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Note

Simultaneous determination of histamine and N^{τ}-methylhistamine in human urine and rat brain by high-performance liquid chromatography with fluorescence detection

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Considerable attention has been given recently to the simultaneous determination of histamine and N^{τ}-methylhistamine [1] in tissues and body fluids. The profile of urinary histamine and N^{τ}-methylhistamine during pregnancy is of interest in connection with the secretion of estrogens and luteinizing hormone [2]. In brain, histamine is catabolized mainly by N-methyltransferase to produce N^{τ}-methylhistamine [3, 4] and has been reported as a neurotransmitter in the central nervous system [5].

Several methods have been reported for the simultaneous assay of the imidazole amines; for example, liquid chromatography after fluorescence derivatization with dansyl chloride [6] and gas chromatography after derivatization with heptafluorobutyrate or N,O-bis(trimethylsilyl)trifluoroacetamide [7].

o-Phthalaldehyde (OPT), which is popular as a fluorescence reagent for amino acids [8] and primary amines [9], reacts rapidly in the presence of 2-mercaptoethanol with histamine and methylhistamines to form fluorescent products. Perini et al. [10] have shown that an amino acid analyzer can be adapted to the determination of histamine and N^{τ}-methylhistamine, and of di- and polyamines by post-column derivatization with OPT.

In the present paper, a simple simultaneous determination of histamine and N^{τ}-methylhistamine by high-performance liquid chromatography (HPLC) (pre-column derivatization with OPT) with fluorescence detection has been developed and is applied to the assay of imidazole amines in human urine and rat brain.

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EXPERIMENTAL

Materials and reagents

Histamine was obtained from Wako (Osaka, Japan), N^{τ}- and N^{π}-methylhistamines were from Calbiochem (La Jolla, CA, U.S.A.) and Cellex-P was from Bio-Rad Labs. (Richmond, CA, U.S.A.). Other chemicals were the same as used in the previous paper [11].

Human urines were obtained from healthy volunteers in our laboratory. Rat brains were obtained from 5—7-week-old male rats (Donryu).

Reaction buffer. The pH of a borate buffer (0.05 *M* sodium tetraborate) was adjusted to 10.0 with 5 *M* sodium hydroxide. A reaction buffer was prepared by mixing 5 ml of the borate buffer, 5 ml of methanol and 0.1 ml of 0.5% (v/v) 2-mercaptoethanol in methanol.

Cellex-P column. Cellex-P (H^+; 0.9 mequiv./g) was purified as described by Kremzner and Wilson [12] with some modifications as follows. Cellex-P (5 g) was washed before use with successive 200-ml portions of 0.1 *M* hydrochloric acid, 1.0 *M* sodium chloride, water, 0.1 *M* sodium bicarbonate, water, 1.0 *M* sodium carbonate, water, 0.1 *M* sodium hydroxide, water, and finally 0.2 *M* sodium phosphate buffer (pH 6.0). Then the Cellex-P was suspended in 0.01 *M* sodium phosphate buffer (pH 6.0) and stored. When required for use, the Cellex-P suspension was poured on to a glass column (150 × 5 mm I.D.) to be 25 mm in height after settling. The column was washed two times with 1.0 ml of the phosphate buffer.

Apparatus

The HPLC system consisted of an Hitachi 635-A liquid chromatograph equipped with a universal injector and an Hitachi 650-10S spectrofluorimeter fitted with a flow-cell unit (cell volume, 18 μl) operating at an emission wavelength of 445 nm and an excitation wavelength of 335 nm. A stainless-steel column (150 × 4 mm I.D.) was packed with LiChrosorb RP-18 (particle size, 5 μm; Japan Merck, Tokyo, Japan) as previously described [11]. An Hitachi-Horiba M-7 pH meter and a Vapor mix S-10 test tube evaporator (Tokyo Rikakikai, Tokyo, Japan) were also used.

Procedure for urine

To urine (1.0 ml) taken in a 5-ml glass stoppered centrifuge tube spiked with N^{π}-methylhistamine (1 nmol) as an internal standard (IS), 5 *M* sodium hydroxide (0.25 ml), *n*-butanol (4.5 ml) and sodium chloride (0.4 g) were added. Histamine and N^{τ}- and N^{π}-methylhistamines were extracted into *n*-butanol with vigorous shaking for 5 min. The butanol layer was separated by centrifugation and a 4-ml portion of the layer was transferred to a glass stoppered test tube containing 0.1 *M* hydrochloric acid (0.5 ml) and benzene (4.0 ml). After 3 min of shaking, the organic layer was discarded.

To the acid layer, 2.5 ml of 0.01 *M* sodium phosphate buffer (pH 6.0) were added. The mixture was adjusted to pH 6.0 with 0.1 *M* sodium hydroxide. The mixture was applied to the Cellex-P column. The column was washed successively with 1.0 ml of the buffer (four times) and 1.0 ml of water. The amines were eluted with 1.2 ml of 0.12 *M* hydrochloric acid. The eluate was adjusted

to pH 8—10 with 0.5 *M* sodium hydroxide and then evaporated to dryness in vacuo at 50°C.

To the residue, 0.2 ml of the reaction buffer and 5 μl of 0.5% OPT in methanol were added and an aliquot (50 μl) of the reaction mixture was applied to the HPLC system. The mobile phase was a mixture of 0.07 *M* disodium hydrogen phosphate (pH 9.45) and methanol (47:53, v/v) and the flow-rate was 0.7 ml/min. The column temperature was ambient.

Procedure for rat brain

A portion (100—500 mg) of whole rat brain spiked with N^{π}-methylhistamine (1 nmol, as IS) was homogenized with 4.0 ml of ice-cold 0.4 *M* perchloric acid in a glass homogenizer. The mixture was centrifuged at 1200 *g* for 15 min. The supernatant (4.0 ml) was transferred to a glass stoppered test tube containing 5 *M* sodium hydroxide (0.5 ml), *n*-butanol (10.0 ml) and sodium chloride (1.5 g). The sample was extracted for 5 min with vigorous shaking. The *n*-butanol layer was separated by centrifugation and an 8.0-ml portion of the layer was transferred to a glass stoppered test tube containing 0.1 *M* hydrochloric acid (1.0 ml) and benzene (15.0 ml); the tube was then shaken vigorously for 3 min, followed by centrifugation (1200 *g*, ca. 4 min). After removal of the organic layer, a portion (0.4—0.9 ml) of the acid layer was transferred to a test tube, and then carried through the same procedure as for urine.

RESULTS AND DISCUSSION

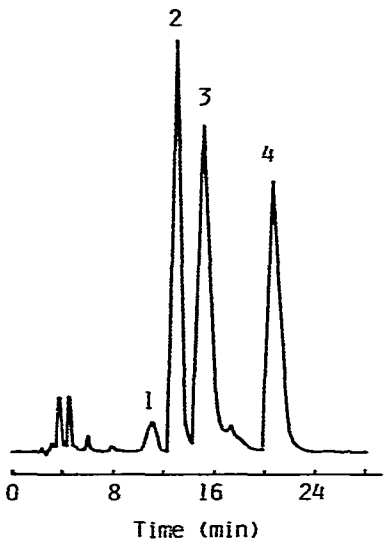
Histamine and N^{τ}- and N^{π}-methylhistamines exhibit fluorescence spectra with an excitation maximum at 335 nm and an emission maximum at 445 nm. The reaction of OPT and 2-mercaptoethanol with these imidazole amines was completed within 30 sec at room temperature and the fluorescent products (OPT derivatives) were stable for at least 60 min under the prescribed conditions [pH 10; solvent, a mixture of methanol and water (50:50, v/v)].

The OPT derivatives of histamine, and N^{τ}- and N^{π}-methylhistamines were completely separated with retention times of 13, 21 and 15 min, respectively; the peak at 11 min was due to ammonia occluded as a contaminant and the peaks at 3.5—8.0 and 17.5 min were caused by the reagent blank (Fig. 1).

The methanol content in the mobile phase had an effect on the retention times and the resolution of the OPT derivatives. At a methanol content higher than 60%, the peaks due to ammonia, histamine and N^{π}-methylhistamine closely overlapped, while a lower concentration of methanol (40%) caused a delay in elution with broadening of the peaks.

The pH of the phosphate solution in the mobile phase affected the capacity factors (k') of the OPT derivatives (Fig. 2). The maximum k' values were obtained at pH 7. Above pH 7, the elution pattern of the OPT derivatives remained unchanged, but the derivatives eluted earlier with increasing pH. Since the column packing degenerated irreversibly at pH values higher than 10, a sodium phosphate solution (pH 9.45) was employed to provide adequate resolution.

The concentration of the phosphate in the mobile phase influenced the peak widths of the OPT derivatives. At a phosphate concentration lower than 0.05



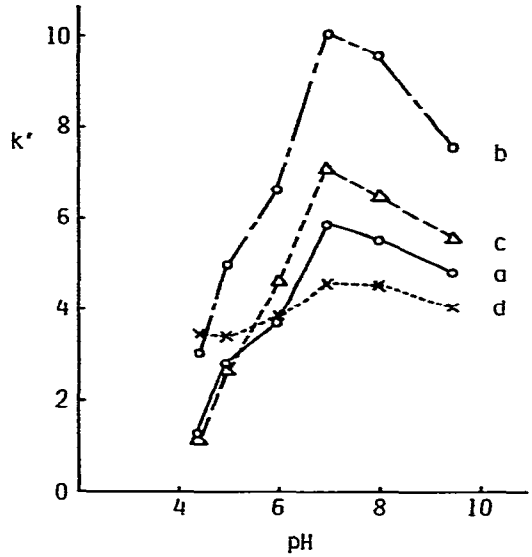


Fig. 1. Chromatogram of the reaction mixture. A mixture of histamine and N^{τ}- and N^{π}-methylhistamines ($1 \cdot 10^{-6}$ *M* each) was treated according to the procedure for urine. Peaks: 1 = ammonia; 2 = histamine; 3 = N^{π}-methylhistamine; 4 = N^{τ}-methylhistamine.

Fig. 2. Effect of pH of the phosphate solution in the mobile phase on the capacity factor (k') of the fluorescent derivatives of (a) histamine, (b) N^{τ}-methylhistamine, (c) N^{π}-methylhistamine and (d) ammonia.

M, the peaks of the derivatives tailed badly, and at concentrations higher than 0.1 *M*, the phosphate separated out in the mobile phase and choked up the HPLC column. Therefore, a concentration of 0.07 *M* was used.

The OPT derivatives of ammonia and some biological amines which elute near to those of the imidazole amines may interfere with the determination of the imidazole amines if present in the sample solution for HPLC. The interfering amines, which were not completely separated by the extraction procedure, could be removed by chromatography on the Cellex-P column. The imidazole amines adsorbed on the column were eluted completely with 0.12 *M* hydrochloric acid. To remove a small amount of contaminating ammonia, the eluate was adjusted to pH 8—10 and evaporated to dryness in vacuo. Care should be taken to avoid contamination with atmospheric ammonia during the assay.

N^{π}-Methylhistamine has not been found in the biological samples, and thus was conveniently used as an internal standard.

The ratios of the peak heights of histamine and N^{τ}-methylhistamine to that of N^{π}-methylhistamine (IS) were plotted against the amounts of each amine. The calibration curves thus obtained were linear up to at least 10 nmol/ml and passed through the origin.

The lower limits of determination (at a signal-to-noise ratio of 2) for histamine and N^{τ}-methylhistamine were 2.0 and 3.8 pmol/ml of urine, and 5.1 and 8.6 pmol/g of rat brain, respectively. Recovery tests were run for urine and rat brain adding known amounts of histamine and N^{τ}-methylhistamine (2 nmol/ml

each for urine and 2.5 nmol/g each for rat brain). Good recoveries (urinary histamine and N^{τ}-methylhistamine, 99 and 102%, respectively; rat brain histamine and N^{τ}-methylhistamine, 94 and 91%, respectively) were obtained.

The precision of the method was examined for histamine and N^{τ}-methylhistamine in urine containing 0.25 and 1.89 nmol/ml, respectively, and in rat brain containing 0.57 and 0.28 nmol/g, respectively. The coefficients of variation were 8.0 and 2.1% for urinary histamine and N^{τ}-methylhistamine ($n = 10$), respectively, and 7.0 and 10.7% for rat brain histamine and N^{τ}-methylhistamine ($n = 4$), respectively.

The amounts of histamine and N^{τ}-methylhistamine in the 24-h urines of 18 healthy persons (9 men, 24—48 years; 9 women, 21—23 years) determined by this method are shown in Table I. The concentrations of histamine and N^{τ}-methylhistamine in rat brain determined by this method were 0.62 ± 0.05 and 0.35 ± 0.05 nmol/g (mean ± S.D.), respectively. These values are in good agreement with published data [13—16].

TABLE I

AMOUNTS OF HISTAMINE AND N^{τ}-METHYLHISTAMINE IN 24-h URINES OF NORMAL PERSONS

Age* (years)	Histamine (μmol)	N^{τ}-Methylhistamine (μmol)
48 (m)	0.14	1.54
41 (m)	0.32	2.31
32 (m)	0.21	1.97
31 (m)	0.60	2.30
29 (m)	0.34	3.43
27 (m)	0.33	2.39
24 (m)	0.52	3.52
23 (m)	0.42	2.52
22 (m)	0.34	1.84
23 (f)	0.15	1.35
22 (f)	0.56	4.17
22 (f)	0.54	1.69
22 (f)	0.45	2.00
22 (f)	0.35	2.22
21 (f)	0.33	0.90
21 (f)	1.14	2.46
21 (f)	0.44	2.88
21 (f)	0.98	2.29
Mean ± S.D.	0.45 ± 0.25	2.32 ± 0.78

*m = male; f = female.

This method is sensitive, rapid and simple and should be useful for the simultaneous determination of histamine and N^{τ}-methylhistamine in biological samples.

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