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Simultaneous Determination of Histamine and *N*^ε-Methylhistamine with High-Performance Liquid Chromatography Using Electrochemical Detection

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Abstract

We have developed a liquid chromatographic method which uses electrochemical detection for the simultaneous quantitation of histamine and *N*^ε-methylhistamine in rat brain. The amines are derivatized with the water-soluble Bolton–Hunter reagent (sulfo B-H). Perchloric acid extracts of rat brains are chromatographed on a strong cation-exchange resin. The eluate is evaporated and allowed to react with sulfo B-H at pH 9.8 at room temperature. The derivatization is complete after 30 s vortexing. The derivatives are purified using a cellulose–phosphate fibrous cation exchanger. They are quantified with an electrochemical detector at a potential of 0.56 V after preoxidizing the sample at 0.47 V. The derivatives of histamine, *N*^ε-methylhistamine, and *N*^α-methylhistamine are completely separated without interfering peaks. Since no *N*^α-methylhistamine was detected in rat brain it was used as an internal standard. The detection limits are 0.1 pmol of histamine and 0.2 pmol of *N*^ε-methylhistamine. The precision of this method is high, with within-run and between-run coefficients of variation of 2–7% and linearity of 0.999. Both histamine and *N*^ε-methylhistamine peak heights increased significantly and selectively after treatment with pargyline. Because of the high sensitivity, accuracy, and precision, the histamine and *N*^ε-methylhistamine contents of single nuclei of the rat hypothalamus can be routinely quantified.

Keywords

histamine; *N*^ε-methylhistamine; *N*^α-methylhistamine; Bolton Hunter reagent; electrochemical detection; hypothalamus

Histamine has been postulated to be a neurotransmitter in the mammalian brain (1). Recently, immunohistochemical studies have described the distribution of histaminergic neurons in the central nervous system of rodents (2,3) with a particularly dense histaminergic innervation in the hypothalamus. The major metabolic pathway for histamine in the brain is methylation (1), and *N*^ε-methylhistamine concentration is an accurate indicator of the turnover rate of neuronal histamine (4,5). Therefore, simultaneous

quantitation of both histamine and *N*^ε-methylhistamine with high sensitivity and accuracy is needed to study the activity of various histaminergic pathways in the brain.

Several methods have been used for the simultaneous detection of histamine and *N*^ε-methylhistamine: HPLC–fluorometric detection after *o*-phthalaldehyde derivatization (6), liquid chromatography or thin-layer chromatography after dansylation (7,8), gas-liquid chromatography (9), and gas chromatography–mass spectrometry (GC-MS)¹ (10). Many of these methods have problems of instability of the derivatives, lack of sensitivity, and high cost or complexity of manipulations. In contrast, liquid chromatography with electrochemical detection (EC) is a low cost, highly sensitive, and specific method for the detection of most monoamines and their metabolites in biological samples (11). Imidazole amines have not been generally quantified with EC, however, because of their low electroactivity.

We have applied derivatization with the Bolton–Hunter reagent for the simultaneous quantitation of histamine and *N*^ε-methylhistamine in rat hypothalamus with EC. The Bolton–Hunter reagent (Fig. 4A, 1a) and its water-soluble sulfo analog (Fig. 4A, 1b) are bifunctional reagents used previously for attaching an iodinated group, a phenol, to proteins through the acylation of lysine side chains (12,13). We now introduce a new application of this reagent which utilizes the electroactivity of the phenolic group for detection of derivatives by electrochemical methods. The amine-reactive portion of the Bolton–Hunter reagent consists of an *N*-hydroxysuccinimide ester, which is a sufficiently mild acylating agent to cause negligible reaction of phenols, even when a large excess of the active ester *N*-hydroxysuccinimide is used. Acylation of an imidazole nitrogen may occur, but the product hydrolyzes rapidly. The general approach of using *N*-hydroxysuccinimide esters as derivatizing reagents for the electrochemical detection of amines has recently been described by us (14).

MATERIALS AND METHODS

Chemicals

Histamine dihydrochloride, *N*^ε-methylhistamine dihydrochloride, and *N*^α-methylhistamine were purchased from Calbiochem. 1-Octanesulfonic acid (Fisher Scientific), EDTA (Fisher Scientific), and sodium acetate (Mallinckrodt) were used to prepare the mobile phase. Sulfosuccinimidyl-3-(4-hydroxyphenyl)propionate, sulfo B–H (Pierce), was used as the derivatization reagent. Double-distilled and deionized water (Millipore ion exchanger) was used throughout. Other commercial chemicals and reagents were of analytical grade and were used without further purification.

Animals

Male Sprague–Dawley rats weighing 300–400 g were obtained from the NIH stock.

¹Abbreviations used: sulfo B–H, sulfosuccinimidyl-3-(4-hydroxyphenyl)propionate = water-soluble Bolton–Hunter reagent; EC, electrochemical detection; GC–MS, gas chromatography–mass spectrometry.

Equipment

A Waters 590 pump was used with a 7125 Rheodyne injector with a 100- μ l loop, and a Coulochem 5100A electrochemical detector (ESA) was equipped with a 5011 analytical cell. The columns used were the Beckman Ultrasphere ODS, 250 $\times$ 4.6-mm i.d. with a Whatman CSKI guard column packed with Waters μ Bondapak C₁₈/Corasil. An SVC 100 H Speed Vac concentrator system (Savant) was used for the evaporation of samples.

Reagents and stock solutions

Aqueous stock solutions of histamine, *N*^ε-methylhistamine, and *N*^α-methylhistamine (1 mM) were prepared in water. They were stored at 4°C for no longer than 2 weeks. Sulfo B-H (0.02 M in water) was made fresh each day. The reaction buffer was prepared by mixing 0.1 M sodium carbonate and 0.1 M sodium bicarbonate (10:1, v/v).

Preparation of tissue for analysis

Rat brains were quickly removed after decapitation. The hypothalami were subsequently dissected according to the procedure of Glowinski and Iversen (15) and immediately frozen and weighed. An entire hypothalamus or a portion thereof was homogenized with 0.5 ml of ice-cold 3% perchloric acid in a glass homogenizer. The homogenizer was rinsed three times with 0.5 ml of 3% perchloric acid. The homogenate and rinses were combined and an appropriate amount of an *N*^α-methylhistamine solution was added as an internal standard. Then the sample was centrifuged at 10,000*g* for 30 min at 4°C. The extract was pipetted into a strong cation-exchange column according to a modification of the method of Yamatodani *et al.* (7,16). Dowex 50 W, dry mesh 100–200 (Sigma) cation-exchange resin, was washed sequentially with excess 2 M hydrochloric acid, water, 2 M sodium hydroxide, and water. The washed resin was equilibrated with 0.2 M sodium phosphate buffer at pH 6.5 and packed into a glass column (5 mm i.d. $\times$ 16 mm). The supernatant of the tissue sample was directly poured into the column without adjusting its pH. After the column was washed with 5 ml of water and 4 ml of 2 M hydrochloric acid, the amines were eluted with 2.5 ml of 3.5 M hydrochloric acid. The eluate was collected into a silanized glass tube and evaporated to dryness.

Derivatization procedure

The dry sample was dissolved in 0.8 ml of water, and 150 μ l of reaction buffer and 100 μ l of a 0.2 M sulfo B–H solution were added. To produce standards, 150 μ l of reaction buffer and 100 μ l of 0.02 M sulfo B–H were added to 1 ml of a standard solution containing known quantities of histamine and *N*^ε- and *N*^α-methylhistamine. The pH of the mixture was between 9.8 and 10.0 when the evaporation of the hydrochloric acid was complete. The samples were shaken vigorously for 30 s using a vortex mixer (Scientific Products). The derivatives were extracted using a cellulose-phosphate fibrous cation exchanger (Sigma) packed into a glass column (17 mm i.d. $\times$ 13 mm) which was sequentially washed with excess 0.1 M sodium hydroxide, water, 0.1 M hydrochloric acid, and finally with enough water to reach a pH between 5 and 6. The reaction mixture was adjusted to pH 5.5 to 6.0 with 0.1 M hydrochloric acid and applied to the column. The column was washed with 5 ml of water and 6 ml of 1.0 mM hydrochloric acid. The derivatives were eluted with 2.5 ml of

0.1 M hydrochloric acid. The first 0.5 ml of the eluate was discarded and the next 2.0 ml was evaporated and stored at 4°C in the dark prior to HPLC analysis. Methanol was added to the dry sample followed by water to a final volume ranging from 110 μ l to 1 ml of 10% methanol solution. One hundred microliters of the solution was injected into the HPLC system.

Chromatographic conditions

The mobile phase consisted of a mixture of 0.14 M sodium acetate and methanol (17:73, v/v) containing 3.89 M 1-octanesulfonic acid and 56 mg of EDTA. The solution was adjusted to pH 3.48 adding glacial acetic acid and degassed under vacuum. The chromatography was carried out at room temperature with a mobile phase flow rate of 1.0 ml per minute. The electrochemical detector was operated in the oxidative screen mode using a response time of 10 s and the flow cell No. 1 potential was 0.47 V. Quantitation was accomplished in flow cell No. 2 at a potential of 0.56 V.

RESULTS

Chromatographic Conditions

The sulfo B-H derivatives of histamine and *N*^ε- and *N*^α-methylhistamine were completely separated with retention times of 8.4, 10.0, and 11.6 min, respectively (Fig. 1). The pH was critical for separation, because the retention times of the derivatives were very sensitive to even minor changes, increasing linearly with increasing pH (Fig. 2). Adding 1-octanesulfonic acid into the mobile phase was important because it selectively affected the retention times of the imidazole amine derivatives, and enabled their separation from reaction products such as *p*-hydroxyphenylpropionic acid. The retention times of histidine and spermidine derivatives that could have interfered with the peaks of the imidazole amine derivatives were 4.2 and 32.6 min, respectively. Thus, they were completely separated from the imidazole amine derivatives.

Derivatization Conditions

Derivatization of imidazole amines with sulfo B-H was carried out at pH 9.8–10.0. The reaction yields for the amines, especially that of histamine, decreased with lowering of the pH of the reaction mixture (Fig. 3). The reaction was completed promptly by vigorous shaking at pH 9.8 at room temperature (Fig. 4B). Because increasing sodium concentration in the reaction mixture decreased the reaction yield, the amount of the reaction buffer was held to the minimum needed to maintain the pH at 9.8. Because the pH of the reaction mixture was important, complete evaporation of hydrochloric acid, the eluate of the Dowex 50 W column, was necessary. Due to reaction products such as *p*-hydroxyphenylpropionic acid derived from excess sulfo B-H producing very large peaks on the chromatograms, the derivatives were further purified employing a cellulose–phosphate fibrous cation exchanger.

To determine the reaction yield for the amines with sulfo B-H in our method, peak heights of standard amines corresponding to 100 pmol after derivatization were compared with those of 100 pmol of pure derivatives. Pure derivatives were made as follows. Histamine dihydrochloride (0.75 g, 4.1 mmol) was dissolved in 60 ml of an equivolume mixture of

methanol and water. Potassium bicarbonate (20 ml, 2 M) and *N*-succinimidyl-3(4-hydroxyphenyl)propionate (1.07 g, 4.1 mmol) were added, and the solution was stirred for 16 h. The reaction mixture was extracted with ether/ethyl acetate (1:1) and three times with *n*-butanol. The butanol extracts were combined, filtered through sodium sulfate, and evaporated leaving a clear oil. The product was crystallized from ethyl acetate/ether, with the first precipitate discarded. The yield was 0.82 g (78%), mp 171–174°C.

Anal

Calcd: $C_{14}H_{17}N_3O_2 \cdot \frac{1}{3}H_2O$ 63.38% C, 6.71% H, 15.84% N. Found: 63.38% C, 6.76% H, 15.77% N. Accurate mass calcd: 259.1321. Found: 259.1322. 3-(4-Hydroxyphenyl)propionyl derivatives of *N*^ε- and *N*^α-methylhistamine were prepared by similar methods. Accurate mass calcd: 273.1477. Found: (*N*^ε-methylhistamine derivative) 273.1459, (*N*^α-methylhistamine derivative) 273.1475. As shown in Table 1, high yields were achieved even for the secondary amine, *N*^α-methylhistamine.

Limit of Sensitivity

The detection limits (defined as signal/noise = 3) of histamine, *N*^ε-methylhistamine, and *N*^α-methylhistamine derivatives were 0.1, 0.2, and 1 pmol of the pure amines, respectively.

Linearity

The linearity of the detector response was studied over the concentration ranges of 0.1 pmol–1 nmol of histamine, 0.2 pmol–1 nmol of *N*^ε-methylhistamine, and 1 pmol–1 nmol of *N*^α-methylhistamine. Each concentration was derivatized independently. The response of the detector to the derivatives of all these amines was linear ($r = 0.999$).

Reproducibility

The precision was investigated by injecting samples containing 10 pmol of each of the amines derivatized independently. Between-run variation was established by derivatization and injection every 3 days for 12 days. Reproducibility was excellent (Table 2).

Stability of the Derivatives

Amounts of 1 ml of 10^{-7} and 10^{-6} M standard solutions of the amines were derivatized and reconstituted into 1 ml of 10% methanol solutions. They were stored at 4°C in the dark and injected every 2 or 3 days. For 10 days after derivatization, the changes in peak heights of the derivatives remained within the between-run variations of the method. Thus, marked stability of the derivatives was demonstrated.

Biological Samples

Histamine and *N*^ε-methylhistamine derivatives could be completely separated from interfering peaks (Fig. 5). No peak was found corresponding to *N*^α-methylhistamine in native rat brain tissue. Thus, based on our and others' findings (8,9), *N*^α-methylhistamine could be used as an internal standard. Though we also found no peak corresponding to *N*^ε-methylhistamine (retention time, 9.4 min) used as an internal standard by other investigators (6,7), we did not employ it because its retention time was similar to the other imidazole

amines. No peaks corresponding to derivatives of histamine, *N*^ε-methylhistamine, or *N*^α-methylhistamine were seen on blank chromatograms obtained from brain samples treated according to our procedure omitting the derivatization step. Preoxidation of the samples in flow cell No. 1 stabilized the baseline and abolished an unidentified peak between histamine and *N*^ε-methylhistamine. The amounts of histamine and *N*^ε-methylhistamine in the rat hypothalamus quantified by this method are 3.01 ± 0.51 and 0.86 ± 0.14 (SD) nmol/g tissue, respectively ($n = 7$). The histamine concentrations were in good agreement with ranges obtained previously by a radioenzymatic assay (17,18) and lower than those measured with HPLC with fluorometric detection (5). The concentration of *N*^ε-methylhistamine was somewhat lower than that found by Hough *et al.*, using GC–MS (19). Our *N*^ε-methylhistamine/histamine ratio was, however, very similar to that in the Oishi *et al.* report obtained with HPLC using fluorometric detection (5). Recovery tests were run as follows: eight rat hypothalami were homogenized with 8 ml of 3% perchloric acid and centrifuged. Internal standard was added to the supernatant and divided into eight aliquots, 750 μ l each. To each of four aliquots, 100 pmol of histamine and *N*^ε-methylhistamine were added. Then all samples were prepurified with a Dowex 50 W column and derivatized. The recoveries of histamine and *N*^ε-methylhistamine obtained were 96 and 86%, respectively. Coefficients of variation for hypothalamic histamine, *N*^ε-methylhistamine, and internal standard obtained from the native tissue were 4.6, 5.2, and 3.8%, respectively ($n = 4$). Due to the high sensitivity and precision of this method the measurement of histamine and *N*^ε-methylhistamine in a single nucleus of rat hypothalamus was possible. Mean histamine and *N*^ε-methylhistamine contents of the median eminence (1.5 ± 0.3 mg tissue, $n = 5$) were 3.7 ± 1.0 and 1.5 ± 0.4 nmol/g tissue, respectively. This histamine concentration is in good agreement with the report of Correa and Saavedra, who used a radioenzymatic assay (20).

Effect of Pargyline on Imidazole Amine Concentrations

As an additional check for the quality of the histamine and *N*^ε-methylhistamine peaks we investigated the effect of the monoamine oxidase inhibitor pargyline on the size of the peaks obtained from the rat hypothalamus. According to the procedure of Oishi *et al.* (5), pargyline hydrochloride solution in 0.9% saline (80 mg/kg ip) was administered 4 h before sacrificing the animals. Control rats were injected with 0.9% saline. The concentrations of both histamine and *N*^ε-methylhistamine increased significantly after pargyline treatment (Figs. 6, 7). The increments were almost identical to those previously reported by Hough *et al.* (4). This pharmacological experiment demonstrated the usefulness of our method for the direct measurement of histamine turnover.

DISCUSSION

The main contribution of the present method is making practical the routine use of HPLC–EC for the simultaneous quantification of histamine and *N*^ε-methylhistamine with high sensitivity and specificity. Our new derivatization method and EC significantly increased sensitivity in comparison with previous fluorometric methods (6,7). This is the first report of the routine application of EC for the detection of imidazole amines. This method should be particularly useful to elucidate the role and functioning of histaminergic neurons in the central nervous system. Though radioenzymatic assay of histamine is even more sensitive, it

does not quantify the methylated metabolites of histamine. The main drawback of our method is that amines and amine derivatives must be purified by cation-exchange chromatography to obtain samples clean enough for the HPLC–EC analysis. Thus, the method is fairly labor intensive, requiring up to 8 h for the complete processing of a series of tissue samples. Recently, Ronnberg *et al.* (21) reviewed several HPLC methods using fluorometric detection for histamine. These methods were based on *o*-phthalaldehyde derivatization. They concluded that cation-exchange chromatography prior derivatization is preferable with these methods as well. The advantage of using a strong cation-exchange resin for the prepurification of the samples is that the quantity of the derivatizing reagent can be markedly reduced. This leads to a dramatic reduction of additional peaks originating from the reaction products in the chromatography.

The Bolton–Hunter reagent has a highly electroactive phenolic ring. Its hydroxy group is close to ideal for quantifying low concentrations of the histamine derivatives with EC. Furthermore, the high stability of the derivatives enables the achievement of excellent precision. *N*^α-Methylhistamine is close to an ideal internal standard for the present procedure to correct for volumetric errors during extraction and injection of samples. It is not present in biological samples; Schippert *et al.* (8) have reported the absence of *N*^α-methylhistamine in the pig gastric mucosa using thin-layer chromatography after dansylation, and Wollin and Navert (9) found no *N*^α-methylhistamine in human urine using gas–liquid chromatography. We also did not find *N*^α-methylhistamine in rat brain. As we have documented, the derivatization and detection of spermidine and histidine are also possible with the present method. Moreover, the method can be applied to the measurement of other amines such as phenylethylamine and to amino acids. We chose the ESA electrochemical detector over the BAS detector due to better sensitivity under optimal operating conditions for each detector. The clear drawback of this choice is a relatively short life span of the detector flow cells (approximately 2 months with 10 samples/dav. 5 days a week).

In conclusion, we have developed a practical method to measure histamine turnover in single nuclei of the rat brain.

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Fig. 1.

Chromatogram obtained by derivatization of standard solution (corresponding to 20 pmol of each standard amine). The numbered peaks are: 1, histamine; 2, *N*^ε-methylhistamine; 3, *N*^α-methylhistamine.

Fig. 2. Relationship between the retention times of derivatives and pH of the mobile phase. The pH was adjusted by changing the volume of glacial acetic acid. ●, Histamine ($r = 0.996$); O, N^{ϵ} -methylhistamine ($r = 0.994$); △, N^{α} -methylhistamine ($r = 0.997$).

Fig. 3.

Effect of pH of the reaction mixture on the reaction yield. The reaction mixture consisted of 1 ml of 10^{-6} M standard solution and 100 μ l of 0.02 M sulfo B-H and reaction buffer. The pH was adjusted by changing the volume and ratio of 0.1 M Na_2CO_3 and 0.1 M NaHCO_3 .

●, Histamine; ○, N^{ϵ} -methylhistamine; △, N^{α} -methylhistamine.

A.

R = H (Bolton-Hunter reagent)

1aR = SO₃⁻Na⁺ (water soluble Bolton-Hunter reagent)1b

B.

Fig. 4.

Derivatization of imidazole amines with sulfosuccinimidy-3-(4-hydroxyphenyl)propionate (water- soluble Bolton–Hunter reagent).

Fig. 5. Chromatogram obtained from rat hypothalamus. Peaks: 1, histamine; 2, N^{π} -methylhistamine; 3, N^{α} -methylhistamine as internal standard (10 nmol/g tissue).

Fig. 6. Chromatograms of rat hypothalami. (A) saline ip; (B) pargyline 80 mg/kg ip. Peaks: 1, histamine; 2, *N^α*-methylhistamine; 3, *N^α*-methylhistamine as internal standard (10 nmol/g tissue).

Fig. 7.

Effect of pargyline on histamine and *N*^c-methylhistamine contents in rat hypothalamus.

Each bar represents the mean $\pm$ SD of four animals. Asterisks indicate significantly different values from saline ip group by Student's *t* test (* $P < 0.05$, ** $P < 0.001$).

TABLE 1

Studies of Reaction Yield

	[A] Peak height of 100 pmol of pure derivative (<i>n</i> = 4) ^a	[B] Peak height of derivative corresponding to 100 pmol of amine (<i>n</i> = 4) ^a	[C] % Recovery of derivative after cellulose-phosphate column (<i>n</i> = 4)	% Calculated reaction yield ^c
Histamine	100 ± 2.9 ^d	89.2 ± 0.0 ^d	90.0	99.1
N ^ε -Methylhistamine	100 ± 1.3	83.9 ± 0.9	88.4	94.9
N ^α - Methylhistamine	100 ± 2.9	86.7 ± 1.6	88.3	98.2

^a Figures indicate relative peak heights (mean peak height of pure derivative of each amine is expressed as 100).

^b Mean recovery of 100 pmol of pure derivative after cellulose phosphate fibrous cation exchanger.

^c Reaction yield = ([B]/[A]) × (100/[C]) × 100%.

^d Mean ± SD.

TABLE 2

Precision of HPLC Analysis for Standard Amines^a

	<i>N</i>	Coefficient of variation (%) ^b		
		Histamine	<i>N</i> ^ε -Methylhistamine	<i>N</i> ^α -Methylhistamine
Within-run	5	2.9	2.7	2.6
Between-run	4	2.7	1.6	6.6

^aSamples injected were 10 pmol of each amine derivatized independently.^bCoefficient of variation = $100 \times (SD/\bar{x})$.