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Note

Histamine assay in tears by fluorescamine derivatization and high-performance liquid chromatography

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Histamine has been found in human tears at a level of about 10 ng/ml [1], an amount significantly increased in allergic conjunctivitis [2]. Histamine release is regarded as responsible for the characteristic clinical pattern of lachrymation, itching, oedema and vasodilation present in allergic conjunctival reactions.

The histamine content of tears has been determined by means of radioenzymatic assay [3—5], which requires extraction procedures and the use of a highly purified enzyme in order to enhance sensitivity [6, 7]. Several methods for histamine assay in biological fluids have been reported, involving gas chromatography [8], low-pressure liquid chromatography [9], mass spectrometry [10, 11] and high-performance liquid chromatography (HPLC) after derivatization of histamine with various reagents [12—16].

The aim of this investigation was to make available a fast and reliable method for histamine determination (1) in a small tear volume (5—10 μl), (2) at picomole levels, (3) without sample pretreatment [17], (4) by fluorescamine derivatization [18], (5) by HPLC technique and fluorimetric measurement of the characteristic fluorophore easily separable from analogous imidazole,

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amino acid and amine derivatives, (6) with a procedure requiring only a few minutes.

The method appears particularly useful since the histamine content of tears can be considered — as an alternative to the currently available irritation tests — as a possible index of conjunctival irritation, and it allows the evaluation of the effects of chemicals, drugs and cosmetics to which subjects have been topically exposed [19].

EXPERIMENTAL

Apparatus

We used a Perkin-Elmer S-3b liquid chromatograph equipped with a Rheodyne 7125 injector valve, an ASVI automatic switching valve and LS-4 fluorimetric detector set at excitation and emission wavelengths of 390 nm and 480 nm, respectively.

A Sigma 15 data station was employed. Separation was achieved with a C_8 (10 μm) Nucleosil or RP-8 (10 μm) Merck column and a pre-column fitting with LiChroprep RP-8 (20—40 μm) using the mobile phase acetonitrile—phosphate buffer (potassium dihydrogen phosphate) 0.004 *M* at pH 3.5 (65:35) in isocratic elution at a flow-rate of 0.5 ml/min.

Reagents and materials

Fluorescamine and histamine were obtained from Sigma (St. Louis, MO, U.S.A.). The HPLC-grade solvents were filtered (0.45 μm) and degassed by sonication before use. Fluorescamine stock solution was 20 mg in 100 ml of acetonitrile; histamine stock solution was 1 mg/ml in water. Sodium borate solution was 0.2 *M* at pH 9.1. All reagents were stored at 4°C.

Sample preparation

Standards. The stock solution of histamine was diluted in water to concentrations ranging from 0.05 to 20.0 ng/ml.

Samples. Tears (20-μl samples) were collected from healthy volunteers and allergic conjunctivitis patients by placing a polystyrene capillary tube in the lower fornix of the conjunctiva.

All samples were kept in polystyrene tubes at −20° C prior to assay.

Sample derivatization

Sodium borate buffer (40 μl) and 10 μl of tears were mixed under vigorous agitation with 50 μl of fluorescamine solution. Aliquots of 20 μl of the resulting solution were injected into the HPLC system.

RESULTS AND DISCUSSION

After fluorescamine derivatization, stopped-flow analysis was performed to obtain accurate excitation and emission wavelength values for the histamine fluorophore, which depend on the mobile phase used (Fig. 1).

Fig. 2 shows representative chromatograms of histamine detection in standard solution (20 ng/ml) and in normal (5.2 ng/ml) and allergic conjunctivitis (50.6 ng/ml) human tear samples. Peaks were characterized by the method of

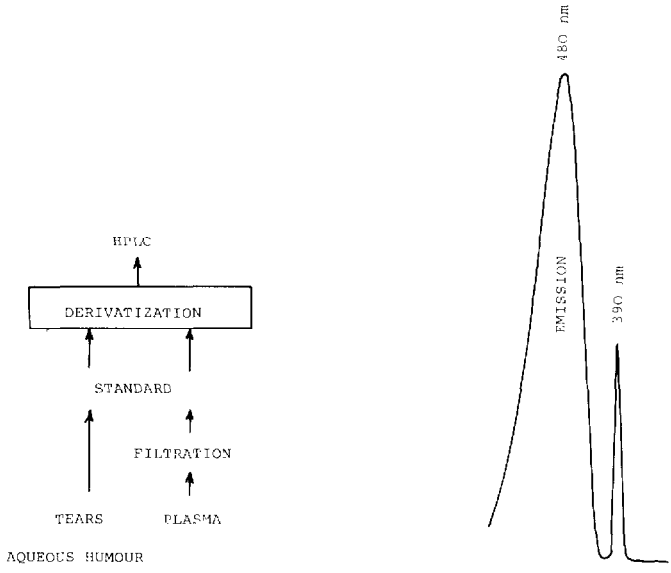

Fig. 1. Sample preparation steps and excitation—emission spectrum of histamine obtained by stopped-flow after fluorescamine derivatization.

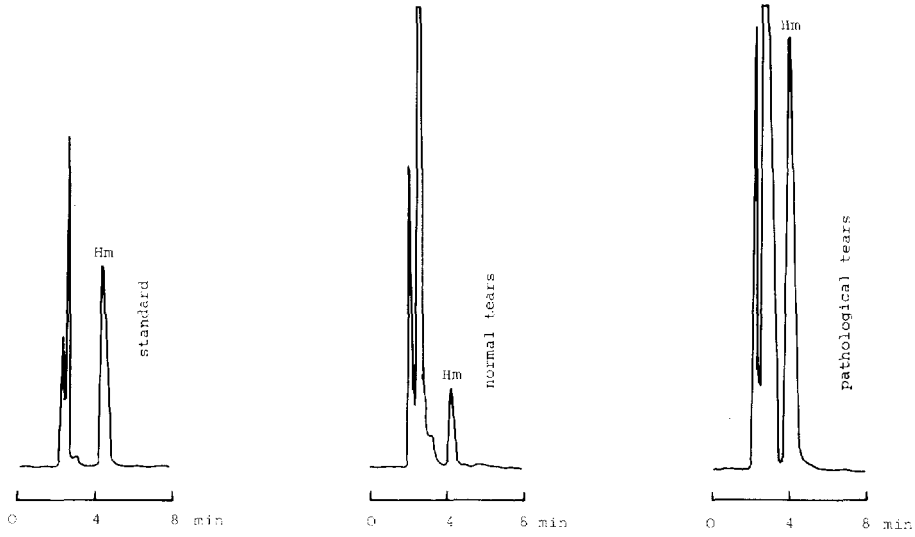

Fig. 2. Chromatograms of histamine (Hm) from standard solution (20 ng/ml) and from normal (5.2 ng/ml) and pathological (50.6 ng/ml) human tear samples. Injection volume 20 μl. Mobile phase: 0.004 *M* phosphate buffer adjusted to pH 3.5 and acetonitrile (35:65) at a flow-rate of 0.5 ml/min.

standard addition and by scanning the excitation and emission spectra of the peak that eluted at the same retention time as the standard using the stopped-flow technique ($k' = 0.91$). Since a reduced injection volume is preferable in routine analysis, where large numbers of samples are handled, the effect of injection volume on resolution was investigated for peaks eluting close to the solvent front. Consequently all detections were carried out by injecting 20 μl of each derivatized sample.

The stability of the histamine fluorophore was tested for at least 3 h by repeated analyses of standard and single tear samples at intervals of 10 min. The reproducibility was 1.5% (relative standard deviation, n = 20).

Fig. 3 shows the calibration curves of histamine in standard solution and added to a human tear sample. The correlation and the similar slopes of the curves confirm the usefulness of the method, which can be applied directly to other biological fluids as reported in Fig. 4.

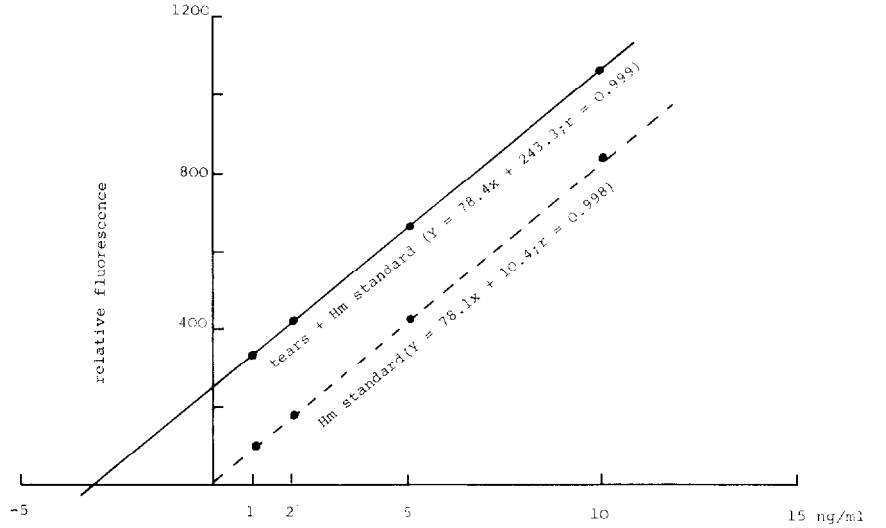

Fig. 3. Calibration curves of histamine (Hm) in standard solution (- - -) and in a human tear sample by standard addition method (——).

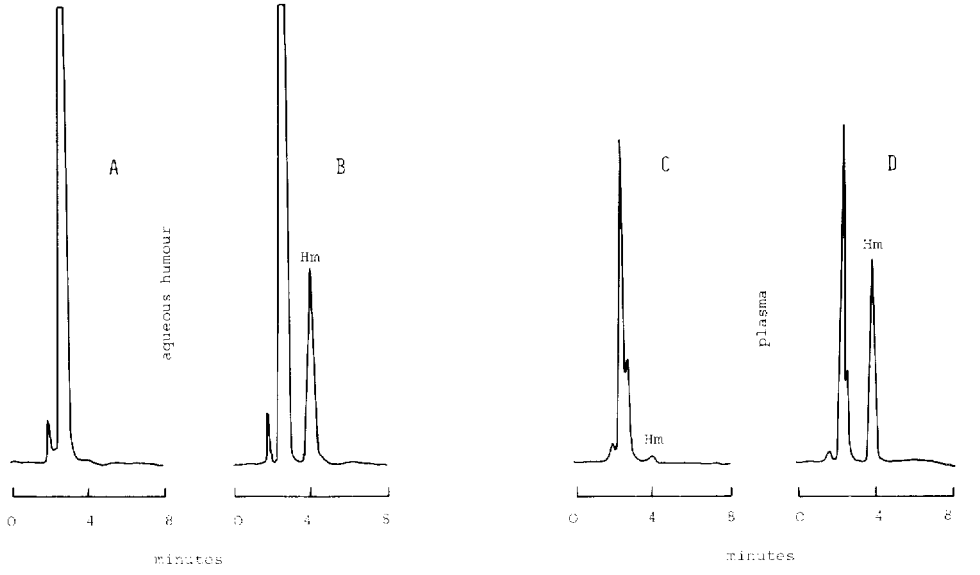

Fig. 4. Chromatograms of 10 μl of human aqueous humour and plasma samples obtained directly (A, C) and by standard addition (B, D) as described in the text. Injection volume 20 μl. Histamine (Hm) detection: not detected in aqueous humour (A), 0.61 ng/ml in plasma (C).

The results show, moreover, that the histamine content of tears can be determined without using deproteinizing agents. The combined action of acetonitrile and the pH of the mobile phase allows retention of the protein fraction in the pre-column system without memory effects [17]. The influence of the pre-column on the capacity factor is negligible; the packing material has to be replaced when the resolution decreases in relation to the number of injected samples. Even though the post-column derivatization reaction has several advantages, the pre-column technique is preferred as it is a simple system suitable for rapid analytical purposes.

The fluorescamine derivatization of histamine occurs rapidly at pH 8.5—9.0 giving a highly fluorescent pyrrolidone derivative. All amino acids and amines also react with fluorescamine; however, in our experimental conditions they were not retained by the column and in particular imidazole derivatives did not interfere with the histamine fluorophore.

The investigation was completed by direct electron-impact mass spectrometry of histamine and its methyl derivatives normally present in biological fluids, before and after fluorescamine derivatization. Preliminary results show (1) the absence of N-methylhistamine in normal human tears, and (2) increased stability of histamine after its fluorescamine derivatization (Bettero et al.) [20].

The present method, as well as other reported HPLC procedures [15, 16], allows selective and sensitive histamine measurements after pre-column derivatization and fluorimetric detection.

The use of fluorescamine—acetonitrile as derivatizing reagent presents several advantages for a rapid and accurate histamine evaluation in biological fluids and particularly in tears where small sample volumes are available: (1) acetonitrile allows sample homogeneity, (2) derivatization occurs instantaneously, (3) fluorophore behaviour can be easily studied by mass spectrometry, (4) only a few microlitres of sample are required, and (5) the total analysis time is less than 5 min.

In conclusion, the method appears particularly suitable for the rapid evaluation of histamine in tears at picomole levels in both allergic or chemically induced conjunctivitis.

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