrates with rapid and simple procedure; reducing carbohydrates tested can be determined at concentrations as low as 0.06–2.25 nmol ml⁻¹ and 0.09–2.40 nmol ml⁻¹ by the methods with 4-methoxybenzamidine and benzamidine, respectively.

Keywords Fluorometric determination, reducing carbohydrate, aromatic amidine, 4-methoxybenzamidine, benzamidine

Many fluorogenic reagents have been proposed for analyses of reducing carbohydrates. The reagents are classified into two groups in regard to their reaction conditions. One is the reagents such as 5-hydroxytetralone¹, *o*-phenylenediamine², resorcinol³ and *N*-(1-naphthyl)ethylenediamine⁴, which all require strongly acidic conditions, and thus most of saccharides are decomposed by acidic hydrolysis. The reagents of the other group are ethylenediamine⁵, taurine⁶, malonamide⁷, 2-ethanolamine⁸, 2-cyanoacetamide⁹ and arginine¹⁰, which allow fluorometric determination of reducing carbohydrates in a weakly alkaline or neutral medium with prolonged heating time longer than 30 min.

During a survey of various amidino or guanidino compounds as the reagents for fluorometric determination of carbohydrates, we found that several aromatic amidines provide fluorescences for reducing carbohydrates under alkaline conditions, as a different type from the reagents mentioned above. The survey was carried out, expecting that ketoalcoholic moiety of carbohydrates might react with amidino and/or guanidino groups to produce fluorescent imidazole derivative(s) under alkaline conditions, since guanidino compounds react with benzoin having a ketoalcoholic moiety to form fluorescent diphenylimidazole derivatives under alkaline conditions.^{11–13} With similar view, Seki and Yamaguchi¹⁴ have recently developed a fluorometric determination of 17-hydroxycorticosteroids by high performance liquid

chromatography using benzamidine as a fluorogenic reagent in the post-column derivatization system.

In this paper, we discuss fluorescent reactivities of various amidino or guanidino compounds to reducing carbohydrates, and further describe optimum reaction conditions for the fluorometric determination of reducing carbohydrates with 4-methoxybenzamidine or benzamidine which gave good results as a fluorogenic reagent among the compounds tested. D-Glucose was employed as a representative compound for the investigations.

Experimental

Reagents

Deionized and distilled water was used throughout. Benzamidine was purchased from Aldrich Chemical Co., Inc., (Milwaukee, WIS, USA). 4-Methoxybenzamidine was synthesized according to the method of Pinner.¹⁵ 3,4-Dimethoxybenzamidine, 2-amidinonaphthalene and 4-amidinopyridine were kindly supplied by Dr. Kina (Dojindo Laboratory, Kumamoto, Japan). All other chemicals were of reagent grade.

Apparatus

Fluorescence spectra and intensities were measured with a Hitachi MPF-4 spectrofluorometer in 10×10 mm

quartz cells; spectral bandwidths of 10 nm were used in both the excitation and emission monochromators. The fluorescence spectra, and excitation and emission maxima are uncorrected.

Analytical procedure for reducing carbohydrate

To 1.0 ml of a sample solution in a test tube, 1.0 ml of an aqueous solution of aromatic amidine (30 mM 4-methoxybenzamidinium or benzamidinium) and 1.0 ml of 1.0 M potassium hydroxide solution are added. The mixture is heated in a boiling water-bath for 3 min for 4-methoxybenzamidinium or for 2 min for benzamidinium, and then cooled in ice-water. The reagent blank is prepared in the same way except that 1.0 ml of the sample solution is replaced with 1.0 ml of water.

The fluorescence intensity is measured at 470 nm emission against 310 nm excitation for the reagent of 4-methoxybenzamidinium, or measured at 470 nm emission against 365 nm excitation for benzamidinium.

Results and Discussion

Screening of amidino or guanidino compounds as fluorogenic reagent

Fluorescence from reducing carbohydrate occurs in reactions with aromatic amidines tested (*eg.* benzamidinium, 4-methoxybenzamidinium, 3,4-dimethoxybenzamidinium, 2-amidinonaphthalene and 4-amidinopyridine) under alkaline conditions with heating, but neither with aliphatic amidine (acetamidinium) nor guanidino compounds tested (*eg.* methylguanidinium and phenylguanidinium). Table 1 shows the fluorescence intensities from D-glucose and those of the reagent blanks, obtained with the aromatic amidines. 4-Methoxybenzamidinium gives the highest intensity for equimolar amount of D-glucose, though benzamidinium also gives fluorescence enough to determine the reducing carbohydrate at sub-nano mole level. The aromatic amidines other than 4-methoxybenzamidinium and benzamidinium are not favorable reagents because of high values of their reagent blanks and besides of low yield of fluorescence from D-glucose. Therefore, 4-methoxybenzamidinium and

benzamidinium were investigated further to establish suitable reaction conditions for analytical method of reducing carbohydrates.

On the other hand, Mikami and Ishida¹⁰ have recently reported that arginine gives fluorescence in the reaction with reducing carbohydrates under neutral conditions similar to those with ethanolamine⁸, *i.e.* heating in a borate buffer (pH 7.5) at higher temperature than 120°C. Thus, guanidino compounds tested, phenylguanidinium and methylguanidinium offer fluorescence for D-glucose provided that the reaction is performed in a neutral borate buffer with heating at 145°C; but the intensity was extremely low in comparison with that given by benzamidinium. On the contrary, the aromatic amidines tested as well as acetamidinium do not permit any development of fluorescence from D-glucose in a weakly alkaline or neutral medium (phosphate or borate buffer), even with heating at a high temperature (145°C).

Determination of D-glucose with 4-methoxybenzamidinium or benzamidinium

With 4-methoxybenzamidinium, the fluorescence spectra produced from D-glucose show an emission maximum at 470 nm with two excitation maxima at 310 and 365 nm (Fig. 1); the intensity at 470 nm emission with irradiation at 310 nm is approximately twice as great as that with irradiation at 365 nm.

On the other hand, benzamidinium gives two different fluorescence spectra for D-glucose depending on the reaction time (Fig. 2). When the reaction time is relatively short (approximately less than 10 min, see in Fig. 4), the fluorescence which has an emission maximum at 470 nm (Fig. 2, b₁) and two excitation maxima at 295 and 365 nm (Fig. 2, a₁), is produced; the intensity at 470 nm emission with irradiation at 365 nm is measured for the determination of reducing carbohydrates. For further reaction periods, another fluorescence occurs which shows an emission and excitation maxima at 370 and 300 nm, respectively (Fig. 2, a₃ and b₃). Such two different fluorescent species are also observed when other reducing carbohydrates (listed in Table 2) are treated with benzamidinium in the same way. From these observations, at least two fluorescent

Table 1 Fluorescence intensities produced by reactions of D-glucose with aromatic amidines and their reagent-blank values

Aromatic amidine	R. F. I. ^a		Excitation maximum/ nm	Emission maximum/ nm
	Test ^b	Blank ^b		
Benzamidinium	100	1.2	365	470
4-Methoxybenzamidinium	550	5.0	310	470
3,4-Dimethoxybenzamidinium	72	17	330	470
2-Amidinonaphthalene	36	61	355	480
4-Amidinopyridine	5	32	360	455

a, The relative fluorescence intensity obtained by the reaction of D-glucose with benzamidinium was taken as 100.

b, Portions (1.0 ml) of 30 nmol ml⁻¹ D-glucose solution and water for test and blank, respectively, were treated according to the analytical procedure, but the reaction time was 2 min for benzamidinium, 2-amidinonaphthalene and 4-amidinopyridine and 3 min for the others. The concentration of each aromatic amidine was 30 mM.

products must be formed for a reducing carbohydrate by the reaction with benzamidine.

In the reaction of D-glucose with 4-methoxybenzamidine, the fluorescence does not arise at a temperature less than 23°C, however, a temperature higher than approximately 70°C allows fluorescence to develop rapidly (Fig. 3). The maximum intensity at 100

°C is achieved by heating for 3 min.

Similar results are also observed in the reaction with benzamidine (Fig. 4), though another fluorescence, with emission and excitation maxima at 370 and 300 nm, respectively, is produced with prolonged reaction time

Fig. 1 Excitation and emission spectra of the final reaction mixture of D-glucose with 4-methoxybenzamidine.

a, excitation spectra; b, emission spectra. a_1 and b_1 : a portion (1.0 ml) of 30 nmol ml⁻¹ D-glucose solution was treated by the procedure recommended. a_2 and b_2 : reagent blank corresponding to a_1 and b_1 .

Fig. 2 Excitation and emission spectra of the final reaction mixture of D-glucose with benzamidine.

a, excitation spectra; b, emission spectra. a_1 and b_1 : a portion (1.0 ml) of 30 nmol ml⁻¹ D-glucose solution was treated as recommended. a_2 and b_2 : reagent blank corresponding to a_1 and b_1 . a_3 and b_3 : a portion (1.0 ml) of 30 nmol ml⁻¹ D-glucose solution was treated as recommended, but the reaction time was 20 min. a_4 and b_4 : reagent blank corresponding to a_3 and b_3 .

Table 2 Fluorescence intensities obtained by the reactions of various reducing carbohydrates with 4-methoxybenzamidine or benzamidine, and their lower limits of determination

Reducing carbohydrate ^a	4-Methoxybenzamidine		Benzamidine	
	R. F. I. ^b	Lower limit ^c nmol ml ⁻¹	R. F. I. ^b	Lower limit ^c nmol ml ⁻¹
D-Glucose	550	0.27	100	0.36
D-Galactose	374	0.40	69	0.52
D-Mannose	480	0.31	92	0.39
D-Fructose	725	0.20	106	0.34
2-Deoxy-D-glucose	83	1.80	16	2.25
D-Glucosamine	105	1.42	17	2.12
D-Galactosamine	204	0.73	34	1.06
N-Acetylglucosamine	325	0.46	49	0.73
L-Rhamnose	253	0.59	36	1.00
L-Fucose	220	0.68	47	0.77
D-Glucose-6-phosphate	330	0.45	67	0.54
D-Glucuronolactone	517	0.29	90	0.40
N-Acetylneuramic acid	115	1.29	15	2.40
D-Ribose	385	0.39	57	0.63
D-Arabinose	360	0.42	58	0.62
D-Xylose	457	0.33	70	0.51
D-Xylulose	535	0.28	94	0.38
2-Deoxy-D-ribose	66	2.25	15	2.40
D-Erythrose	2400	0.06	403	0.09
DL-Glyceraldehyde	2330	0.06	263	0.14
Maltose	567	0.26	100	0.36
Gentiobiose	583	0.25	110	0.32
Lactose	320	0.47	68	0.53

a, Portion (1.0 ml) of 30 nmol ml⁻¹ solution of each compound was treated with 4-methoxybenzamidine or benzamidine according to the procedure recommended. b, The relative fluorescence intensity obtained by the reaction of D-glucose with benzamidine was taken as 100. c, Defined as the concentration which gives a fluorescence intensity twice the blank.

Fig. 3 Effects of reaction time and temperature on the fluorescence development in the reaction of D-glucose with 4-methoxybenzamidine.

Portions (1.0 ml) of 30 nmol ml⁻¹ D-glucose solution were treated as recommended for various reaction times at different reaction temperatures. a, 145°C; b, 100°C; c, 70°C; d, 50°C. e-h: reagent blanks corresponding to a-d, respectively.

Fig. 4 Effects of reaction time and temperature on the fluorescence development in the reaction of D-glucose with benzamidine.

Portions (1.0 ml) of 30 nmol ml⁻¹ D-glucose solution were treated as recommended for various reaction times at different reaction temperatures. a and i, 145°C; b and j, 100°C; c and k, 70°C; d, 50°C. a-h: measured at 470 nm emission with irradiation at 365 nm. i-n: measured at 370 nm emission with irradiation at 300 nm. e-h and l-m: reagent blanks corresponding to a-d and i-k, respectively.

as demonstrated in Fig. 2. The latter fluorescence is apparently less intense, over the reaction times examined at any temperature, than the former one with maximum intensity at 470 nm emission and 365 nm excitation.

Consequently, to obtain an intense fluorescence from reducing carbohydrate, a short reaction time at 100°C (3 min for 4-methoxybenzamidine and 2 min for benzamidine) is selected in the analytical procedure. In this case, the final reaction mixture must be cooled quickly in ice-water at the end of the time in order to

Fig. 5 Effect of potassium hydroxide concentration on the fluorescence development in the reaction of D-glucose with benzamidine and 4-methoxybenzamidine.

Portions (1.0 ml) of 30 nmol ml⁻¹ D-glucose solution were reacted with the reagent of (a) benzamidine or (b) 4-methoxybenzamidine according to the procedure recommended with various concentrations of potassium hydroxide. c and d: reagent blanks corresponding to a and b, respectively.

stop the reaction. This treatment is also an aid in stabilizing the produced fluorescence for at least 1 h. When the reaction mixture is allowed to stand at room temperature (23 °C), the fluorescence intensity decreases gradually with time at the rate of about 20% h⁻¹ in both the reactions.

For developing fluorescence from reducing carbohydrates, both the reactions with 4-methoxybenzamidine and benzamidine should be carried out in an aqueous potassium or sodium hydroxide solution. The maximum intensity from D-glucose is attained by 1.0 M potassium hydroxide in both the reactions (Fig. 5). Sodium hydroxide at the concentration of 1.0 M gives slightly weak fluorescence, approximately 80% of that given by 1.0 M potassium hydroxide, in each reaction.

A concentration of benzamidine at 25 mM or greater, or of 4-methoxybenzamidine at 30 mM or greater provides a maximum constant fluorescence from D-glucose, but higher concentrations of each reagent result in increasing values of the reagent blank; 30 mM each solution was used in the analytical procedure.

The calibration graphs obtained by both the analytical procedures with 4-methoxybenzamidine and benzamidine were linear over the concentration range 0.1–200 nmol ml⁻¹ of D-glucose. The precision was established by repeating 20 analyses at the same time on 30 nmol ml⁻¹ D-glucose. The coefficients of variation were 1.9 and 2.1% for the methods with 4-methoxybenzamidine and benzamidine, respectively.

Fluorescences from other carbohydrates in the reaction with 4-methoxybenzamidine or benzamidine

All of the reducing carbohydrates tested can fluoresce under the reaction conditions established for both the

reagents. Their relative fluorescence intensities and the minimum determinable concentrations are listed in Table 2. The excitation and emission spectra of the fluorescences from these reducing carbohydrates are very similar in shape and maxima to those of the fluorescence from D-glucose and are not characteristic of individual carbohydrates, in each case using 4-methoxybenzamidine or benzamidine.

On equimolar basis, 4-methoxybenzamidine gives fluorescence intensities approximately 5.5 times higher than those given by benzamidine for all the reducing carbohydrates tested, though its reagent-blank value is also 4 times higher than that of benzamidine. Among the reducing carbohydrates tested, 2-deoxyaldoses and aminosugars fluoresce rather weakly, and D-erythrose (a tetrose) and DL-glyceraldehyde (a triose) fluoresce intensely in each reaction. In consequence, lower determinable limits of the carbohydrates with 4-methoxybenzamidine and benzamidine are given by 0.06–2.25 nmol ml⁻¹ and 0.09–2.40 nmol ml⁻¹, respectively.

Non-reducing carbohydrates including glycosides, alditols and aldonic acids such as sucrose, raffinose, stachyose, α -methyl-D-glucoside, D-glucose-1-phosphate, D-sorbitol, D-inositol, D-glucosaccharo-1,4-lactone and neuramic acid- β -methylglucoside give no fluorescence, except that gluconic acid gives fluorescences with 66 and 16% intensities of that given by an equimolar amount of D-glucose in the reactions with 4-methoxybenzamidine and benzamidine, respectively.

Feature of the reagents

4-Methoxybenzamidine and benzamidine permit fairly selective determination of reducing carbohydrates since these reagents do not provide fluorescences for 17 different L- α -amino acids, aldehydes (formaldehyde, acetaldehyde and benzaldehyde), keto acids (α -ketoglutaric acid, phenylpyruvic acid and *p*-hydroxyphenylpyruvic acid), carboxylic acids (acetic acid and formic acid) and nucleosides (adenosine and cytidine). These reagents also offer advantage of rapidity in reaction over other fluorogenic reagents so far reported for the determination of reducing carbohydrates. Thus, both the reagents may be successfully used for automatic detection system after chromatographic separation; 4-methoxybenzamidine in particular will be the

greater reagent in the system because it gives very high yield of fluorescence for reducing carbohydrates. This work is in progress in our laboratory.

The chemical structure of fluorescent product(s) in the reaction of reducing carbohydrates with 4-methoxybenzamidine or benzamidine remains unknown. The further study should be required for the mechanism of the fluorescence reactions with these aromatic amidines.

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