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Evaluation of BCMA as a New Color Reagent for the Choline and Spermine Tests

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Abstract. *bis*[3-*bis*(4-Chlorophenyl)methyl-4-dimethylaminophenyl]amine (BCMA) was tested as a color reagent for the enzymatic choline and spermine tests for the identification of semen in stains. The sensitivity as a function of optical density with BCMA was about 10-fold higher than that with the 4-aminoantipyrine (4AA)-phenol system and about 5-fold higher than that with the 4AA-N-ethyl-N-(3-methylphenyl)-N′-acetylethylenediamine (EMAE) system. However, the high sensitivity of the BCMA did not appreciably contribute to sensitive detection of choline in the stains with the naked eye, because of the absorption maximum (755 nm) close to infrared wavelength of the color developed from BCMA. The choline test with BCMA observed with the naked eye showed that the method did not exceed that with the previous 4AA-EMAE system in sensitivity, specificity and stability. BCMA could not be used for the qualitative detection of spermine in stains because of its too low content in small seminal samples. BCMA seems more useful for quantitation of substrates by spectrophotometry than for discrimination of its color with the naked eye.

Key words : Sex, Semen identification, Choline test, Spermine test, Seminal stains, BCMA

Introduction

The detection of choline and spermine seems useful for the identification of semen in stains as preliminary tests because these small molecules are much more resistant to putrefaction, aging and heat. The original crystal methods by Florence[1], Barberio[2] and Puranen[3] should be replaced by new enzymatic tests[4~6], because the classical methods are relatively time-consuming and sometimes not reliable[7].

Recently, *bis*[3-*bis*(4-chlorophenyl)methyl-4-dimethylaminophenyl]amine (BCMA) (Japanese patent No. 59-182361, Kyowa Medex Co., Ltd., Tokyo) has been developed as a new chromogenic reagent for the assay of hydrogen peroxide coupled with peroxidase reaction. Its chemical structure and reaction mode are shown in Fig. 1. This reagent produces a light green color with an absorption maximum at 755 nm (Fig. 2) and shows much higher sensitivity than the traditional 4-aminoantipyrine (4AA)-phenol system or 4AA-N-ethyl-N-(3-methylphenyl)-N′-acetylethylenediamine (EMAE) system. In the present study, we have tested this new reagent for detection of choline and spermine in human seminal stains.

Materials and Methods

1. *Materials*

BCMA blended with a detergent (0.122 g BCMA/g solid) and EMAE were kindly donated by Kyowa Medex Co., Ltd., Tokyo; choline oxidase (15.7 U/mg) was purchased from Toyobo Co., Ltd., Osaka; 4AA from Wako Pure Chemical Industries, Ltd., Osaka; and N-2-hydroxyethylpiperazine-N′-ethanesulfonic acid (HEPES) and choline-HCl from Sigma Chemical Co., St. Louis, Mo. Oat seedling polyamine oxidase was prepared as described previosuly[8]. Other common chemicals used were of the highest purity commercially available.

Various human body fluids, inculding semen, and juices of vegetables and fruits were dropped or smeared on filter paper (Tokyo Roshi No. 2, Tokyo). They were allowed to dry at room temperature and cut into small pieces (2 × 5 mm). Seminal stains kept at room temperature for 1 month to 28 years in our laboratory were also used.

2. *Methods*

1) Standard assay for choline with BCMA

One milligram of horseradish peroxidase, 2.5 mg

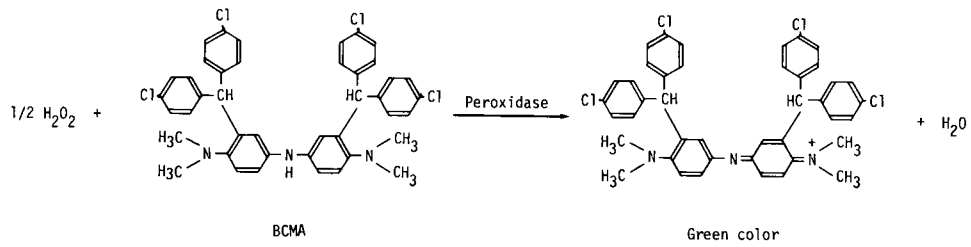

Fig. 1. Reaction of the BCMA system.

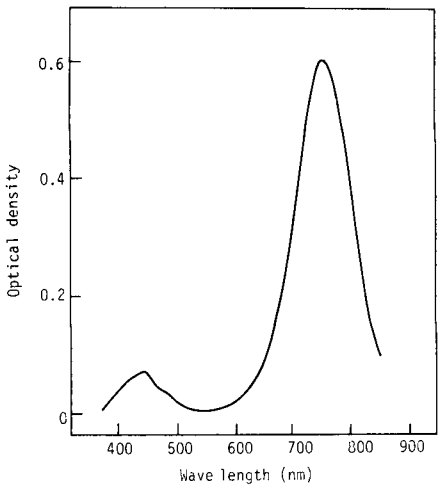

Fig. 2. Absorption spectrum of the product with the BCMA system. Twenty nanomoles of choline were reacted with choline oxidase and peroxidase in the presence of BCMA in 3.0 ml of the reaction mixture.

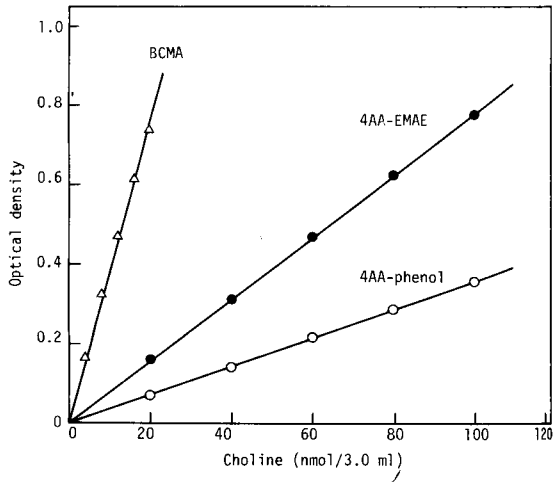

Fig. 3. Calibration curves with the systems of BCMA, 4AA-EMAE and 4AA-phenol as a function of various concentrations of choline. The incubation mixture was 3.0 ml.

choline oxidase and 5 mg BCMA with a detergent were dissolved in 20 ml of 0.05 M HEPES buffer (pH 6.5). This solution should be prepared just before use. For qualitative observation of its color, 0.5-1.0 ml of the above solution was added to a stain and incubated at 37°C for 30 min. For quantitative analysis, 2.0-3.0 ml of the solution was incubated and the resulting color intensity was measured at 755 nm on a spectrophotometer. It was confirmed that 30 min of incubation gave the maximal absorption, showing that the substrate was completely consumed within this period.

2) Assays with 4AA-phenol or 4AA-EMAE system

The quantitative assays of choline or spermine with 4AA-phenol or 4AA-EMAE system were carried out with potassium phosphate buffer (final 0.17 M, pH 7.4) as described previously[6),9)].

Results

Figure 3 shows calibration curves with the BCMA, 4AA-EMAE and 4AA-phenol systems as a function of various concentrations of choline present in test tubes. The sensitivity measured with optical density with BCMA was about 10-fold higher than that with the 4AA-phenol system and about 5-fold higher than that with the 4AA-EMAE system. The detection limit with the BCMA system was about 3 nmol/tube which gave an optical density of 0.1.

To check sensitivity of the qualitative detection of choline in stains, five seminal samples were serially diluted, dropped on filter paper and dried to make small seminal stains, which were then treated by the standard assay for choline with BCMA with the naked eye. As a result, all samples were positive up to a 4-8 fold dilution.

Seminal stains left at room temperature for various periods were subjected to the present qual-

Table 1. Choline test with BCMA observed with the naked eye on human seminal stains of various ages

Age of stain	Tested, *n*	Positive, *n*	Negative, *n*
1 month to 1 year	15	15	0
1 year to 2 years	19	19	0
3 years to 5 years	23	22	1
6 years to 10 years	12	12	0
11 years to 28 years	15	9	6

Table 2. Choline test with BCMA observed with the naked eye on the stains of various human body fluids

Body fluid	Tested, *n*	Positive, *n*	Negative, *n*
Semen	52	52	0
Blood	10	0	10
Saliva	11	0	11
Nasal discharge	7	0	7
Tears	4	0	4
Sweat	7	0	7
Breast milk	8	0	8
Vaginal fluid	27	2	25
Urine	25	0	25
Feces	11	0	11

itative method. The result is shown in Table 1. Except for one sample being negative from 3 to 5 years, all samples were positive up to 10 years of aging. From 11 to 28 years, some samples gave negative results.

Table 2 shows the results on various human body fluids. Except for semen, 2 out of 27 vaginal fluid samples were positive, but its color was much weaker than that of semen.

Table 3 shows the results on stains made by various common vegetables and fruits likely to be encountered in forensic science work. Although a majority of the samples were negative, some stains of vegetables, such as broad bean, lettuce, cauliflower and broccoli, gave positive results, although the color intensities were much weaker than that of semen. All fruit stains were negative.

Quantiative analyses of choline in seminal stains were also made with 2.0 ml of the BCMA solution. Small seminal stains (2 × 5 mm) gave 0.8-1.2 of optical density.

We tried to measure spermine in small seminal stains with the BCMA solution in the presence of oat seedling polyamine oxidase. The experiment gave an optical density of 0.08-0.24. This result means that the qualitative spermine test with the BCMA system observed with the naked eye is not suitable for routine analysis because stain samples with relatively low contents of spermine will largely be negative.

Table 3. Choline test with BCMA observed with the naked eye on the stains of vegetable and fruit extracts

Sample	Tested, *n*	Positive, *n*	Negative, *n*
Cabbage	2	0	2
Carrot	2	0	2
Radish	2	0	2
White potato	2	0	2
Barley malt	2	0	2
Green pepper	2	0	2
Spinach	2	0	2
Onion	2	0	2
Kidney bean	3	0	3
Green pea	3	0	3
Broad bean	2	2	0
Cucumber	2	0	2
Lettuce	2	1	1
Cauliflower	3	3	0
Broccoli	1	1	0
Grape	2	0	2
Grapefruit	3	0	3
Loquat	3	0	3
Melon	3	0	3
Mandarin orange	3	0	3
Watermelon	3	0	3
Banana	2	0	2
Mango	3	0	3
Pineapple	3	0	3
Papaya	1	0	1
Tomato	2	0	2
Kiwi fruit	1	0	1
Strawberry	2	0	2
Apple	2	0	2
Plum	3	0	3
Peach	2	0	2

Discussion

In the present study, we have introduced BCMA, a new chromogenic reagent into identification of semen in stains. It is based on the oxidase-dependent production of hydrogen peroxide; the hydrogen peroxide-dependent reaction of BCMA in the presence of peroxidase produces a light green color (Figs. 1 and 2). The advantage in the use of

BCMA is its high sensitivity when the optical density of its product is measured spectrophotometrically. The BCMA system gave the sensitivity about 10-fold higher than that of the 4AA-phenol system and about 5-fold higher than that of 4AA-EMAE system (Fig. 3). In our previous study[4] on the detection of seminal spermine, we used 2'-7'-dichlorofluorescin for the peroxidase system. This reagent gave enormously high sensitivity; its detection limit was less than 1.0 nmol/tube. However, unfortunately, it suffered from its autoxidation especially by light. The scientific level of research on the hydrogen peroxide detecting system in Japan seems most advanced in the world. It is expected that new and more advantageous systems will be developed in Japan in the future.

We have shown that the BCMA system can also be used for detecting choline in human seminal stains with the naked eye (Table 1-3). Although the system gave very high sensitivity by instrumental analysis (Fig. 3), its wavelength of absorption maximum (755 nm) is close to an infrared range; the color tone is not ideal for the observation with the naked eye. This is the reason why the sensitivity observed with the naked eye with BCMA system was lower than we expected. Thus the usefulness of the BCMA system for qualitative observation did not exceed that of our previous 4AA-EMAE system[5] in sensitivity, specificity and stability (Tables 1-3). However, for quantitative measurements of its optical density, the BCMA system seems to be the best choice at the present time.

The most classical reagent for detecting hydrogen peroxide is 4AA-phenol developed by Emerson[10] in 1943. This system gives a red color with an absorption maximum at 508 nm. The sensitivity of this system is fairly low (Fig. 3); 25 nmol of polyamine in 1.65 ml of an incubation mixture give an optical density of only about 0.1[9]. The detection of choline in seminal fluid[11] and its stains[12] by use of this system was reported. In addition to the low sensitivity of the system, the method is not suitable for bloody samples because of its red color. We therefore recommend the 4AA-EMAE system[5] for qualitative detection of choline in seminal stains with the naked eye, and the present BCMA system for quantitative analysis of choline and spermine in seminal fluid.

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新しい発色試薬BCMAのコリンならびにスペルミンテストにおける評価

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抄録 最近本邦で開発された過酸化水素検出発色試薬bis〔3-bis（4-chlorophenyl）methyl-4-dimethyl-aminophenyl〕amine（BCMA）を，精液証明法であるコリンならびにスペルミンテストに利用し，従来の4-アミノアンチピリン（4AA）-フェノール系や4AA-N-ethyl-N-(3-methylphenyl)-N′-acethylethyl-enediamine（EMAE）系と比較検討を行つたので報告する．吸光度測定によれば，BCMA系における感度は4AA-フェノール系の約10倍，4AA-EMAE系の約5倍の感度を有した．しかし，精液斑のコリン検出を肉眼で判定する場合にはBCMAによつて生ずる色素は明るい緑色で，むしろ赤外に近いため，高い吸光度の割には色調は強く感じられず，精々従来の4AA-EMAE系とほぼ同様な感度を有していた．精液斑中スペルミン検出をBCMAを用いて試みたところ，定量でも0.08～0.24の吸光度であり，肉眼判定では感度はさらに低くなり実用には不適であることが分つた．BCMAは肉眼判定よりむしろ吸光度測定による定量に威力を発揮するものと思われる．