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Note

Determination of histamine in plasma by high-speed liquid chromatography

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The fluorometric method so far described for determination of histamine in biological samples is based on the reaction of histamine with *ortho*-phthalic aldehyde (OPT) in an alkaline medium to form a fluorescent product, which is converted by acidification to a more highly fluorescent and stable species [1]. Although fluorescent products formed from many amines of biological importance, with OPT in alkaline media, are destroyed by the acidification, spermidine and a few amino acids give acid-stable fluorescent products with the same procedure [2—4]. Therefore, for the assay of histamine in biological samples by the OPT method, a special purification technique such as ion-exchange chromatography has been required prior to reaction with OPT [5—9].

Recently, we found that the fluorophores of histamine and spermidine with OPT could be separated successfully by reversed-phase high-speed liquid chromatography (HSLC) though their structures remained unknown. In this paper, HSLC is used effectively for the determination of histamine in plasma. This method gives reliable results and is readily performed with 1 ml of plasma containing as little as 0.2 pmoles of histamine.

EXPERIMENTAL

Chromatographic apparatus, and preparation of the column and the mobile phase

The liquid-chromatographic system consisted of a Hitachi 635 Liquid Chromatograph, a Hitachi 203 Spectrophotofluorometer equipped with a Hitachi flow-cell unit (cell volume, 20 μl) and a Hitachi 056 Recorder (chart speed, 2.5 mm/min). The fluorescence intensity was monitored at the emission wavelength of 450 nm with the excitation wavelength set at 350 nm.

A stainless-steel column (15 × 0.4 cm, I.D.) was packed by the slurry tech-

nique: to 10 ml of a mixture of dioxane, tetrachloromethane and tetrabromoethane (40:40:20), 1.0 g of LiChrosorb RP-18 (5 μm; Merck, Tokyo, Japan) was added. After the slurry was poured into the packing reservoir, chloroform was made to flow through the column at 150—300 kg/cm^2 for 1 h. Then, the mobile phase described below was made to flow at the rate of 0.5 ml/min for 30 min. The column thus packed can be used for more than 300 injections with only a small decrease in the theoretical plate number. The system was operated at room temperature.

The mobile phase was a mixture of 0.2 *M* NaCl solution and methanol (55:45) adjusted to pH 3 with 0.1 *M* HCl, by measurement with a Hitachi-Horiba M-7 pH meter.

Chemicals and reagents

All of the chemicals and solvents used were of reagent grade. *n*-Butanol, benzene and methanol were redistilled before use. An NaCl-saturated sodium hydroxide solution was prepared by adding 30 g of NaCl to 100 ml of 1 *M* NaOH.

Standard solution

A stock solution (1×10^{-3} *M* histamine) was prepared by dissolving 184 mg of histamine·2HCl (Wako, Osaka, Japan) in 100 ml of distilled water. The working solution (5×10^{-8} *M* histamine) was prepared by diluting the stock solution with distilled water to use within a day.

Analysis of plasma

To 1.0 ml of plasma placed in a 10-ml centrifuge tube, 1.0 ml of 0.4 *M* $HClO_4$ was added. The mixture was stirred well and centrifuged at 1200 *g* for 10 min. A 1.0-ml sample of the supernatant solution was transferred to a 10-ml glass-stoppered shaking tube containing 0.3 ml of 5 *M* NaOH, 0.4 g of NaCl and 2.5 ml of *n*-butanol. The contents of the tube were shaken for 5 min on a mechanical shaker. After centrifugation, the aqueous phase was aspirated, followed by the addition of 1.5 ml of NaCl-saturated sodium hydroxide solution to remove contaminants such as amino acids. The contents of the tube were again mixed for 3 min. After centrifugation, 2.0 ml of the organic phase were transferred to another 10-ml glass-stoppered shaking tube containing 0.25 ml of 0.1 *M* HCl and 2.0 ml of benzene. The mixture was shaken for 3 min. After centrifugation, 0.1 ml of the acidic aqueous phase was transferred to a small test-tube, to which 20 μl of 1 *M* NaOH and 5 μl of 1% OPT—methanol solution were successively added to develop fluorescence. After mixing for exactly 4 min, 10 μl of 0.95 *M* H_2SO_4 was added to stop the fluorescence reaction. A 100-μl volume of the mixture was injected for HSLC—fluorescence analysis. As the standard, 1.0 ml of histamine working solution described above was used instead of plasma. Concentration of plasma histamine was calculated as follows:

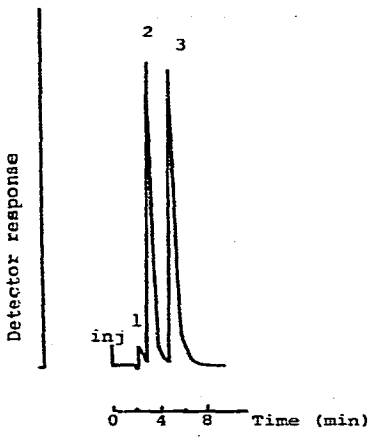
$$\text{Histamine concentration (ng/ml)} = \frac{111}{1000} \times \frac{\text{peak height of sample (mm)}}{\text{peak height of standard (mm)}} \times \left[\begin{matrix}\text{concentration of histamine} \\ \text{standard solution (pmole/ml)}\end{matrix}\right]$$

where $C_5H_9N_3 = 111$.

RESULTS AND DISCUSSION

As shown in Fig. 1, OPT-histamine fluorophore (OPT-his) and OPT-spermidine fluorophore (OPT-spd) formed in the reaction mixture were completely separated, with retention times of 5.0 and 3.0 min, respectively. The peaks observed at the retention time of 2.0—3.0 min were due to the reagent blank. Since the reagent blank showed a weak fluorescence, overlapped with the fluoroscence of OPT-his in the range of 400—480 nm, the separation of OPT-his from the fluorophore of the reagent blank greatly enhanced the sensitivity of the method for the determination of histamine by OPT reagent.

When the methanol content of the mobile phase decreased, the retention time of OPT-his was delayed considerably with broadening of the peak, though the peaks of OPT-spd and the reagent blank remained unchanged. On the other hand, if the methanol content was more than 60%, the peaks of OPT-his and OPT-spd became overlapped. The pH of the mobile phase and the presence of NaCl in the mobile phase were found to affect the peak width of OPT-his. When the pH was made greater than 3 and NaCl was not added, the peak of OPT-his tailed badly.



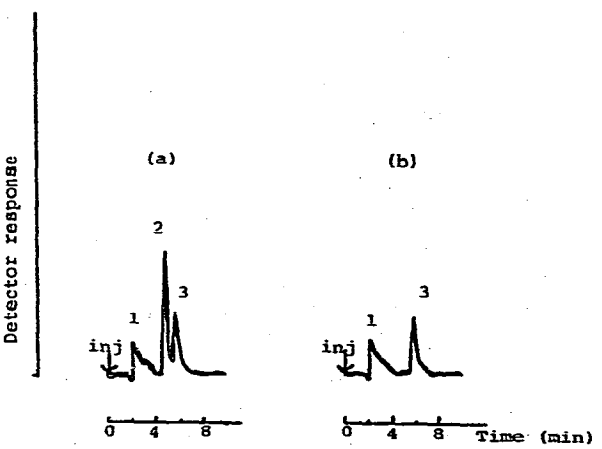


Fig. 1. Separation of OPT-his and OPT-spd. A mixture of 1×10^{-7} *M* histamine and 1×10^{-4} *M* spermidine was used. Peaks: 1, OPT reagent blank; 2, spermidine; 3, histamine.

Fig. 2. Chromatograms from analysis of plasma sample under the prescribed conditions. (a) = plasma (0.84 ng/ml histamine); (b) = reagent blank. Peaks: 1, OPT reagent blank; 2, histamine; 3, unidentified peak caused by the extracting solvents.

Fig. 2 shows typical chromatograms obtained with plasma and the reagent blank under the given conditions. The peak observed at the retention time of 6.0 min was caused by the extracting solvents.

There was a linear relationship between the fluorescence intensity (peak height) and the concentration of histamine in the range of 0.2—100 pmole/ml. The histamine content of plasma might be calculated by the ratio of the peak height of sample to that of a standard solution. The limit of sensitivity of the method was 0.2 pmoles of histamine, which corresponded to 22.2 pg/

ml when calculated in terms of sensitivity for plasma. The sensitivity was defined as the amount giving a signal-to-noise ratio of 2.

n-Butanol is a good solvent for the extraction of histamine and other biogenic amines from tissues and biological fluids deproteinized by perchloric acid [1]. By washing the *n*-butanol extract with salt-saturated alkaline solution, amino acids can be removed. The specificity of the present method was examined for some biogenic amines. Polyamines, catecholamines and indoleamines did not interfere even if added to plasma in amounts more than 5 times normal.

The recovery of histamine was checked by adding known amounts of histamine (5—50 pmole/ml) to pooled plasma. Recoveries were 96 ± 4%. The precision of the method was examined by performing 20 assays on pooled plasma containing 25 and 50 pmole/ml histamine. The standard deviations were 1.34 and 2.4, respectively. The coefficients of variation were 4.9% for 50 pmole/ml and 5.6% for 25 pmole/ml histamine, respectively.

The amount of histamine in the plasma of 10 healthy men (26—29 years) determined by this method was 0.61 ± 0.16 ng/ml, which was in good agreement with 0.62 ± 0.3 ng/ml and 0.69 ± 0.26 ng/ml which were described by Graham et al. [7] and Lorenz et al. [9], respectively.

The present HSLC method for the determination of plasma histamine has the following advantages over other methods, including the enzymatic isotopic method [10]: analyses are carried out rapidly with small amounts of sample and the sensitivity can be greatly improved.

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