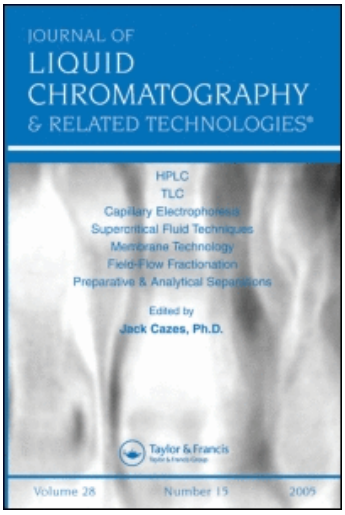

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FLUOROMETRIC DETERMINATION OF HISTAMINE IN BIOLOGICAL FLUIDS AND TISSUE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

High-pressure liquid chromatography was used to separate the fluorescent adduct formed from the reaction of histamine with o-phthalaldehyde (OPT) from other biogenic amines in tissue, cerebrospinal fluid (CSF), sweat and urine samples. Using off-column derivatization and isocratic elution techniques fluorescent OPT adducts can be detected in the low picogram range. Perchloric acid extracts of tissue samples from Aplysia california and urine specimens collected from healthy adult males, including internal standard, were derivatized with OPT buffer, pH 9.5 and extracted with ethylacetate to increase sensitivity and stabilization of the fluorescent adduct prior to chromatography. Sweat and CSF samples were reacted with OPT buffer and aliquots of this mixture injected directly onto the chromatographic column (μBondapak CN) with methanol/0.08 mol/liter acetic acid (52/48 by volume) as the mobile phase. Assay of pooled urine containing added histamine (1 μg/ml) gave a with-in run coefficient of variation of 2.5%. The use of o-phthalaldehyde as an off-column HPLC derivatization agent for fluorometric determination of low-levels of biogenic amines is rapid, sensitive and easily adapted to routine use in a clinical or neurobiological laboratory.

INTRODUCTION

Histamine, known to be a mediator of immediate hypersensitivity reactions (1-3), is also believed to be a neurotransmitter in the central nervous system of mammals (4-6) and invertebrates (7). In addition, histamine formation in animals is markedly stimulated by a number of

different stress conditions including chemical compounds, endotoxins, and of particular importance to this laboratory, hypo- and hyperthermic environments (8).

The concentration of histamine and similar biogenic amines, in tissue samples and body fluids can be estimated using several techniques. Fluorescence methods, including automated procedures (9-11), have been used but lack sensitivity and specificity. Newer and more sensitive procedures include complex assays in which double-isotope derivative (12) and radioenzymatic methods (13, 14) are used.

An alternative approach to biogenic amine analysis, which maintains adequate sensitivity for small volume biological samples, utilizes high-performance liquid chromatography (HPLC) and derivatization of primary amines with o-phthalaldehyde (OPT) prior to chromatographic separation and fluorescent detection. This technique was originally applied to urinary catecholamines (15) using HPLC and a μBondapak C_{18} reversed-phase column. Davis et al. (16) has shown this procedure to be applicable for the measurement of seven biogenic amines in plasma and tissue samples using a two-step gradient elution program and a μBondapak/phenyl column. In addition this procedure can be extended to at least 17 biogenic amines, including polyamines and a convenient internal standard, using a C_{18} reversed-phase column and gradient elution program (17).

In all the above cases, however, histamine is eluted near the solvent front and in the vicinity of other endogenous biogenic amines. The purpose of this study is to demonstrate that analysis of histamine can be achieved using off-column fluorescent derivatization with OPT, a simple isocratic mobile phase, and a μBondapak CN column for separation of the histamine-OPT fluorescent adduct. The method is applicable to the determination of histamine in tissue homogenates obtained from Aplysia california, a marine invertebrate, nervous tissue, cerebrospinal fluid obtained from healthy,

adult monkeys, and sweat obtained from healthy, human, male subjects undergoing hyperthermal stress. In addition, the applicability of this technique to urine analysis will also be demonstrated.

MATERIALS AND SOLUTIONS

The mobile phase used for quantitative determination of histamine was methanol/0.08 mol/liter acetic acid (pH 2.9) mixture (52/48 by vol.). All solutions were made with de-ionized, glass-distilled water. The mobile phase was filtered through a 0.45 μm filter and degassed immediately before use.

O-phthalaldehyde, 3,4-dihydroxybenzylamine hydrogen bromide, ethyl acetate, taurine, histamine and all amino acids were obtained from Aldrich Chemical Co., Milwaukee, WI. All other biogenic amines were obtained from Calbiochem, La Jolla, CA. Mercaptoethanol was obtained from Sigma Chemical Co., St. Louis, MO. Standard solutions (100 mg/liter) of each biogenic amine, including internal standard (3,4-dihydroxybenzylamine), were prepared by dissolving these compounds in methanol. All solutions were filtered through a 0.45 μm filter before use.

O-phthalaldehyde derivatizing reagent was prepared fresh daily by dissolving 160 mg of OPT in 3 ml ethanol and 0.2 ml 2-mercaptoethanol under a well-ventilated hood. This solution was added to 100 ml of 0.4 mol/liter boric acid which had been adjusted to pH 9.5 with potassium hydroxide.

METHODS

Liquid Chromatography

A Model 204 Liquid Chromatograph with Models 6000 A Solvent Delivery System and U6K Universal Injector (Waters Associates, Inc., Milford, MA) was used for chromatography. An isocratic mode was employed and the eluent was monitored with a Model J4-7461 Aminco Fluoromonitor (American Instruments Co., 8030 Georgia Ave., Silver Spring, MD) with a Corning 7-60 primary filter (excitation maximum at 340 nm) and a Wratten 2A

secondary filter. Chromatograms of the biogenic amine - OPT adducts were recorded with a model B-5211-15 strip chart recorder (Houston Instruments, Austin, TX). Peak height, area and retention time were determined by a programmable computing integrator (Supergrator-3, Columbia Scientific Industries, Austin, TX). A Model 1021A Electronic Filter (Spectrum Scientific Corp., Newark, DEL) was placed in series between the fluorescent detector and recorder for baseline noise correction. The cut off frequency of the filter was set at 0.05 Hz and the gain adjusted as needed for each determination (usually set at 2 or 5). Chromatographic conditions are described in the figure legends. A Waters μBondapak CN reversed-phase column was used for all analysis and flushed daily with methanol (Burdich and Jackson Laboratories, Inc., Muskegon, MI).

Sample Preparation and Derivatization

Chromatograms of histamine and other biogenic amine standards were obtained by reacting 100 μl of OPT derivatizing reagent with various amounts (usually 20 ng) of standard. After 2 min incubation at room temperature, various amounts of distilled water (e.g. 100 μl) were added for purposes of dilution, and aliquots of this mixture were injected onto the reversed-phase column.

For nervous tissue samples from A. california, ganglia and auricles were removed from the animal, cleaned of as much connective tissue and nerve fiber tracts as possible, blotted dry and weighed. Single pinnules with their attachment to the efferent vessel wall were dissected from the gill, washed in sea water to remove mucous and ink, dryed and weighed. All samples consisted of pooled tissue from 3 to 10 animals. After weighing, samples were placed in 0.1N perchloric acid on ice, homogenized by hand in ground glass homogenizers, and frozen until use.

Frozen tissue samples, and pooled urine specimens collected as previously described (15), were thawed at room temperature, mixed thoroughly, and centrifuged at 3000 x g for 5 min. To 1 ml of the perchloric acid

extract, or urine specimen, 40 µl of 1N NaOH, 1 ml of OPT derivatizing reagent, and 200 ng of internal standard were added. After 2 min of gentle shaking, 2 g of sodium chloride were added and the mixture extracted with 3 ml of ethyl acetate. The ethyl acetate fraction was then extracted with 2 ml of 0.05 mol/liter dibasic sodium phosphate buffer, pH 10 (16). The ethyl acetate fraction was placed in 5 ml tapered-cone screw-cap vials (K-74900, Kontes, Vineland, NJ) and reduced to 100 µl under dry nitrogen and stored at 4°C until analysis. Prior to chromatography, the ethyl acetate extract containing the derivatized biogenic amines was brought to room temperature and aliquots injected onto the reversed phase column for liquid chromatographic determination.

For cerebrospinal fluids and sweat samples, 200 µl of fluid was reacted directly with 200 µl of OPT reagent and 20 µl of 1N NaOH. Aliquots of this mixture were directly injected onto the reversed-phase columns. Histamine and other biogenic amines were identified on the basis of retention times by comparison with standards and by co-chromatography of standards with biological samples. Histamine content for tissue and urine samples was calculated from peak area ratios using internal and external standards. For example, histamine content for tissue samples was calculated from the following equation:

$$\frac{\text{ng}}{\text{mg tissue}} = \frac{(A_{HI}/A_{IS})_{sample}}{(A_{HI}/A_{IS})_{std}} \times \frac{\text{ng HI std}}{\text{injection volume (µl)}} \times \frac{100\ \mu l}{1\ ml\ HClO_4} \times \frac{\text{Total homogonized tissue volume (ml } HClO_4)}{\text{Tissue wt (mg)}}$$

For cerebrospinal fluids and sweat, histamine content was calculated by direct comparison of histamine peak area in the sample to a standard calibration curve (Figure 3).

RESULTS

Figures 1 and 2 show the fluorescent response obtained for histamine, selected biogenic amines and amino acids following derivatization with excess OPT. No fluorescent response is observed if distilled water is substituted for biogenic amine or amino acid. Using the derivatization

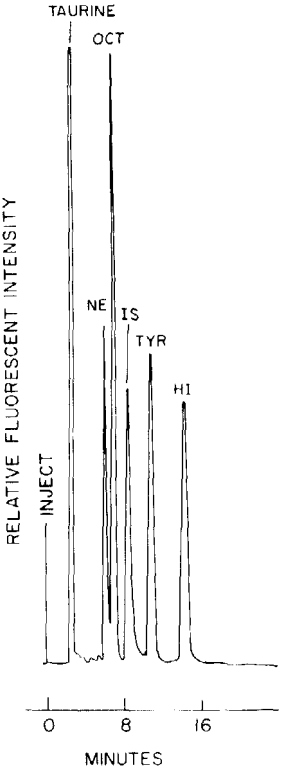

Figure 1. Chromatogram of fluorescent OPT-amine adducts for histamine and selected biogenic amines.

Twelve nanograms of each biogenic amine were derivatized with excess OPT derivatizing reagent, pH 9.5. Column, μBondapak CN: eluent, methanol/0.08 mol/liter acetic acid (52/48 by volume, pH 2.9): flow rate, 1.2 ml/min; temperature , 23°C; sample volume, 20 μl; fluorescence detection, x 100, excitation maximum 340 nm (Corning 7-60 primary filter), Wratten 2A secondary filter, atten x 100: electronic filter, cut-off frequency 0.05 Hz, Gain x 1.0: NE, norepinephrine; OCT, octopamine; IS, internal standard, 3,4-dihydroxybenzylamine; TYR, tyramine; HI, histamine.

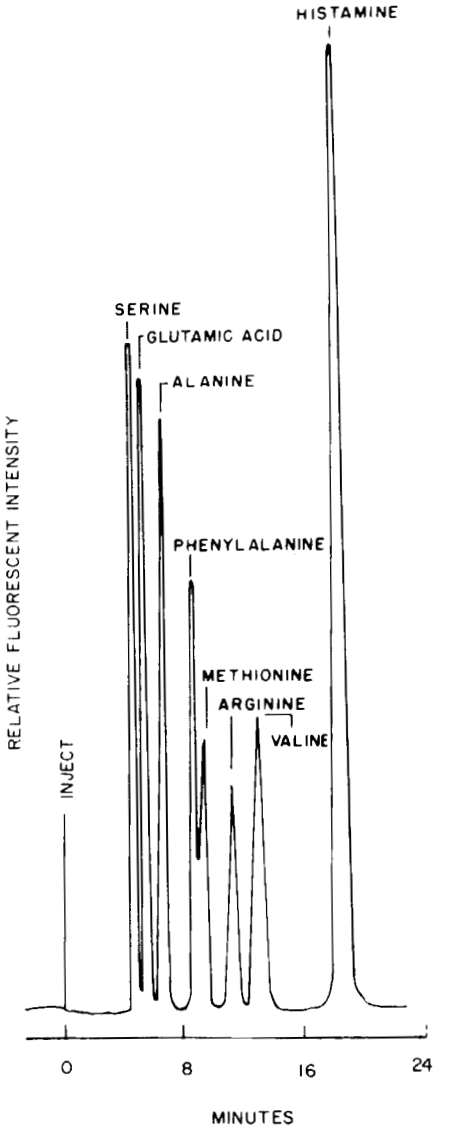

Figure 2. Chromatogram of fluorescent OPT-amine adducts for histamine and selected amino acids.

Twenty-five nanograms of each amino acid and histamine were derivatized with excess OPT derivatizing reagent, pH 9.5. Flow rate, 1.0 ml/min: Other chromatographic conditions are given in Figure 1.

procedure previously outlined, the limit of detection is approximately 50 pg for the amines shown in Figures 1 and 2. In addition the following biogenic amines and amino acids were shown not to interfer with the histamine assay: L-dopa, normetanephrine, dopamine, phenylethanolamine, 3-o-methyldopamine, leucine, cystine, glycine, threonine, lysine, aspartic acid, histidine, and tryptophan.

Linear response curves were obtained for histamine and two other biogenic amines at concentrations from 250 pg to 9 ng injected into the column (Figure 3). This range can be extended to 30 ng and is more

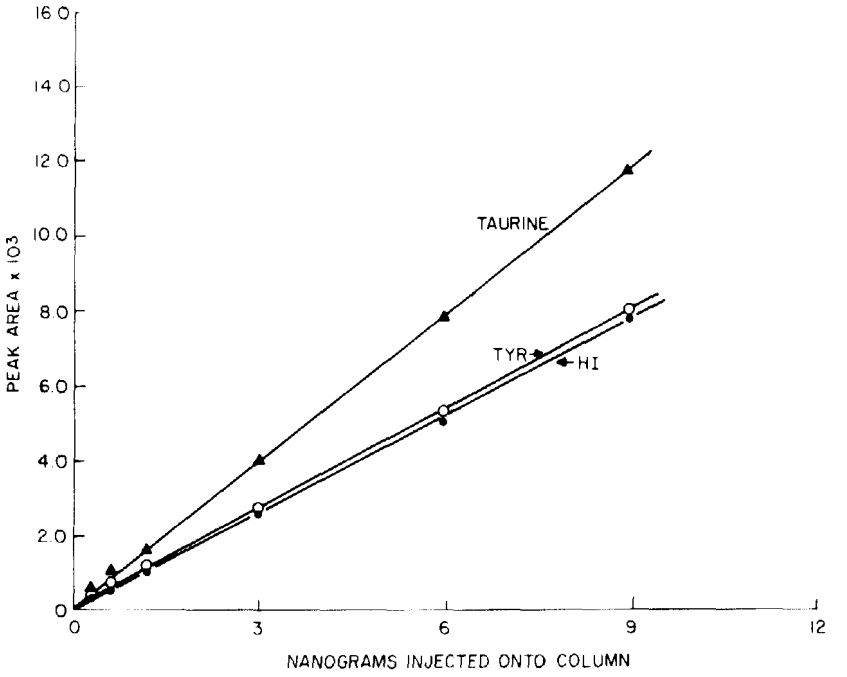

Figure 3. Linearity of detection for histamine and related biogenic amines.

Sample volume, 10 µl containing indicated amounts of biogenic amines. Chromatographic conditions and abbreviations are given in Figure 1.

than adequate for the analysis of histamine at concentrations expected to be found in the samples previously described.

Figures 4-7 illustrate the chromatograms obtained for Aplysia tissue (pedal ganglia), cerebrospinal fluid, sweat, and pooled urine, respectively.

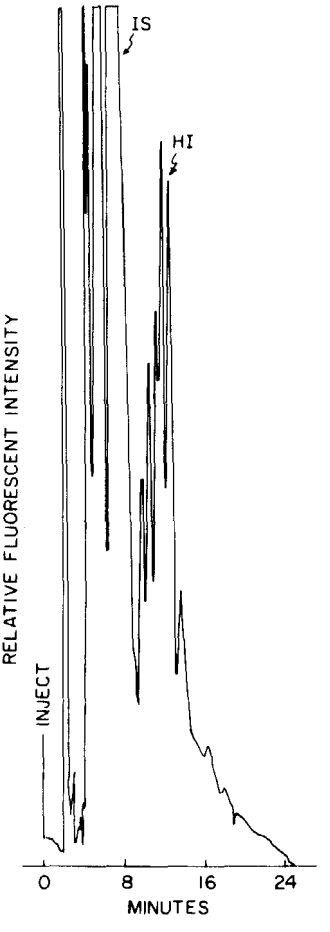

Figure 4. Chromatographic separation of biogenic amine-OPT adducts of Pedal ganglia homogenate from Aplysia california.

Sample volume, 2 µl; 1 ml of $HC10_4$ extract of Pedal ganglia from 3 animals was extracted and derivatized by the above procedure; flow rate, 1.2 ml/min: fluorescent sensitivity, x 30, electronic gain x 2: all other chromatographic conditions are the same as given in Figure 1. HI content calculated to be 2.6 pmol/mg tissue.

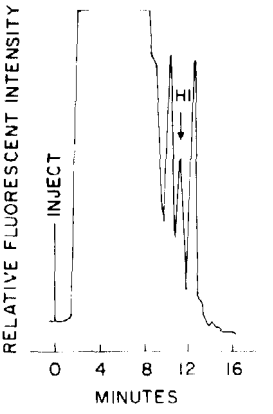

Figure 5. Chromatogram obtained following derivatization of cerebrospinal fluid (monkey) with OPT.

Sample volume, 25 µl: Two hundred µl of CSF was reacted with 200 µl of OPT buffer and 20 µl 1N NaOH. Flow rate, 1.5 ml/min: fluorescent sensitivity, x 30, electronic gain x 2: all other chromatographic conditions are the same as Figure 1. HI content calculated to be 14.3 ng/ml.

Data on within-run precision were determined by processing 10 aliquots of pooled urine, containing 1 µg/ml of added histamine, through the complete procedure during a single day. The histamine concentration was determined to be 1.020 ± 0.025 (SD), C.V. 2.5%, indicting an approximate endogenous histamine level of 20 ng/ml. For five other urnie specimens collected from healthy adult males, the histamine level varied from 15 ng/ml to 65 ng/ml, within the previously reported range expected for urinary histamine excretion (18). Recovery of internal standard added to tissue and urine samples was 62.5% ± 4.2% (mean ± S.D., n=10).

Table 1 lists the histamine concentrations found in major Aplysia california ganglia analyzed by the above procedure. Histamine levels found in sweat samples collected from six healthy adult males undergoing hyperthermal stress ranged from 16.7 ± 5.2 ng/ml (mean ± S.D.) to 132 ± 11.5 ng/ml with a mean of 77.5 ng/ml. In a similar manner the histamine level in cerebrospinal fluid collected from a healthy adult monkey (macaca mulatta) was determined to be 13.4 ± 4.4 ng/ml (mean ± S.D., n=7).

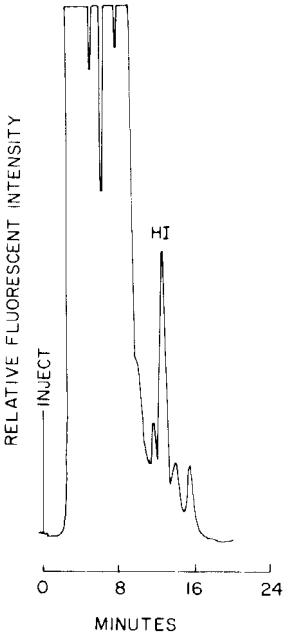

Figure 6. Chromatogram obtained following derivatization of sweat (human) with OPT.

Sample volume, 50 µl: Derivatization procedure described in text and Figure 5. Flow rate 1.2 ml/min: fluorescent sensitivity, x 30, electronic gain x 2: All other chromatographic conditions are the same as Figure 1. HI content calculated to be 40.0 ng/ml.

DISCUSSION

The combination of reversed phase high-performance liquid chromatography and off-column fluorescent derivatization with o-phthalaldehyde provides a rapid, sensitive means for histamine analysis in biological fluids and tissue. Additionally, the use of a µBondapak CN Column provides increased resolution of histamine from other important primary amines in these complex biological samples without the use of multi-component or gradient elution techniques. For more complex biological matrices such as urine or tissue samples, extraction and concentration of the amine-OPT adduct with ethylacetate, provides a further increase in sensitivity as well as stablization of the fluorescent adduct.

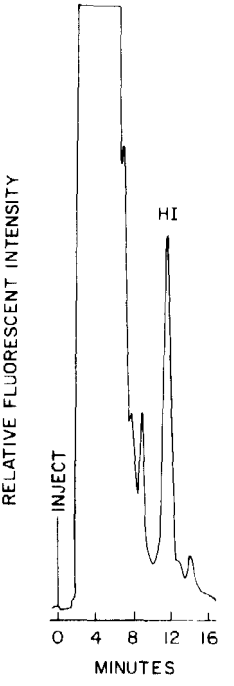

Figure 7. Chromatogram obtained following derivatization of pooled urine containing added histamine (1 µg/ml).

Sample volume 50 µl: flow rate 1.5 ml/min: fluorescent sensitivity, x 100, electronic gain x 2: all other chromatographic conditions are the same as figure 1. HI content calculated to be 1.025 µg/ml.

TABLE 1

Histamine Concentrations (pmol/mg wet wt) of Aplysia california ganglia

ABDOMINAL	0.82 ± 0.06[a]
PLURAL	0.30 ± 0.06
PEDAL	2.50 ± 0.14
CEREBRAL	0.08 ± 0.02
BUCCAL	1.50 ± 0.30
AURICLES	0.16 ± 0.02
PINNULES	0.19 ± 0.02

[a] MEAN ± SD, n=3

While measurement of content alone is not adequate to determine if a substance is biologically important (e.g. a neurotransmitter or thermoregulatory agent), a knowledge of content and distribution is of critical importance. As such, the application of this technique to other biologically active primary amines has great promise. The analysis of octopamine, a biogenic amine that in invertebrate nervous systems may function as a neurotransmitter, is being investigated by this laboratory. Additional studies have shown that taurine and most primary amino acids can be detected at picogram sensitivites using this technique.

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