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Note

Rapid thin-layer chromatographic–densitometric determination of histamine in tuna

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Cases of poisoning by partially spoiled scombroid fishes have been known for a number of years, but the causative agent has not been completely defined. Several reports[1–3] suggest that histamine is responsible, or at least that its level might be used as an indicator of the existence of the toxic agent or agents[4–6].

In 1956 Code and McIntire[7] reviewed some chemical methods for the determination of histamine. Included were colorimetric procedures involving the coupling of histamine to a diazotized aromatic amine or to dinitroflurobenzene, and an isotope dilution technique involving the coupling of histamine with labeled pipsyl chloride followed by the addition of unlabeled dipipsyhistamine and recrystallization to constant activity. These techniques were superseded by fluorometric methods which were reviewed by Shore[8] and have been modified by Staruszkiewicz *et al.*[9]. Such methods allowed greater sensitivity and specificity compared to the older colorimetric methods. The fluorometric methods are based on the coupling of histamine with O-phthalaldehyde (OPT) at high pH to form a fluorescent product which rearranges upon acidification to form an even more highly fluorescent and stable fluorophore[10]. The acid stability of the histamine–OPT fluorophore lends additional specificity to the procedure, as a number of other primary amines couple with OPT in a basic medium to form Schiff base products which, however, are broken down to their constituents upon acidification. OPT forms acid-stable reaction products with a few other substances besides histamine; among them, for example, histidine which occurs in large amounts in tuna[11]. Thus, the procedures for the separation of histamine from these substances are important, but have been time-consuming and laborious.

Thin-layer chromatography (TLC)–densitometry has been used widely for lipid analysis (for example, Blank *et al.*[12]) and for histamine and its metabolities by Schwartzman[13] and Schwartzman and Halliwell[14]. However, as described, these methods take 4 to 5 h for TLC development. The method presented in this report does not require preliminary column purification of the extract and is a quick and simple method for determination of histamine in fish.

EXPERIMENTAL

Materials and methods

Pre-coated silica gel glass plates (Sil G-25, 20 × 20 cm, 0.25 mm thick, with-

out gypsum; Brinkmann, Burlingame, Calif., U.S.A.) were used. Reagents employed were: reagent-grade methanol (Mallinckrodt, St. Louis, Mo., U.S.A.); reagent-grade ammonium hydroxide (Allied Chemical, Morristown, N.J., U.S.A.); ninhydrin (Nutritional Biochemicals, Cleveland, Ohio, U.S.A.); histamine·2 HCl and histidine·HCl (Sigma, St. Louis, Mo., U.S.A.). Also, a TLC densitometer Model 530 with 570 nm filter (Photovolt, New York, N.Y., U.S.A.), and a Waring blender Model 5012 with Waring mini-jar MC2 (Waring, Winsted, Conn., U.S.A.) were used.

Standard solutions were prepared from histamine·2 HCl and histidine·HCl that had been held in a desiccator. Amounts of 20 mg of each were dissolved in 4 ml of distilled water, and diluted to a final volume of 20 ml with methanol (solution A). Further dilutions were prepared as indicated in Table I.

TABLE I

PREPARATION OF STANDARD SOLUTIONS

Solution	*Preparation*	*Histamine (μg per 10 μl)*	*Histidine (μg per 10 μl)*
A		6.0	8.1
B	4 ml of A + 1 ml of methanol	4.8	6.5
C	3 ml of A + 2 ml of methanol	3.6	4.9
D	2 ml of A + 3 ml of methanol	2.4	3.2
E	1 ml of A + 4 ml of methanol	1.2	1.6
F	0.5 ml of A + 4.5 ml of methanol	0.6	0.8

For calibration, 10 μl of each of the standard solutions (A to F) are spotted on a TLC plate (which had been activated in an oven at 110° for 1 h and stored in a desiccator), and dried under nitrogen. The TLC plate is developed with methanol–ammonia (20:1) solution (freshly prepared) in a multi-plate TLC glass tank (9¼ × 4 × 8 in.) for 70 min at room temperature, dried in an oven at 90° for 8 min immediately after development, removed, sprayed with ninhydrin solution (3% in methanol), and developed for 20 min at room temperature. This achieves uniform color and maximum intensity. The plate is scanned in the densitometer and the areas of the trapezoid peaks (Fig. 1) are measured and plotted against concentrations.

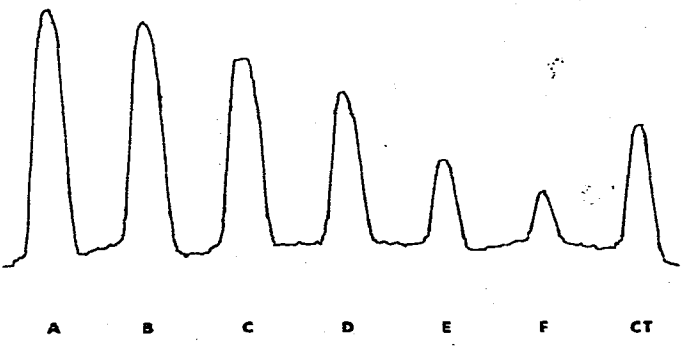

Fig. 1. Densitometer scan of histamine spots on the TLC plate shown in Fig. 2.

The sample of fish or canned fish (whole can) is comminuted in a food blender, and 10 g are transferred to the mini-jar of a high-speed blender[15]. A 50-ml volume of methanol is added, the whole blended for about 2 min, and transferred to a 100-ml volumetric flask. The lid and blender jar are rinsed with methanol and the rinsings are added to the flask. The flask is then heated in a water-bath to 60° and allowed to stand at this temperature for 15 min with occasional shaking, cooled to room temperature, adjusted to volume with methanol to 100 ml, and filtered.

Methanol extracts of the sample (10–50 μl depending on the amount of histamine) are spotted together with 2 spots of standard solution on the same plate and developed as described above.

Each determination of histamine was calibrated against standards because of slight variations in chromatographic conditions between runs. The standard deviation was less than 7%.

RESULTS AND DISCUSSION

Since histamine was well separated from other constituents (Fig. 2), the TLC–densitometry method did not require the preliminary purification steps which are required for other fluorometric[8,9] or colorimetric[7] methods. About ten samples could be applied on one plate. The purple color developed within minutes after spraying with ninhydrin and was stable for at least two months at −4°, in contrast to a less

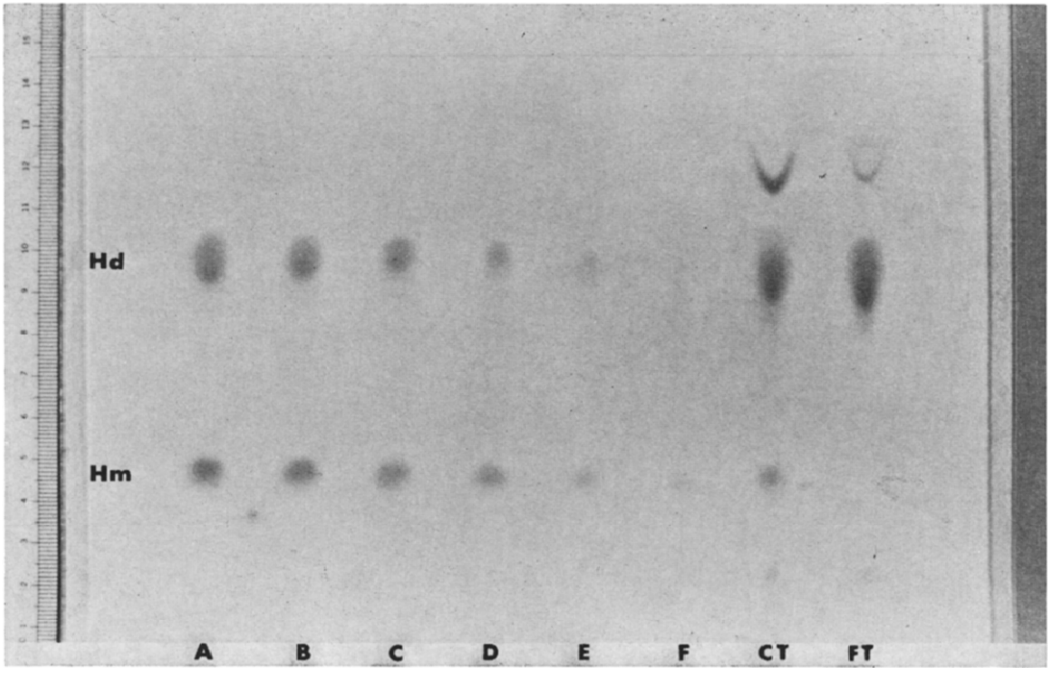

Fig. 2. TLC of histamine (HM; $R_F = 0.32$) and histidine (Hd; $R_F = 0.48$) from standard mixtures, solutions A–F (see text) and from methanol extracts of tuna. CT is a methanol extract of a canned tuna known to have caused illness, and FT is an extract of fresh tuna.

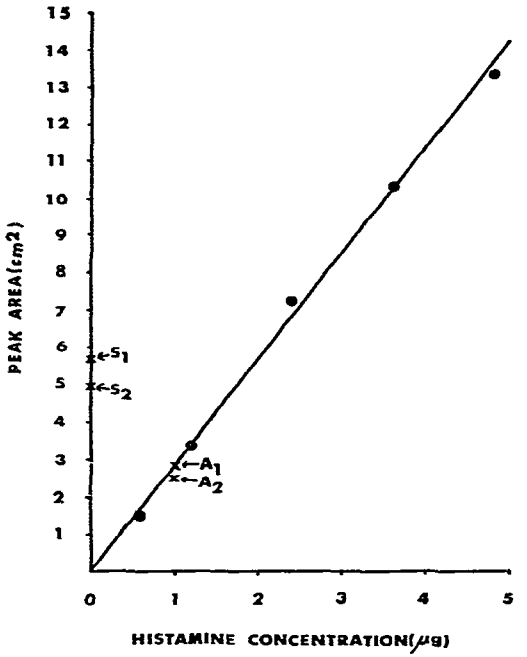

Fig. 3. Relationship between histamine concentration and densitometer peak area. S_1 = calibrated peak area of the sample; S_2 = experimental peak area of the sample; A_1 = peak area of the standard on original calibration curve; A_2 = peak area of the standard on sample plate.

stable diazo stain[16]. The quantitative determination of histamine was performed with the densitometer in the dark.

Calculation of the amount of histamine in the extract was made by interpolation of the standard curve after correction of the area of the peak from the extract based on the area obtained from the standard on the same plate. The corrected peak area (S_1) is obtained by multiplication of the area of the sample (S_2) times A_1/A_2, where A_2 is the area for the standard on the same sample plate and A_1 is the area for the same amount of standard from the original calibration curve. In Fig. 3 for example:

$$S_1 = S_2 \times \frac{A_1}{A_2} = 4.9 \text{ cm}^2 \times \frac{2.9}{2.5} = 5.7 \text{ cm}^2$$

From the value of S_1, the amount of histamine in the sample applied to the plate was 2 μg. The sensitivity of the method is 0.1 μg, and the working range up to 5 μg.

With a limited series the rates of recovery of pure histamine added to samples of fish before extraction was 58 $\pm$ 3%. Recoveries could be improved to 90–95% by modifying the procedure as follows: extract the ground fish for 15 min with 50 ml methanol at 90°, centrifuge, wash the pellet twice with methanol, re-extract at 90° with more methanol and wash again. The combined methanol extracts and washes are filtered through dry Whatman No. 42 filter paper and brought to volume.

This method avoids several time-consuming steps, such as ion-exchange column

chromatography and derivatization in the fluorometric method. The operation of the TLC–densitometry measurement of histamine was performed with the same TLC plate without any sample transfer after the methanol extraction. For many purposes visual inspection of the spot was sufficient to enable an estimate of the histamine level. If the sample is a liquid, such as tissue juice or bacteria broth, it can be directly applied to the TLC plate. The possibilities of using the method for the identification of histidine-decarboxylating bacteria and for the determination of histidine are being studied.

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