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HISTAMINE IN TISSUE: DETERMINATION BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AFTER CONDENSATION WITH *o*-PHTHALDIALDEHYDE

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SUMMARY

Histamine was determined by reversed-phase high-performance liquid chromatography in perchloric acid extracts after condensation with *o*-phthaldialdehyde. Fluorescence was monitored at 360 nm excitation and 450 nm emission wavelengths after elution with mixtures of 0.1 *N* acetic acid containing 0.1% pentanesulfonic acid and acetonitrile. The detection limit was 0.9 pmol of histamine. The histamine content was determined in rat whole brain, medulla oblongata, dorsal and ventral spinal cord, dorsal and ventral skin of the hind paw, stomach, ileum, rectum, lung and a hind-quarter perfusate, and compared to published data. The advantages of the described method over other methods are (A) rapid analysis in an automated system, (B) no selective extraction procedure is necessary, and (C) interfering substances are easily separated from the histamine fluorophore.

INTRODUCTION

The fluorimetric method for the estimation of histamine in biological samples is based on the reaction with *o*-phthaldialdehyde in alkaline medium to form a fluorescent product which is converted to a more fluorescent and stable product by acidification [1]. Much effort has been given to avoiding interference from other naturally occurring substances, mainly histidine and spermidine [2—4] by specific purification techniques such as ion-exchange chromatography [5—9] or selective extraction into isoamyl alcohol [10] prior to the reaction with *o*-phthaldialdehyde. Thin-layer chromatography as well as paper, thin-layer and gel electrophoresis [11, 12], or high-speed liquid chromatography [13], have also been used to identify the histamine fluorophore from amongst other, interfering substances.

With the development of novel chromatographic methods another tool

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has become available to circumvent such laborious extraction procedures. Recently, high-performance liquid chromatography (HPLC) has been employed for the separation of various biogenic amines after condensation with *o*-phthaldialdehyde [14]. This method involves extensive sample clean-up and stepwise gradient elution over 80 min. The present paper describes a simple and rapid method for the determination of histamine after condensation with *o*-phthaldialdehyde using HPLC under isocratic elution conditions.

EXPERIMENTAL

Reagents and materials

L-Histamine dihydrochloride, L-histidine hydrochloride (pyrogen-free and histamine-free), sodium imidazole-4-acetate and spermidine trihydrochloride were obtained from Serva (Heidelberg, G.F.R.); *o*-phthaldialdehyde was from E. Merck (Darmstadt, G.F.R.), and sodium 1-pentanesulfonate from Fluka (Buchs, Switzerland). All other reagents were of analytical grade (E. Merck). All solvents were degassed in an ultrasonic bath prior to use.

Stock solutions

Standards of L-histamine, L-histidine, imidazoleacetic acid, and spermidine in concentrations of 1 mg ml^{-1} were dissolved in 0.2 *N* perchloric acid. *o*-Phthaldialdehyde was dissolved in methanol (1% w/v).

Apparatus, column, and mobile phase

The HPLC system consisted of an Altex 110A pump with pulse dampener, a Kontron ASI-45 autosampler with a Rheodyne injection valve, a reversed-phase column (Waters μBondapak C_{18}, 300 × 3.9 mm, particle size 10 μm), and a Kontron SFM 23 fluorescence detector. Chromatogram recordings and all calculations were performed on a Shimadzu CR1A integrator.

The mobile phase consisted of 0.1 *N* acetic acid containing 0.1% pentanesulfonic acid; acetonitrile concentrations ranged from 15 to 25% (Fig. 1). In extracts containing high concentrations of interfering substances, such as those from nervous tissue, the separation of the histamine fluorophore was optimised by use of a more polar elution medium (15% acetonitrile). The retention time of the histamine fluorophore was about 12 min, the time of the HPLC analysis was 30 min. To avoid overloading of the column by non-polar substances which adhere to the column, particularly after several sample injections, the chromatography was interrupted 15 min after each sample injection and 500 μl of acetonitrile were injected. Impurities adhering to the column were washed out with the front peak of acetonitrile. Further equilibration was carried out for 13 min prior to the injection of the next sample. In all tissue samples listed in Table I, except nervous tissue, the concentrations of interfering substances were found to be so low that a sufficient separation of the histamine fluorophore was achieved by the use of a non-polar elution medium (mobile phase containing 20—25% acetonitrile). In this instance, the retention time of the histamine fluorophore was 5—6 min and the time of the HPLC analysis could be shortened to 15 min. Column wash during the automated analysis was performed by injection of 500 μl

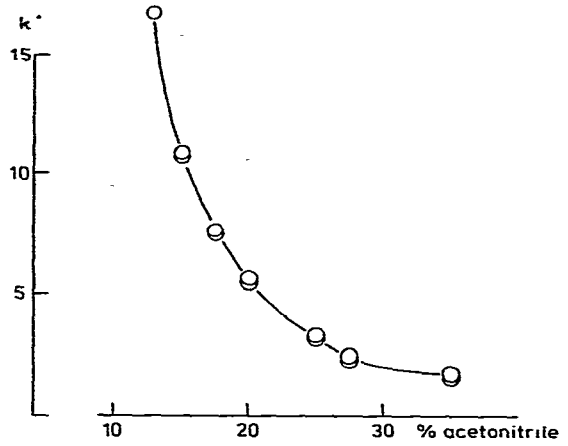

Fig. 1. k' values for the histamine—o-phthaldialdehyde fluorophore in 0.1 N acetic acid, 0.1% pentanesulfonic acid—acetonitrile mixtures at a flow-rate of 2.5 ml min^{-1}, fluorescence detection at 360 nm excitation and 450 nm emission wavelengths.

TABLE I

HISTAMINE CONTENT OF DIFFERENT RAT TISSUES COMPARED WITH PUBLISHED DATA

Data are expressed as the mean ± S.E.M., n as indicated, references in the square brackets.

Tissue	n	Histamine (nmol g^{-1})			
		HPLC	Radioenzymatic assay	Fluorimetric assay	Bioassay
Whole brain	5	0.75 ± 0.03	0.72 [17] 0.54 [16]	0.47 [21] 0.68 [20] 0.50 [21] 0.41 [22] 0.43 [24]	0.65 [4] 0.48 [18] 0.51 [23]
Medulla oblongata	5	0.50 ± 0.08		0.27 [24]	
Dorsal spinal cord	5	0.45 ± 0.01			
Ventral spinal cord	5	0.34 ± 0.03			
Hind paw, dorsal skin	10	195 ± 38	198.9 [17]		590.4 [18]
Hind paw, ventral skin	10	164 ± 11			
Stomach, antrum	6	106 ± 8	320.6 [17]		326.1 [10]
Stomach, fundus	6	104 ± 15	59.9 [17]		137.2 [18]
Ileum	6	71 ± 5			50.0 [18]
Rectum	6	75 ± 5			22.7 [18]
Lung	6	76 ± 9			153.5 [10] 32.7— 99.0 [18]
Hind-quarter perfusate	10	1.05 ± 0.20			0.82 [19]

of acetonitrile after ten samples followed by an equilibration period of 13 min prior to the injection of the next sample.

No satisfactory results in the separation of the histamine fluorophore were

obtained by the use of heptanesulfonic acid instead of pentanesulfonic acid or without ion-pair reagent, due to tailing and peak broadening.

Extraction of tissues

Sprague—Dawley rats of either sex, weighing about 250 g, were killed by cervical dislocation. The brain, the medulla oblongata, the spinal cord, the hind-paw skin, the stomach, a portion of the ileum (10 cm in length, close to the ileocoecal junction), and the rectum were excised. Tissue samples were frozen in liquid nitrogen, weighed, and pulverised using a Braun—Melsungen Dismembrator (1 min, 10 mm amplitude). The powder was taken up in approximately 5—10 volumes of 0.2 *N* perchloric acid and dispersed by ultrasonication for 1 min. The samples were centrifuged (1000 *g*, 10 min) and the supernatants stored at —20°C.

Extraction of perfusates

Perfusion of the isolated hind-quarter of the rat was performed according to the method of Erjavec et al. [15]. Sprague—Dawley rats of either sex (200—300 g) were used. The aorta and the vena cava inferior were cannulated at the level of the iliolumbar arteries and perfused with oxygenated Krebs—Ringer solution containing gelatine at a flow-rate of 2.2 ml min^{-1} (for details see ref. 15). The venous outflow was collected in periods of 5 min at 0°C. The samples were acidified by the addition of 17 µl of concentrated perchloric acid (70%, w/v) per ml of sample (final concentration 0.2 *N*). Protein precipitates were removed by centrifugation (1000 *g*, 10 min). The supernatants were stored at —20°C.

Estimation of histamine

Samples of 1 ml volume were made alkaline by the addition of 0.4 ml of 1 *N* sodium hydroxide. Derivatisation was performed by adding 0.3 ml of *o*-phthaldialdehyde stock solution followed by vigorous mixing. This *o*-phthaldialdehyde concentration was found to be sufficient for quantitative derivatisation in all investigated tissue extracts under the described conditions. This was checked using an internal standard of 3630 pmol of histamine. The samples were allowed to react with the *o*-phthaldialdehyde for 4 min. Then the fluorophore was converted to a more fluorescent and stable product by acidification with 0.1 ml of 3 *N* hydrochloric acid. Since the spermidine standard and all tissue samples contained a precipitate after acidification, all samples were centrifuged (1000 *g*, 10 min); 500 µl of the supernatant were directly injected into the HPLC system. Fluorescence was monitored at 360 nm excitation and 450 nm emission wavelengths [1]. The detection limit was 0.9 pmol histamine per 500 µl of injected sample at a signal-to-noise ratio of 3:1.

RESULTS AND DISCUSSION

Analysis of standards

As shown in Fig. 2A, the standards of L-histamine used produced three peaks after condensation: a major peak at 12.3 min retention time and two

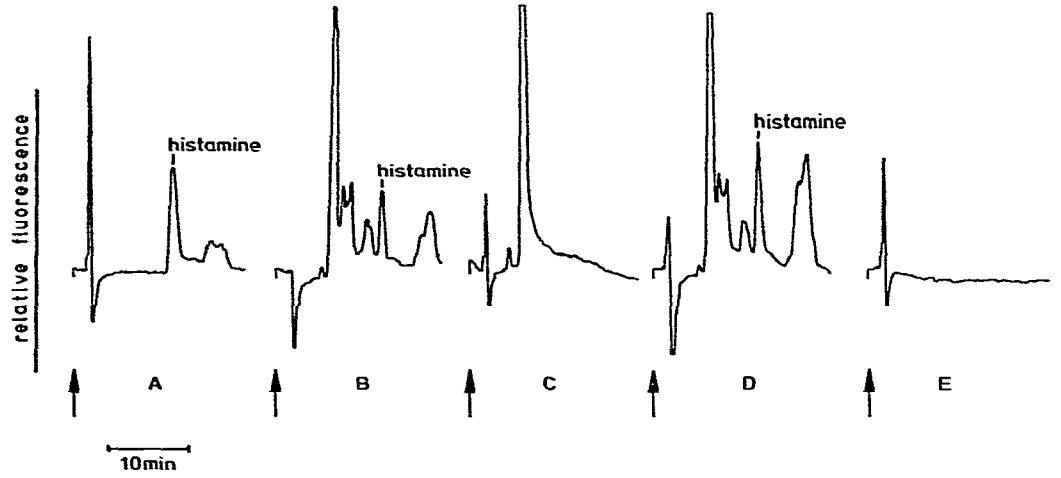

Fig. 2. Chromatograms of standards: (A) L-histamine (25.0 pmol); (B) L-histidine (16.1 nmol); (C) spermidine (19.4 nmol); (D) mixture of histamine (12.5 pmol), L-histidine (8.1 nmol), and spermidine (9.7 nmol); (E) solvent. All substances were diluted in 0.2 *N* perchlorid acid. Injection volume was 500 μl. The mobile phase consisted of 0.1 *M* acetic acid, 0.1% pentanesulfonic acid and 15% acetonitrile at a flow-rate of 2.0 ml min^{-1}. Attenuation for these chromatograms: position "high" of the fluorimeter, integrator input 32 mV full scale. The arrows indicate the injection of samples.

smaller ones with retention times of 16.8 and 18.0 min. The peak areas of the two minor peaks were proportional to the peak area of the main peak at all concentrations investigated. The minor peaks seemed to be by-products formed by the condensation procedure since they occurred even in biological samples. All calculations refer to the major peak of fluorescence which was linearly dependent on the histamine concentration up to 2270 pmol ml^{-1} (linear regression, $r^2 = 0.99$).

The condensation product of L-histidine showed at least eight peaks (Fig. 2B). The main peak which seemed to correlate with the L-histidine content had a retention time of 6.9 min. Three other peaks corresponded to histamine and histamine by-products as inferred from internal standardisation, indicating that the standard L-histidine contained impurities of histamine. However, the histamine content of the standard substance was found to be less than 0.05%. Standards of spermidine showed a single peak at a retention time of 7.3 min (Fig. 2C). Standards of imidazoleacetic acid, even in concentrations of 20 nmol per sample, showed no fluorescence by this method.

The peaks of histidine and spermidine may overlap each other but the histamine fluorophore is always separated from all of these peaks (Fig. 2D). The reproducibility of the assay was tested by eighteen repeated injections of 18.8 pmol of histamine obtained from a single derivatisation. The first injection was performed not earlier than 30 min after derivatisation because a slight initial decline of fluorescence was observed within this period. The following seventeen injections were made at intervals of 30 min. The mean of the calculated peak areas showed a standard deviation of 8.3%. This indicates reproducibility of the analysis and the stability of the histamine fluorophore for at least 9 h, which agrees with the results of Davis et al. [14].

Analysis of tissue and perfusate extracts

In agreement with others [3, 4, 10, 11] we found the ratio of histamine to interfering substances, mainly spermidine, to be highest in brain and other nervous tissues. Kremzner and Pfeiffer [3] reported that rat brain contains 1000 times more spermidine than histamine. Michaelson [4] reported a spermidine to histamine ratio of 500:1 in nervous tissues. However, even under these unfavourable conditions, the histamine fluorophore could be completely separated from interfering substances like spermidine and histidine using a more polar elution medium (mobile phase containing 15% acetonitrile), as shown in Fig. 3A. The retention time of the histamine fluorophore was about 12 min, and the last peak obtained had a retention time of 25 min.

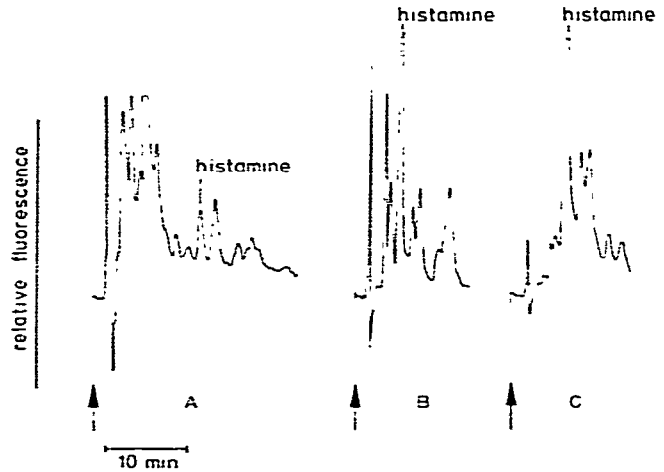

Fig. 3. Chromatograms of rat tissue samples and perfusates of the isolated rat hind-quarter. (A) Medulla oblongata (8.1 pmol), mobile phase contained 15% acetonitrile, flow-rate 2.0 ml min^{-1}, integrator input 32 mV full scale. (B) Perfusate of the rat hind-quarter (20.5 pmol), mobile phase contained 20% acetonitrile, flow-rate 2.5 ml min^{-1}, integrator input 32 mV full scale. (C) Dorsal skin of the hind paw (2.9 nmol), mobile phase contained 20% acetonitrile, flow-rate 2.5 ml min^{-1}, integrator input 128 mV full scale. The arrows indicate the injection of samples.

All other tissues were found to contain far fewer interfering substances than nervous tissue. A sufficient separation of the histamine fluorophore from interfering substances was achieved with a less polar elution medium (mobile phase containing 20—25% acetonitrile). Under these conditions the duration of the HPLC analysis could be shortened to 15 min (Fig. 3B and C).

The content of histamine in some rat tissues and in the perfusates from the isolated rat hind-quarter measured by HPLC is within the range of values obtained by other methods (Table I). The detection limit of 0.9 pmol enabled the measurement of histamine even in a crude homogenate of the rat medulla oblongata, as shown in Fig. 3A.

The described HPLC method for the determination of histamine in biological samples has the following advantages over other methods including gas chromatography—mass spectrometry [25] and radioenzymatic assays [16,

17]: (A) analyses can be carried out rapidly in an automated system after a simple sample preparation and derivatisation with *o*-phthaldialdehyde; (B) interfering substances are easily separated from the histamine fluorophore; (C) no specific extraction procedure is necessary; and (D) no loss of histamine seems to occur.

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