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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PLASMA AND BRAIN HISTAMINE WITHOUT PREVIOUS PURIFICATION OF BIOLOGICAL SAMPLES: CATION-EXCHANGE CHROMATOGRAPHY COUPLED WITH POST-COLUMN DERIVATIZATION FLUOROMETRY

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SUMMARY

A highly sensitive and specific method was developed for the determination of histamine in biological materials by high-performance liquid chromatography with a cation exchanger and an automated Shore's fluorometric detection system. Since substances causing interference in Shore's *o*-phthalaldehyde method, such as ammonia, histidine, spermine and spermidine, were completely separated on the column and their fluorescent intensities were much less than that of histamine in this detection system, histamine could be determined by injecting a perchloric acid extract of human plasma or mouse brain tissue directly onto the column without any previous purification procedure. The lower limit of detection of histamine by this method is 0.05 pmol, and the within-day and day-to-day variations in plasma histamine assay are less than 3%. The plasma histamine level in normal human subjects was found to be 4.0 ± 1.6 pmol/ml (mean ± S.D., n=20). A good linear correlation was obtained between values for the histamine contents of mouse brain tissues determined by this method and by a radioenzymatic method with a purified histamine-N-methyltransferase preparation. The histamine levels of whole brain, hypothalamus, thalamus, brain stem and frontal cortex in male ddY mice were 367 ± 38, 1143 ± 70, 414 ± 66, 196 ± 47 and 467 ± 91 pmol/g of wet tissue (mean ± S.D.), respectively.

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INTRODUCTION

Accurate estimation of histamine in biological materials, especially in plasma and brain tissues, is very difficult mainly because its content is much lower than those of interfering substances present in extracts [1]. With the development and subsequent improvements of the enzymatic radioisotopic assay using histamine-N-methyltransferase and [^{3}H]-S-adenosylmethionine, sensitive determination of histamine is now possible [1], but inter-laboratory variation [2] and problems in the specificity [3] of the method have been pointed out.

High-performance liquid chromatography (HPLC) combined with fluorometry has also been used for histamine assay [4—9], but these methods require troublesome sample clean-up and/or a derivatization procedure before the determination. Shore's *o*-phthalaldehyde method [10] is a sensitive and relatively specific fluorometric assay for histamine. By combination of this fluorometric method with chromatography on a cation exchanger, we developed a method of post-column derivatization HPLC using a continuous-flow reaction system [11]. In the method described in the previous paper, partial purification of histamine from biological samples by Dowex 50 column chromatography was necessary before its injection onto the HPLC column. However, we subsequently found that such purification could be avoided by stepwise elution and use of optimal conditions in the post-column derivatization system.

In this paper, we describe an HPLC method for determination of histamine in human plasma and mouse brain tissues without preliminary clean-up of the sample; i.e., by direct injection of the deproteinized sample of plasma or brain tissue onto the HPLC column.

EXPERIMENTAL

Chemicals

Histamine diphosphate was obtained from Sigma (St. Louis, MO, U.S.A.). Potassium dihydrogen phosphate, *o*-phthalaldehyde, sodium hydroxide and sulphuric acid of super-special grade were purchased from Wako (Osaka, Japan). Other chemicals were of analytical-reagent grade and were used without further purification. Glass-distilled water purified further with a Milli-Q system (No. XD20 115 84WN, Japan Millipore, Tokyo, Japan) was used throughout.

Chromatographic apparatus and conditions

Fig. 1 shows a diagram of the chromatographic system. Each plunger of a quadruple plunger pump (Model SF-4, Toyo Soda, Tokyo, Japan) served independently to deliver the mobile phase and reagents. A stainless-steel column (100 × 6 mm I.D.) packed with a cation exchanger (TSK gel SP-2SW, 5 μm, Toyo Soda) was eluted with 0.25 *M* potassium dihydrogen phosphate at a flow-rate of 0.6 ml/min. After elution of histamine, the mobile phase was changed to 0.5 *M* potassium dihydrogen phosphate with a three-way solenoid valve (Model MTV-3-M6, Takasago Electric, Nagoya, Japan) equipped with a digital timer. The sample injector was a Rheodyne Model 7125 with a 100-μl sampling loop. The eluate from the column was mixed first with 0.1% *o*-phthalaldehyde solution (1 g of *o*-phthalaldehyde dissolved in 10 ml of methanol and made up

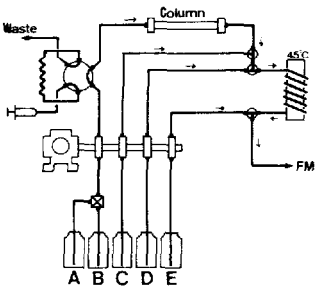

Fig. 1. Flow diagram of the HPLC system. Column: TSK gel SP-2SW, 100 × 6 mm I.D., particle size 5 μm. Mobile phase: (A) 0.25 *M* potassium dihydrogen phosphate; (B) 0.50 *M* potassium dihydrogen phosphate; flow-rate, 0.60 ml/min. Reagents: (C) 0.1% *o*-phthalaldehyde, flow-rate 0.15 ml/min; (D) mixture of 4 *M* sodium hydroxide and 0.2 *M* boric acid, flow-rate 0.16 ml/min; (E) 10% sulphuric acid, flow-rate 0.16 ml/min. FM = fluoromonitor, excitation wavelength 360 nm, emission wavelength 450 nm.

to 1000 ml with distilled water) at a flow-rate of 0.15 ml/min, and then a mixture of 4 *M* sodium hydroxide and 0.2 *M* boric acid was added (0.16 ml/min) to adjust the reaction mixture to pH 12.5. The solution was mixed in a reaction coil made of polytetrafluoroethylene tubing (5 m × 0.5 mm I.D.) wound round an aluminium heating block (5 cm O.D., regulated at 45 ± 0.1°C) and then 10% (v/v) sulphuric acid was added (0.16 ml/min) with mixing in a short reaction coil (1 m × 0.5 mm I.D.). The pH of the final reaction mixture was 3.0.

The fluorescence intensity was measured at 450 nm with excitation at 360 nm (slit widths for both excitation and emission, 10 nm) in a spectrofluorometer (Model 650-10LC, Hitachi Seisakusho, Tokyo, Japan) equipped with a 90-μl square flow cell, a recorder, and a chromatographic data processor (Model C-R3A, Shimadzu, Kyoto, Japan).

Sample preparation

Human blood. Blood was drawn from the antecubital vein of healthy non-allergic adults into a heparinized plastic syringe with a No. 18 gauge needle. The blood was promptly transferred to an ice-chilled plastic centrifuge tube containing 0.2 *M* sodium ethylenediaminetetraacetate (10 μl/ml of blood). The tube was inverted to mix the contents, and then plasma was separated by centrifugation at 3000 *g* for 10 min at 4°C. The plasma within ca. 1 cm above the buffy coat was not used to avoid possible contamination with leucocytes. To 1.0 ml of the plasma, 50 μl of 60% perchloric acid were added with vigorous mixing and the mixture was stored at −40°C. For analysis, the frozen mixture was centrifuged at 10 000 *g* for 30 min at 4°C. Then 100 μl of the clear supernatant were injected directly onto the HPLC column.

Mouse brain. Male ddY mice of six weeks old were obtained from KARI (Osaka, Japan) and were kept at 24°C under a standard light—dark cycle (8:00—20:00) for at least one week. Mice were sacrificed by decapitation at 14:00 h. The whole brain, or the hypothalamus and other regions of the brain, were isolated quickly, washed with cold sterile saline, blotted with filter paper, weighed on an electric balance, and promptly homogenized in 5–10 vols. of cold 3% perchloric acid containing 5 m*M* sodium ethylenediaminetetraacetate

by sonication for 10 sec in an ice-bath, and the homogenate was stored at −40°C. For analysis, the frozen homogenate was centrifuged at 10 000 *g* for 30 min at 4°C, and 10—100 μl of the clear supernatant were injected directly onto the HPLC column.

Radioenzymatic assay of brain histamine

We examined the correlation of the values obtained by the present HPLC method with those obtained by the enzymatic radioisotopic method of Verburg et al. [3] with a preparation of histamine-N-methyltransferase purified by DEAE cellulose chromatography. Perchloric acid extracts of several regions of mouse brain were adjusted to pH 7.8 by dropwise addition of 1 *M* potassium hydroxide, and briefly centrifuged to remove insoluble potassium perchlorate, and 50 μl of the supernatant were used for the assay.

RESULTS AND DISCUSSION

As shown in Fig. 2, representative interfering substances in the Shore's fluorometric assay were well separated from histamine. Because the mobile phase was changed from 0.25 *M* to 0.5 *M* potassium dihydrogen phosphate, strongly basic substances, such as spermidine, spermine and serotonin, were eluted together after histamine. Table I summarizes the retention times and relative fluorescence intensities of several interfering substances. Of these, putrescine had the closest retention time to histamine, but its fluorescence intensity was much weaker (0.005%) than that of histamine, and in practice its presence in samples of plasma or brain tissues did not interfere with the determination of histamine.

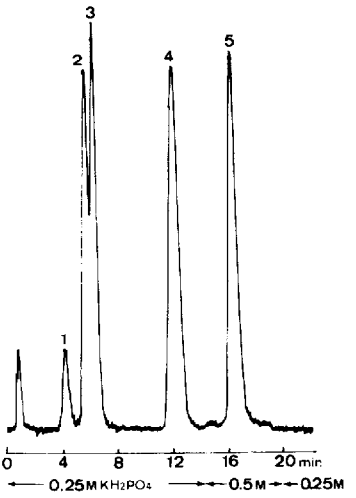

Fig. 2. Separation of histamine. The mobile phase in the column was changed from 0.25 to 0.5 *M* potassium dihydrogen phosphate at 15 min of elution. Peaks: 1 = reduced glutathione (250 pmol); 2 = histidine (125 pmol); 3 = ammonia (50 nmol); 4 = histamine (5 pmol); 5 = spermidine (1 nmol).

TABLE I

RETENTION TIMES AND RELATIVE FLUORESCENCE INTENSITIES OF HISTAMINE AND SOME INTERFERING SUBSTANCES

Substance	Retention time (min)	Relative fluorescence intensity*
Glutathione	4.5	2
Histidine	6.0	4
Ammonia	6.8	0.01
Putrescine	11.0	0.005
Histamine	12.0	100
Spermidine	17.0	0.5
Spermine	17.0	0.02
Serotonin	17.0	0.2

*Peak heights obtained by analysis of appropriate amounts of each substance were compared with the calculated peak heights of the same amounts of histamine. Values are expressed as percentages of that of the histamine peak.

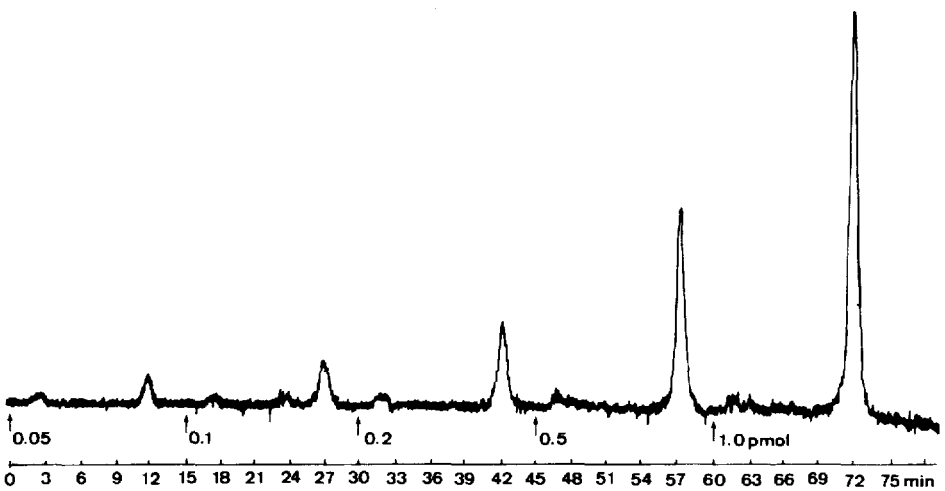

Fig. 3. Chromatogram of standard histamine. Standard samples containing 0.05—1.0 pmol of histamine in 100 μl of 3% perchloric acid were injected at 15-min intervals. Histamine was eluted 12 min after injection of each sample.

Fig. 3. shows a chromatogram of authentic histamine (0.05—1 pmol injected). From the chromatogram, the lower limit for detection seemed to be ca. 0.05 pmol. The linear regression equation and correlation coefficient between the amount injected (x) and the peak height (y) in the range 0.05—10 pmol were $y = 17.99x + 1.52$ and 0.9998, respectively. We examined the reproducibility of the method by injecting 47 samples of 2 pmol of histamine in 100 μl of 3% perchloric acid at 20-min intervals. The coefficient of variation in the 47 repeated assays was only 0.78%.

Fig. 4. shows a typical chromatogram for determination of histamine in

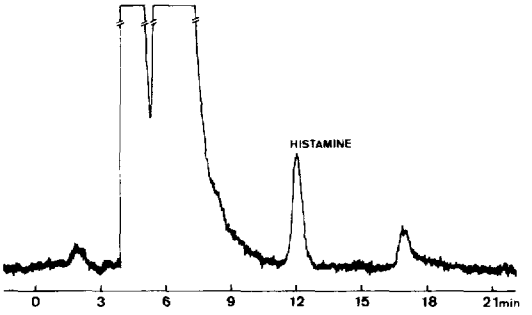

Fig. 4. Chromatogram of a plasma sample. Deproteinized plasma (100 μl) was injected directly onto the HPLC column. The concentration of histamine in the plasma was calculated as 3.70 pmol/ml from this chromatogram.

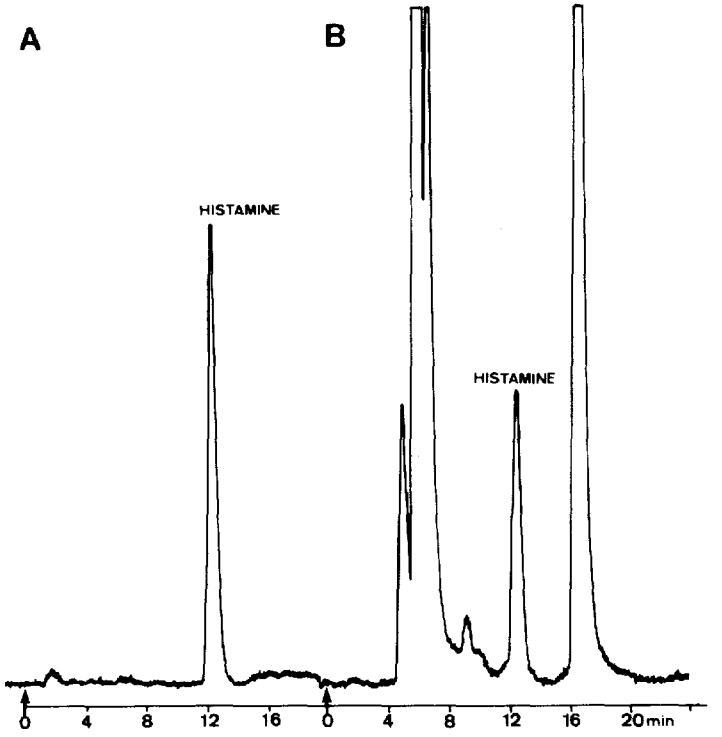

Fig. 5. Chromatogram of a brain sample. (A) Elution pattern of standard histamine sample (5 pmol in 100 μl). (B) Elution pattern of a deproteinized extract of mouse hypothalamus injected directly onto the HPLC column. The amount of sample injected corresponded to 2.65 mg of hypothalamus, and the content of histamine was calculated as 1192 pmol/g of wet tissue.

normal human plasma. When 2.5 pmol of histamine were added to 1 ml of pooled plasma, the overall recovery was 98.5 ± 2.5% (mean ± S.D., n=5). The within-day and day-to-day coefficients of variation in separate determinations on pooled plasma were less than 3% (n=5). The plasma level of histamine in healthy non-allergic adults was 4.0 ± 1.6 pmol/ml (mean ± S.D., n=20).

TABLE II

HISTAMINE CONTENTS OF MOUSE BRAIN

Region	Histamine content (mean ± S.D.) (pmol/g of wet tissue)	n
Whole brain	376 ± 38	10
Hypothalamus	1143 ± 70	5
Thalamus	414 ± 66	5
Brain stem	196 ± 47	5
Frontal cortex	467 ± 91	5

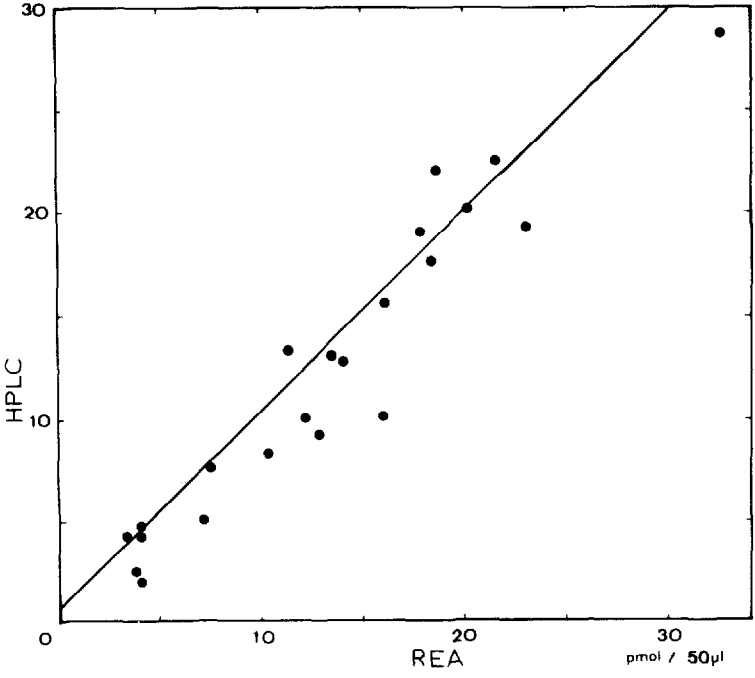

Fig. 6. Correlation between histamine values in brain extracts determined by the HPLC method and by the radioenzymatic method (REA). Samples (50 µl) of the same deproteinized extracts of several parts of mouse brain (n=22) were assayed by both methods. Values are expressed in picomoles of histamine in 50 µl of extract. The linear regression equation is $y = 0.948x + 1.468$ (correlation coefficient = 0.951).

Fig. 5 shows a typical chromatogram of histamine in an extract of the hypothalamus of the male mouse. The mean histamine contents of the hypothalamus, thalamus, brain stem, frontal cortex and whole brain were 1143 ± 70, 414 ± 66, 196 ± 47, 467 ± 91 and 376 ± 38 pmol/g of wet tissue, respectively (mean ± S.D., Table II).

The correlation between the HPLC method and a radioenzymatic method was examined by assays of the samples from the same tissue extracts of several

parts of mouse brain (n=22). As shown in Fig. 6, there was a good linear correlation between the two assay methods, and the linear regression equation was calculated as $y = 0.948X + 1.468$ (r=0.951).

Histamine has been determined by HPLC mainly by reversed-phase column chromatography with pre-column fluorescence labelling of histamine with *o*-phthalaldehyde in the presence of 2-mercaptoethanol [4—6, 9] or Shore's *o*-phthalaldehyde condensation method [8]. These methods require extensive clean-up of samples for good resolution and high sensitivity in assay of histamine in biological materials, especially plasma and brain tissues. However, such procedures are troublesome and may result in low reproducibility.

Previously we reported a post-column derivatization HPLC method using a cation exchanger and an automated flow system for the reaction used in Shore's fluorometric method [11]. In that method we used a sample clean-up procedure on a small column of Dowex 50 to remove several interfering substances, so in this work we tried to simplify the assay procedure to obtain an HPLC system that did not require sample purification. The stationary phase used here, TSK gel SP-2SW, was in principle the same cation exchanger as TSK IEX-510 used previously. However, by introduction of stepwise elution with simplified mobile phases, the histamine peak could be separated from front peaks and strongly basic substances, such as spermine and spermidine, within a reasonable time. In addition, use of optimal conditions for the post-column flow reaction system was very effective for resolution of the histamine peak from the huge front peaks. The optimal conditions for the reaction were in good agreement with those reported by Allenmark et al. [12]. Thus 10—100 μl of perchloric acid extract of plasma or brain tissue could be injected directly onto the HPLC column, and at least 0.05 pmol (ca. 5 pg) of histamine could be determined without any interference. The histamine concentrations in human plasma and mouse brain tissues determined by this method were in good agreement with reported values [13, 14].

So far we have analysed more than 2000 samples of biological materials, including plasma and brain tissues, without any trouble. One assay can be completed within 20 min and one column can be used for more than 500 analyses. This system will also be useful for routine use in clinical laboratories.

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