TECHNICAL NOTE

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A Medicolegal Study on Enzymic Fluorometry of Choline in Human Semen

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ABSTRACT: Studies have been conducted on an enzymic fluorometric method based on an initial rate of reaction for the determination of choline. The reaction system consists of choline oxidase coupled to peroxidase and homovanillic acid. Concentrations of choline as low as 0.1 nmol could be detected by this procedure. The concentration of free choline in normal semen was 18.7 to 29.5 μmol/mL. Free choline in other body fluids was negligible. The choline concentrations in seminal stains maintained at room temperature were not changed during a 30-day period. Those concentrations in seminal fluids kept at room temperature were detected until at least the fifth day.

KEYWORDS: pathology and biology, choline, semen, sex, body fluid, enzymic fluorometry

It is well-known that normal semen contains a considerable amount of free choline which can be demonstrated by the classical Florence test [*1-2*]. This test is based on crystallization of choline, and has been used for presumptive identification of semen. Choline is more stable under various chemical conditions than other seminal enzymes [*3-6*], and its colorimetric determination as a means of presumptive test for the presence of semen has been shown to be advantageous in the medicolegal field [*7-8*].

In this paper we developed an enzymic fluorometric method for the microdetermination of the presence of free choline in semen, and also demonstrate the changes in concentration of choline in both seminal fluid and stains at room temperature.

Materials and Methods

Samples

Vaginal contents from women who had not had sexual intercourse for at least two weeks were collected in the Department of Obstetrics and Gynecology of Hokkaido University Hospital. Normal semen, serum, saliva, and urine were collected from healthy male individuals.

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The saliva was centrifuged at 3000 rpm for 10 min and the supernatant was used as the sample. All specimens were kept at −20°C until assayed.

The three different samples of seminal fluid in clear glass vials were allowed to stand at room temperature for ten days and the level of choline in each aliquot was measured at two-day intervals. Three other different seminal fluids (5 μL each) were placed on pieces of gauze (1 by 1 cm), and left to dry at room temperature. Each seminal stain was soaked in physiological saline and centrifuged at 3000 rpm for 10 min before assay. Changes in the level of choline in each supernatant were then observed every five days.

Procedure for Determination of Choline

The principle of the enzymic assay procedure is illustrated in Fig. 1. The free choline in the sample is first oxidized by choline oxidase (COD) to produce hydrogen peroxide and betaine. The hydrogen peroxide that is produced oxidizes and condenses homovanillic acid (HVA) in the presence of peroxidase (POD) to generate stoichiometrically a fluorescent compound. The fluorescent intensity was measured at a λ_{ex} = 310 nm and a λ_{em} = 426 nm using a Hitachi 650-10S fluorescence spectrophotometer.

Nonfluorescent water (Luminasol® water) was used for preparing all reagents or diluting samples and 0.1*M* Tris-hydrochloric acid buffer (pH 7.4) for making the reagent solutions. The standard assay procedure was carried out as follows. Into a cuvette (1 cm in width) were pipetted 1.0 mL of the buffer, 0.2 mL of HVA (10 nmol), 0.1 mL of POD (4 units), and 0.1 mL of either the standard solution or the sample, and mixed well with a pencil-type mixer. The cuvette was set into the fluorescence spectrophotometer. The excitation maximum was adjusted to 310 nm and the emission maximum to 426 nm. After the baseline became stable within several minutes and was adjusted to a zero intensity, 0.1 mL of COD (0.4 units) was added and immediately mixed. At about 30 s after the addition of the COD solution, an initial rate of change of fluorescence (ΔF/min) was recorded, and calibration plots were likewise made. All procedures were carried out at room temperature (25°C).

Chemicals

Choline oxidase [EC1.1.99.1] from Alcaligenes sp. (14 units/mg of protein) and horseradish peroxidase [EC1.11.1.7] (252 purpurogallin units/mg) were purchased from Toyobo Co., Japan. Homovanillic acid was obtained from Sigma Chemical Co., US. Choline chloride, Luminasol water (nonfluorescent water), and all other reagents were purchased from Wako Pure Chemical Industries, Ltd., Japan.

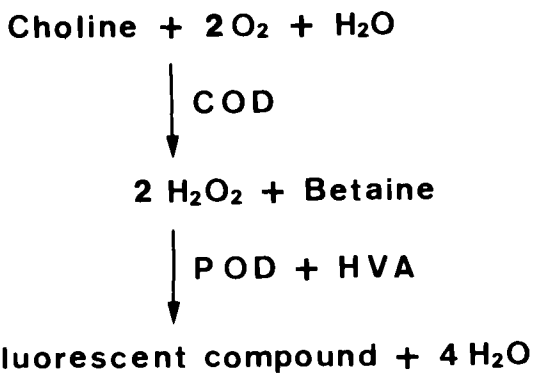

FIG. 1—*The principle of the enzymic fluorometry of choline: COD: choline oxidase, POD: peroxidase, and HVA: homovanillic acid.*

Results

The use of nonfluorescent water in the reaction system was necessary for stability of the recorder baseline. If either the concentration of HVA as substrate or pH of the buffer was increased, the baseline became unstable and high, probably because of autooxidation of HVA. Therefore, the pH of the buffer and the concentration of HVA were adjusted to 7.4 and 0.05m*M* (10 nmol/0.2 mL), respectively. By this assay system the initial rate of change of fluorescence (ΔF/min) was found to be directly proportional to the choline concentration. At the concentrations of 0.2 to 1.6 nmol of choline, analysis could be performed within 2 to 3 min based on the initial rate method.

When the concentration of the standard choline chloride solution was varied as shown in Fig. 2, there was a linear relationship between the concentration of the choline chloride and the fluorescent compounds. Four units of peroxidase and 0.4 units of choline oxidase were enough to convert 1.6 nmol of choline to the corresponding fluorescent products. A level of free choline as low as 0.1 nmol in the assay system could be detected. The coefficient of variation of intra- and inter-assays ($n = 10$) was 2.2 and 3.2%, respectively.

The concentration of free choline in various biological fluids is summarized in Table 1. The level of free choline in normal semen was 18.7 to 29.5 μmol/mL. Only a trace amount of choline was detected in vaginal contents and urine. No choline was detected in serum and saliva (Table 1).

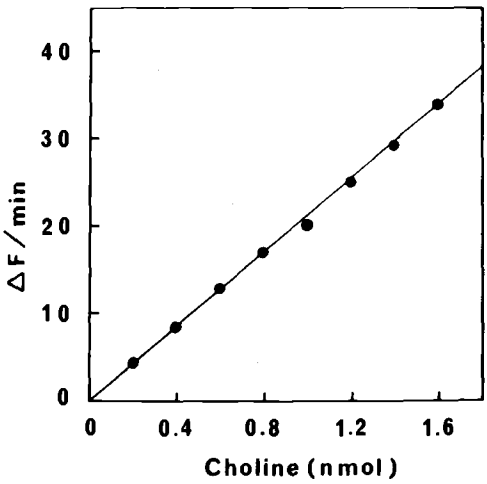

FIG. 2—*The calibration curve for free choline chloride.*

TABLE 1—*Concentration of choline in body fluid.*

Body Fluids	No. of Samples	Choline, μmol/mL
Semen	10	24.2 ±1.72[a]
Vaginal content	8	0.025 ±0.0088
Urine	10	0.019 ±0.0051
Saliva	10	ND[b]
Serum	10	ND

[a]Mean ± standard error.
[b]Not detected.

The amount of choline recovered which had been added to a constant volume of semen and urine is shown in Tables 2 and 3. The semen and urine used were diluted 4000- and 200-fold, respectively, with the buffer. Choline chloride was added in the assay system in amounts as indicated in Tables 2 and 3. The recovery of the choline added to the semen and urine was 99.3 ± 1.84 and 97.6 ± 0.72%. Their coefficient of variation was 1.85 and 0.74%, respectively.

The time course of the level of choline in the seminal fluids and stains was also observed. The changes in the choline levels in three different seminal fluids allowed to stand at room temperature for ten days are shown in Fig. 3. This indicates that the amount of choline gradually decreased on a daily basis but was detectable until at least the fifth day. On the other hand, the choline levels in three different seminal stains maintained at room temperature were not changed during a thirty-day period (Fig. 4).

Discussion

In this paper we describe a method for the determination of trace amounts of free choline in biological fluids using an enzymic fluorometry. Its application to forensic science practice is evident. This method is based on the initial rate of change of fluorescence (ΔF/min) produced by the oxidative condensation of homovanillic acid using the enzymes, choline oxidase, and peroxidase. Accuracy and precision were achieved. The sensitivity of this method was approximately 200 times greater than that of a previously described enzymic colorimetric method [7].

As shown in Table 2, good recovery of the choline was achieved. This indicates that there was no inhibition of the choline oxidase-peroxidase enzyme mixture which is specific for free choline [7]. However, the amount of choline recovered that had been added to urine was slightly decreased.

Normal semen contained a considerable amount of choline; the level was found to be 18.7 to 29.5 μmol/mL. At least 0.01 μL of seminal fluid was needed to determine the content of free choline. As little as 0.1 nmol of choline could be measured by this method. In other biological fluids the free choline levels were practically negligible. The choline levels in seminal stains were very stable and unchanged during a 30-day period. The concentration of choline

TABLE 2—*Recovery of choline in semen.*

Added, μmol/mL (Choline Chloride)	Found, μmol/mL	Difference	Recovery, %
0	16.7	...	...
8	25.1	8.4	101.2
16	32.2	15.5	98.5
24	40.2	23.5	98.8
32	47.2	30.5	96.9
40	57.3	40.6	101.1

TABLE 3—*Recovery of choline in urine.*

Added, μmol/mL (Choline Chloride)	Found, μmol/mL	Difference	Recovery, %
0	0.034	...	...
0.4	0.418	0.384	96.3
0.8	0.814	0.784	97.6
1.2	1.200	1.166	97.2
1.6	1.575	1.541	96.4
2.0	1.954	1.920	97.7

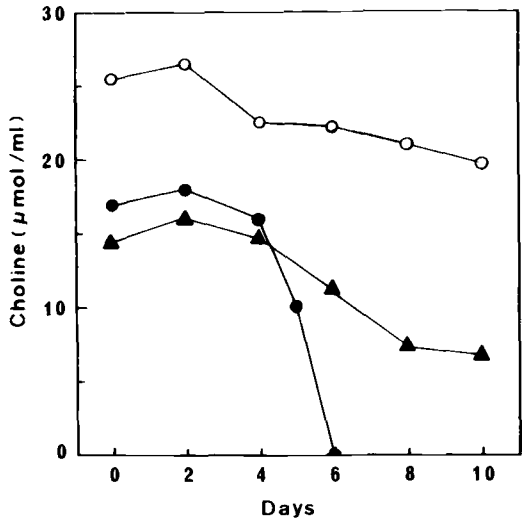

FIG. 3—*The change in the choline concentration in seminal fluids at room temperature during ten days.*

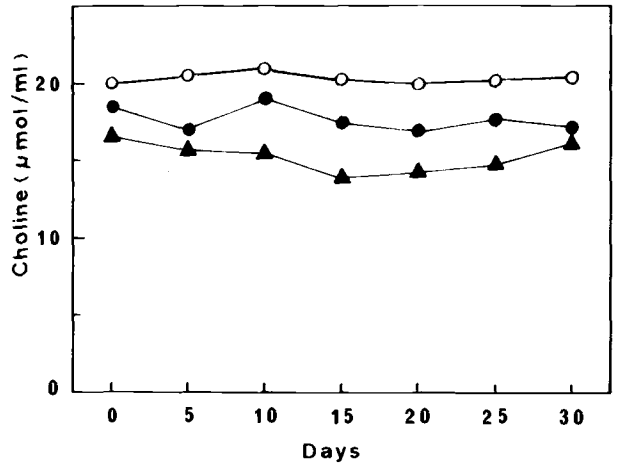

FIG. 4—*The change in the choline concentration in seminal stains at room temperature during 30 days.*

in seminal fluid, on the other hand, was gradually decreased although the reduction rate among the samples was different. It would appear that the choline was metabolized or decomposed or both by microorganisms proliferating in the vial during incubation. However, the choline levels in seminal fluids were maintained until at least the fifth day (Fig. 4).

The enzymic fluorometric method reported here is very useful to detect trace amounts of choline. Where no spermatozoa are found, particularly in vaginal contents from victims of sexual offenses, the demonstration of certain concentrations of choline can serve as presumptive evidence of semen.

Acknowledgments

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References

[1] Beeman, J., "Notes on the Identification of Seminal Stains by Means of the Florence Reaction," *Archives of Pathology*, Vol. 34, No. 5, Nov. 1942, pp. 932-933.

[2] Polson, C. J. and Gee, D. J., *The Essential of Forensic Medicine*, 3rd ed., Pergamon Press, Oxford, 1973, pp. 507-512.

[3] Kind, S. S., "The Acid Phosphatase Test," in *Methods of Forensic Science*, Vol. 3, A. S. Curry, Ed., Wiley Interscience, New York, 1964, pp. 267-287.

[4] Adams, E. C. and Wraxall, B. G., "Phosphatase in Body Fluid: the Differentiation of Semen and Vaginal Secretion," *Forensic Science*, Vol. 3, No. 1, Jan./Feb. 1974, pp. 57-62.

[5] Nakanishi, K., "Studies on γ-Glutamyl Transpeptidase of Semen From the Medicolegal Aspects. Part 1. γ-Glutamyl Transpeptidase in Human Seminal Fluid," *The Japanese Journal of Legal Medicine*, Vol. 30, No. 4, July 1976, pp. 281-287.

[6] Suzuki, O., Oya, M., Katsumata, Y., Yada, S., and Higashide, K., "A Medico-Legal Study on Diamine Oxidase in Human Semen," *The Japanese Journal of Legal Medicine*, Vol. 33, No. 1, Feb. 1979, pp. 1-6.

[7] Takatori, T., Tomii, S., and Tanaka, T., "The Determination of Choline in Human Semen by the Enzymic Method," *Forensic Science International*, Vol. 17, No. 1, Jan./Feb. 1981, pp. 79-84.

[8] Suzuki, O., Matsumoto, T., Oya, M., Katsumata, Y., and Asano, M., "A New Enzymatic Method for the Demonstration of Choline in Human Seminal Stains," *Journal of Forensic Sciences*, Vol. 26, No. 2, April 1981, pp. 410-415.

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