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Direct detection of endogenous histamine in rat peritoneal mast cells by in-capillary derivatization high-performance capillary electrophoresis

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Abstract

A simple method for the detection of endogenous histamine in rat peritoneal mast cells was evaluated using on-line mode in-capillary derivatization high-performance capillary electrophoretic (ICD-HPCE) techniques, which were previously developed by our group [S. Oguri et al., J. Chromatogr. A, 787 (1997) 253–260]. The method involves a suspension of peritoneal mast cells ($1 \cdot 10^6$ cells/ml of saline) collected from a male Wistar rat (eight weeks of age), which are directly introduced into the capillary tube from the anodic end by hydrostatic injection (at 25 cm height, for 2–20 s). When a high-voltage potential (25 kV) is applied to the capillary, which is already filled with the run buffer containing both a lysing reagent (SDS, sodium dodecyl sulfate,) and a derivatizing reagent (OPA, *o*-phthalaldehyde; NAC, *N*-acetylcysteine), histamine in the mast cells was detected at high-sensitivity level without further procedures. During ICD-HPCE, the mast cells injected in the capillary were lysed with the lysing reagent, free histamine released from the cell was labeled with the derivatizing reagent, and its derivative was electromigrated, separated and detected with a fluorescence detector (excitation wavelength at 340 nm, emission wavelength at 450 nm) in a fused-silica capillary (75 cm effective length×50 μm I.D.). The run buffer used was a 20 m*M* phosphate–borate buffer (pH 10) containing 20 m*M* SDS, 2 m*M* OPA and 2 m*M* NAC. This method was also examined with regard to the possibility of its use for determination of histamine at the single mast cell level.

Keywords: Derivatization, HPCE; Histamine

1. Introduction

Recently, the high-performance capillary electrophoretic method has come to be a useful and powerful separation technique, and now there are many applications that use this method [1,2]. To date, however, high-performance capillary electrophoresis (HPCE) still does not hold the position as the most representative method among analytical separation techniques. One reason is that the sensitivity of this method is relatively low by comparison with high-performance liquid chromatography (HPLC), which is the most widely employed methods at present.

But many analytical chemists, nevertheless, have been concentrating their energies on the development

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of HPCE methods and their applications, because it is thought that HPCE has many potential advantages as an analytical method. The most important thing is that HPCE development should not try to reproduce or emulate the results obtainable by HPLC; rather, HPCE technology should be developed with a view to those types of analyses for which it, and it alone, is suitable.

Along these lines, some exciting challenges using HPCE were presented in the early 1990s. Manz et al. [3] first demonstrated the ''down-sizing'' of HPCE by means of a micro-fabricated chip and evaluated it for the separation of amino acids. After that, planar-glass microchip capillary electrophoresis devices were applied to ultra-high-speed DNA sequencing or to research in other fields [4–6]. In 1993, Kanbara and Takahashi [7] assembled a multiple-sheathflow capillary array DNA analyzer, which is presently being developed for microchip application by Woolley and Mathies [8,9]. In other HPCE applications, Gilman and Ewing [10] reported on a single mammalian cell analyzed by HPCE equipped with a laser-induced fluorescence detector and by on-column derivatization. Here it is important to note that the assay of the contents of a single cell can be achieved by HPCE, but not by HPLC.

Histamine, which is well known as one of the chemical mediators in connection with allergies, is primarily found in mammalian mast cells. Measurements of histamine in the mast cells of biological samples are usually performed by bioassay [11], fluorocolorimetry [12], enzyme immunoassay [13], HPLC [14–16], and so on. However, these methods can only supply an averaged figure based on a group of cells being analyzed, and are incapable of analyzing single cells. In addition, individual cells in biological samples are not equivalent to each other. Therefore, it is very important to develop assay methods for single cells in order to help elucidate biological functions.

Along these lines, we developed a new HPCE method named ''in-capillary derivatization (ICD) HPCE'', and demonstrated the use of this method [17–21]. This method holds out the possibility, the authors believe, of performing an analysis on single cells. In this paper we describe our research efforts to adapt this ICD-HPCE method to the direct detection and determination of endogenous histamine in rat peritoneal mast cells.

2. Experimental

2.1. Reagents and materials

Bovine serum albumin (BSA), sodium dodecyl sulfate (SDS) and OPA (*o*-phthalaldehyde) employed in this study were of biochemical reagent grade, and the NAC (*N*-acetylcysteine) was of superfine reagent grade. Sodium pentobarbital (Nembutal) was commercially obtained from Dainippon Pharmaceutical Co. (Osaka, Japan). Histamine and all other chemicals used were of the highest or HPLC grade commercially available. These reagents were purchased from Wako (Tokyo, Japan), and used without further purification. All aqueous solutions were prepared by using water purified with a Milli-Q purified system (Millipore, Milford, MA, USA). A 0.2 m*M* histamine standard solution was also prepared by dissolving with water. A buffer solution was prepared by mixing equal amounts of 20 m*M* sodium dihydrogenphosphate and 20 m*M* sodium tetraborate; pH was adjusted to 10 with 1 *M* NaOH. These solutions were stored at room temperature prior to use. Run buffer solutions for ICD-HPCE were prepared as follows: 26.8 mg of OPA, 32.6 mg of NAC and 576.8 mg of SDS were dissolved together with 100 ml of the 20 m*M* phosphate–borate buffer (pH 10). A portion of each solution was filtered with a disposable syringe-filtering unit, DISMIC-13cp (Advantec, Tokyo, Japan). These run buffers were prepared just before use.

2.2. Animals

Male Wistar rats were purchased from Nippon SLC (Shizuoka, Japan), and were housed under conditions of free access to water and regular chow (CE-2, Chubukagakushizai, Nagoya, Japan) at 25±1.0°C prior to experiments.

2.3. Apparatus

The HPCE system consisted of a Jasco Model CE-800 (Jasco, Tokyo, Japan) equipped with a FP-920 fluorescence detector (Jasco), with the flow cell-unit for HPLC replaced by the capillary cell-unit for HPCE. All data were printed out with an intelligent data processor, Model 807-IT (Jasco). A capillary tube of fused-silica (75 cm effective length×50 μm

I.D.) was used throughout the work. The window (1.0 cm) for detection was made by removing a polyimide coating at a position 25 cm from the cathodic end. Sample solutions were introduced into the capillary tube from the anodic end by hydrostatic injection by raising the tube 25 cm higher than the level of the cathodic electrode for 10 s. Electropherograms were recorded by monitoring the fluorescence intensity at 450 nm (excited at 340 nm). A voltage potential of 25 kV was applied throughout the work.

2.4. On-line mode ICD-HPCE

Two reservoirs were placed in the HPCE system, one at the anodic site and another at the cathodic site. The anodic and cathodic reservoirs contained 1 ml of run buffer containing the derivatizing reagent (2 m*M* OPA/NAC) and additive (20 m*M* SDS) and 10 ml of 20 m*M* phosphate–borate buffer (pH 10), respectively. Before a sample solution was injected into the capillary at the anodic end, any remaining residue in the capillary was swept out with the run buffer and subsequently filled with the same buffer by suction at the cathodic end. When the HPCE system was not in use, the run buffer in the capillary tube and in both reservoirs was replaced with 20 m*M* phosphate–borate buffer (pH 10) or water.

2.5. Measurement of plug length at inlet end of capillary tube

A dye solution consisting of 10 mg/ml of Brilliant Blue solution was introduced to the anodic end of the capillary by the same hydrostatic injection method as described in the previous section. The length of the blue zone thus produced was then measured against a white background after removing approximately 2 cm of the polyimide coating from the capillary tube.

2.6. Preparation of rat peritoneal mast cells

Mast cells were collected from the peritoneal cavity of a male Wistar rat (eight weeks of age) by the method described by Hirai et al. [22]. The rat was anesthetized with ether and was killed by cutting the carotid artery. Then 20 ml of Tyrode's solution (137 m*M* NaCl, 2.7 m*M* KCl, 1.0 m*M* $MgCl_2$, 1.8 m*M* $CaCl_2$, 0.4 m*M* NaH_2PO_4, 11.9 m*M* $NaHCO_3$ and 5.6 m*M* D-glucose) was injected into the peritoneal cavity. After massaging the abdomen for 3 min, the suspended solutions of mast cells in Tyrode's solution were collected from the peritoneal cavity. The solutions were centrifuged at 150 *g* at 4°C for 10 min and the supernatant was pipetted off. A 2-ml volume of saline was added and suspended solution was layered on 4 ml of BSA–saline solution whose density and pH were adjusted to 1.068 and 7.0, respectively. After centrifugation (110 *g* at 4°C for 10 min), the bottom layer containing mast cells was washed twice with 4 ml of saline, and once again centrifuged at 100 *g* at 4°C for 10 min. The mast cells were suspended in the same medium at about $1 \cdot 10^6$ cell/ml. The purity of the mast cells was about 80~90% as observed with an Olympus phase contrast microscope.

3. Results and discussion

3.1. Determination of histamine in mast cell by ICD-HPCE method

To analyze a single cell by HPCE, the method developed by Gilman and Ewing usually involves the three following steps: (1) an individual cell is injected into the front end of a separation capillary by carefully handling the capillary tube with a micromanipulator under a microscope, which is used as a chamber to lyse the cell and to label the contents in the cell; (2) a derivatization reagent is injected for lysing the cell and labeling analytes at the same place in the capillary and; (3) subsequently, the cell contents are electromigrated and detected with a laser-induced fluorescence detector [10]. Although this method is a good and proven technique, it is rather complicated to perform.

In our study, a simpler method was employed, e.g., one using on-line mode ICD-HPCE as described in the previous section. The strategy employed is illustrated in Fig. 1, and involved the following stages: In step 1, a separation capillary of ICD-HPCE was filled with a run buffer containing both a lysing reagent for mast cells and a derivatization reagent (OPA/NAC) for histamine; subsequently a suspension of mast cells was directly injected by hydrostatic injection method in the usual manner for HPCE; when these two condition had been met, a

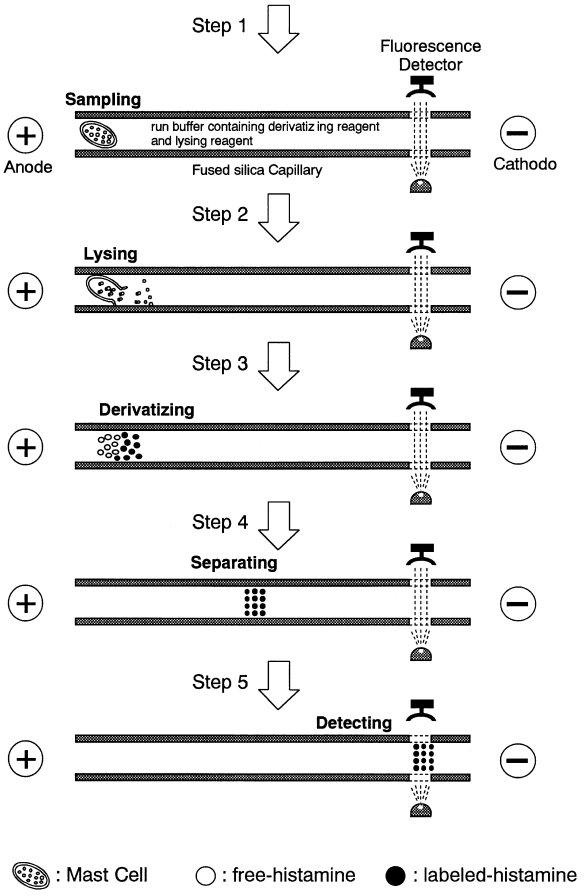

Fig. 1. Schematic representation of the method for determination of endogenous histamine levels in rat peritoneal mast cells. In step 1, the suspension of mast cells is directly injected hydrostatically into the capillary, which is filled with a run buffer consisting of 2 m*M* *o*-phthalaldehyde (OPA), 2 m*M* *N*-acetylcysteine (NAC), 20 m*M* sodium dodecyl sulfate (SDS), and 20 m*M* phosphate–borate buffer (pH 10). After that, in steps 2 and 3, a voltage potential of 25 kV was applied to the capillary. The cells were lysed with SDS in the run buffer (step 2), free histamine was liberated from the cells and was labeled with OPA/NAC, the derivatizing reagent in the run buffer (step 3); the derivative was then electromigrated and separated (step 4) and finally the derivative was detected with a fluorescence detector (step 5).

high voltage (25 kV) was applied and electrophoresis was carried out (steps 2–5). During the ICD-HPCE, after mast cells were lysed (step 2), the histamine which was liberated from the cells was labeled with OPA/NAC (step 3), the tagged histamine was electromigrated (step 4) and was detected (step 5) in the capillary. The results obtained in steps 3–5 of this procedure have already been described under the terminology of ICD-HPCE [17]; what is new in this study is the presence of a suitable lysing reagent already mixed with the run buffer.

3.2. Additives for lysing mast cells and ICD-HPCE

To find a suitable lysing reagent for this method, several additives were investigated by the following procedures. First, 50 μl of a suspension (10^6 cells/ml of saline) of rat peritoneal mast cells was separately mixed with 50-μl volumes of acetone, ethanol, 0.025% (w/v) Triton X-100 and 20 m*M* SDS in a 0.6-ml polypropylene micro-centrifuge tube. An aliquot of each mixture was immediately set on the stage of a microscope and the shapes of the mast cells were observed at approximately 400× magnification to compare the differences of cell shapes before and just after treatment with each additive. While making each observation of the effect on cell structure of each additive, the corresponding mixture containing the additive in question was immediately centrifuged and subsequently an aliquot of the supernatant was analyzed using ICD-HPCE in order to evaluate the levels of free histamine released from the lysed mast cells. The electropherograms shown in Fig. 2a–e were obtained as a result of treatment by SDS, ethanol, acetone, 0.025% (w/v) Triton X-100 and saline (blank), respectively. The peak of histamine was recognized at a migration time of about 10 min, and was identified by comparing the changes of the peak-area counts obtained from the supernatant for SDS before and after being spiked with histamine.

In Fig. 2a–e, the peak-area counts of histamine were the highest for SDS, in comparison with other lysing reagents, and the shape of the mast cells appeared remarkably changed immediately after SDS was added to the suspension. In Fig. 3a and b, photographs show the shape (a) before and (b) just after treatment with SDS. As the results of these, histamine was sufficiently released from the mast cells by lysing with 20 m*M* SDS.

The use of SDS as the lysing reagent has the advantage that SDS is commonly employed as the run buffer in MEKC (micellar electrokinetic chromatography), and, therefore, poses no further complications for electric current flow. Based on the above-

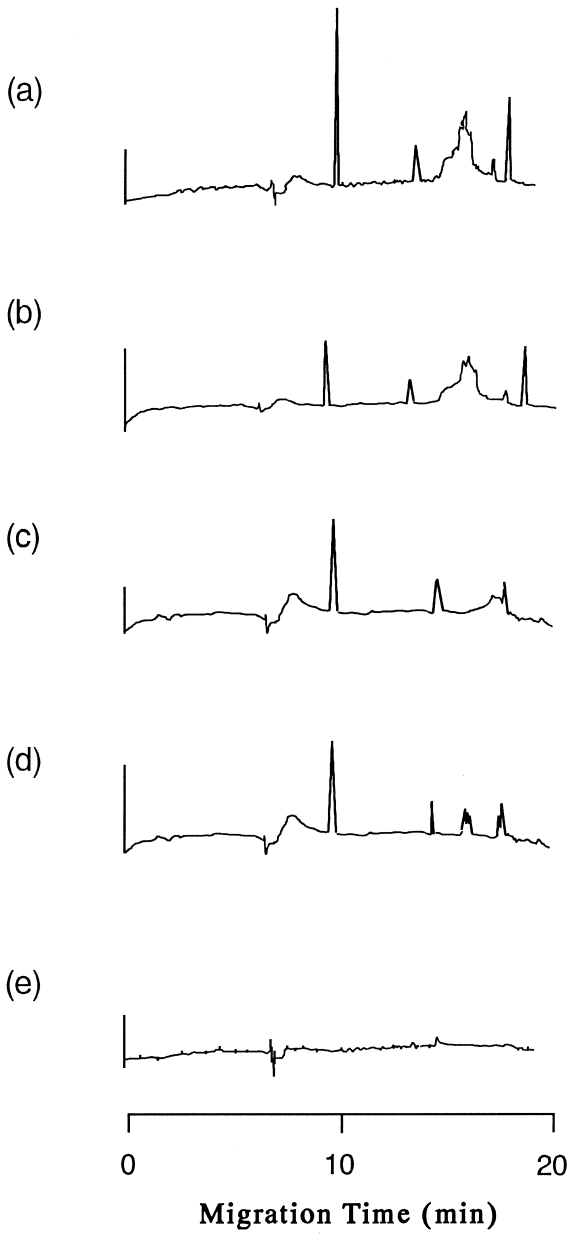

Fig. 2. Typical ICD-HPCE electropherograms of treatments of rat peritoneal mast cells with (a) SDS, (b) ethanol, (c) acetone, (d) 0.025% (w/v) Triton X-100 and (e) saline (blank). The injection time and the run buffer were 10 s and 2 m*M* OPA–2 m*M* NAC–20 m*M* SDS–20 m*M* phosphate–borate buffer (pH 10), respectively. The other conditions are described in the Sections 2.3 and 2.4.

mentioned results, we chose a run buffer composed of 2 m*M* OPA, 2 m*M* NAC and 20 m*M* SDS in 20 m*M* phosphate borate buffer (pH 10) for this study. Given all of the above conditions, it was shown that this system has the capability of clearly distinguishing the presence of histamine relative to that of other amines potentially present (cadaverine, putrescine, sepermidine and tyramine) [20].

3.3. The comparison of peak-area counts and mass of histamine

To estimate the amount of histamine in rat mast cells, the injection volume of the separation capillary of the ICD-HPCE system was determined by measuring a plug-length of the capillary when a 10 mg/ml of Brilliant Blue solution was hydrostaticaly injected at a height of 25 cm over the time range 2–30 s. The results of the relationship between the plug-length and the injection time (from 2 to 30 s at 25 cm height) are shown in Fig. 4. In addition, Fig. 4 also shows the scale of injection-volumes in the capillary calculated on the basis of the plug-length and the internal radius (50 μm) of the capillary. The injection volume or plug-length increases with increasing time of hydrostatic injection. Under these conditions, the plug-length was not affected by the contents already present in the capillary, such as the concentration of run buffer (10 to 100 m*M*) or by run buffers with or without the presence of SDS.

3.4. Peak-area count vs. injection time or mass of histamine

The relationship between peak-area count and injection time, or peak-area count and the mass of histamine as calculated on the basis of an injection volume of 0.2 m*M* standard histamine solution was studied by a quadruplicate analysis (i.e., four separate readings were taken for each sample). As indicated by the straight-line graph in Fig. 5, the relationship between the peak-area count versus injection volume or mass of histamine is linear. The linearity was calculated by the least squares regression method for $y=ax+b$, where y is the peak-area count of the histamine peak and x is the mass of histamine or injection time when the 0.2 m*M* standard histamine solution was used. The correlation

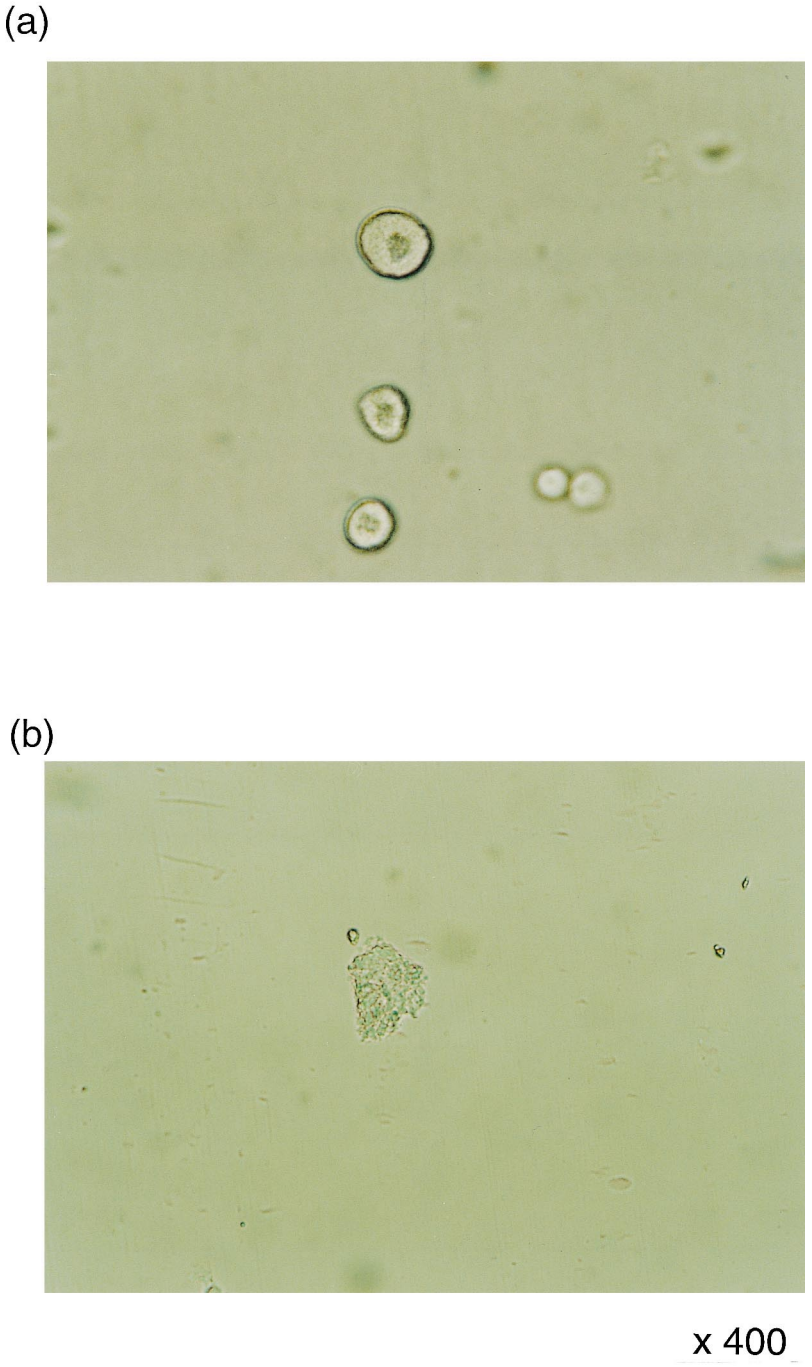

Fig. 3. Photograph of typical rat peritoneal mast cells (a) before, and (b) just after treatment of the suspension of mast cells with equal amounts of 20 m*M* SDS saline at approximately 400× magnification.

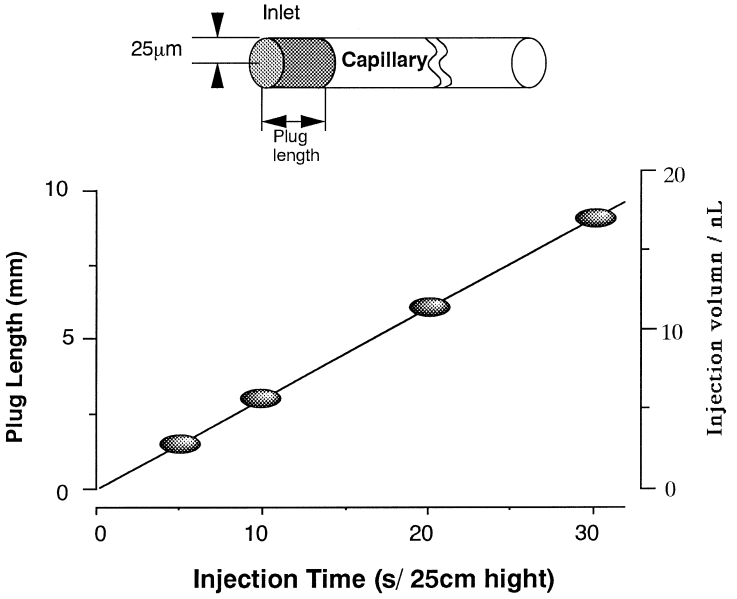

Fig. 4. Effect of injection time on plug-length of the capillary. The data express averages obtained by a quadruplicate analysis of a 10 mg/ml Brilliant Blue solution. See Section 2.5 for further explanation.

factor (r) for this is 0.999, indicating very good linearity. Reproducibility tests were also carried out using the same standard solution with a 10 s injection time. The relative standard deviations (RSDs) ($n=10$) of the migration times and peak-area counts of histamine were 0.3% and 1.3%, respectively, very close to values previously reported [18].

From these results, the lower limit of detectability of histamine was determined to be ca. 10 amol or 1.1 pg. The mass of histamine in a rat peritoneal mast cell was previously reported as 23 to 40 pg/cell [23] or 31.5±1.9 pg/cell [24]. Considering the lower limit of detection of the present method, it appears that this method could certainly be used to detect the level of endogenous histamine in a single mast cell.

3.5. Measurement of histamine in rat peritoneal mast cells

Measurement of histamine levels in rat peritoneal mast cells was tried using the present method. When the suspension of mast cells ($1\cdot10^6$ cells/ml of saline) was directly injected for 10 s, a typical ICD-HPCE electropherogram was produced as shown in Fig. 6. The peak of histamine migrated for 10.3 min and was sufficiently separated from other peaks; the profile here is almost the same as that of Fig. 2a–d.

Although numerous species were present from the broken cells, few peaks were observed beyond those expected in the electropherograms. The following reasons may be considered: (1) using the OPA/thiol system as the reagent can detect only amino compounds, but no other compounds present in the broken matrix; (2) in general, the fluorescence intensity of OPA/thiol derivatives of low-molecular-mass amino compounds, such as histamine and amino acids, are higher than those of high-molecular-mass amino compounds such as peptides and proteins, and finally; (3) low-molecular-mass amino compounds, except for histamine, if present at all, exist at very low concentration levels in mast cells.

To estimate the number of cells introduced into the capillary, the injection time was varied as mentioned above. When a 0.2 mM standard histamine solution was used as described, the relationships between the injection time and the peak-area counts were linear as shown in Fig. 5. By contrast, when the suspension of 10^6 cells/ml of saline was injected in the same manner as for Fig. 5, the results

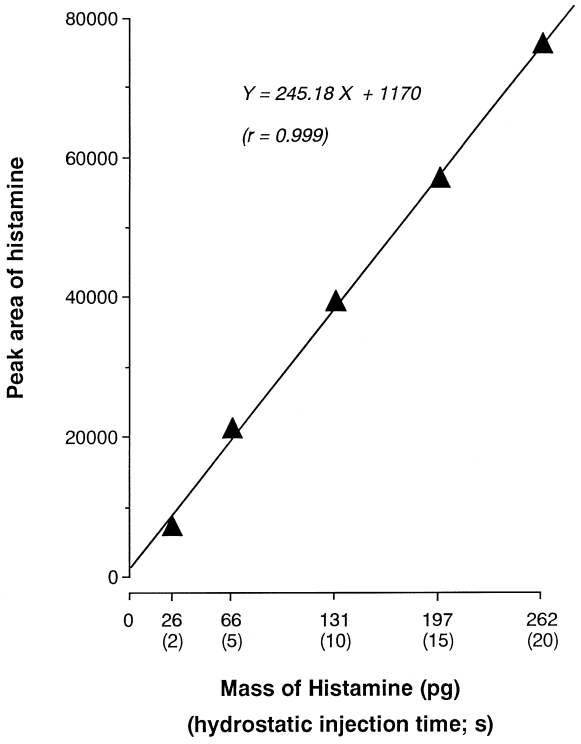

Fig. 5. Effect of mass of histamine on peak-area count. Each solid triangle plots the average of a quadruplicate ICD-HPCE analysis. Values in parentheses express injection times. ICD-HPCE conditions are the same as in Fig. 2.

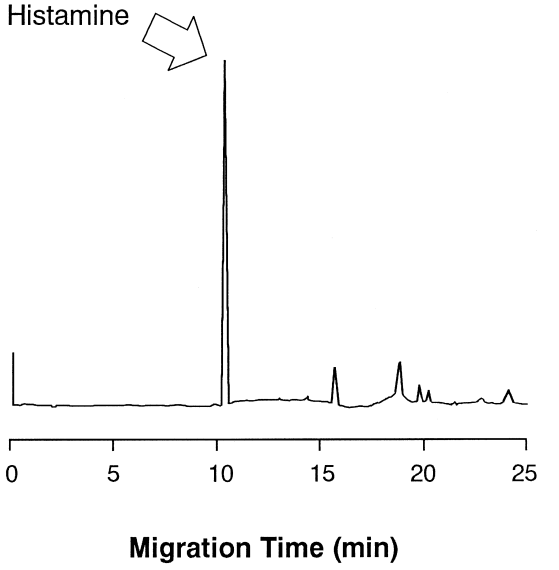

Fig. 6. Typical ICD-HPCE electropherogram of the suspension of mast cells injected directly for 20 s. The arrow indicates the position of the peak of histamine on the ICD-HPCE electropherogram. Other conditions are the same as in Fig. 2.

shown in Fig. 7 indicate a line like a ''staircase'', not a straight line as in Fig. 5. In the case of a 2-s injection time, the volume of sample injected into the capillary was estimated at 1.2 ml, in which a single cell as assumed to be present. On the *y*-axis corresponding to 2 s in Fig. 7, solid triangles were plotted at 0 (non-detected, abbreviated as ''n.d.''), 13 and 23 pg of histamine. The last two data items are roughly equal to the level of endogenous histamine in the rat peritoneal mast cell [23,24]; thus, we concluded that it was likely that a single cell was present in the capillary. Then, a ''staircase line'', having 30 pg of height/step was drawn on Fig. 7 for reference. The histamine levels were then analyzed at 0–1 cell for 2–5 s, 1–2 cells for 10 s and over 2 cells for 15–20 s injection times. However, the theoretical number of cells thought to be injected does not seem to fit the raw data, especially at 15 or 20 s. It is probably safe to conclude that the actual number of mast cells

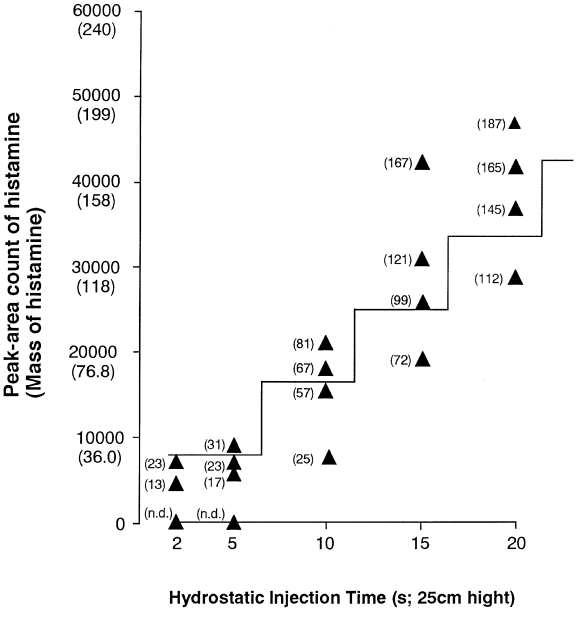

Fig. 7. The effect of varying the injection time on the amount of measurable histamine. Each solid triangle was obtained by application of the present method to the suspension of mast cells and each value in parentheses expresses the mass of histamine (in pg). The ICD-HPCE conditions are the same as in Fig. 2.

injected is, in fact, smaller than the theoretical number, because it is hard to inject the cells into the capillary when the hydrostatic injection method is used. Under these conditions it is impossible to know the exact number of cells injected. Unfortunately, our laboratory lacks a microscope equipped with a micromanipulator. Were such equipment to be used to introduce just a single mast cell into the capillary of the ICD-HPCE system, then we strongly believe this method would easily allow for the determination of the amount of histamine in a single mast cell.

4. Conclusion

In this paper, we described a simpler technique for the determination of histamine in rat peritoneal mast cells by ICD-HPCE with fluorescence detection. The biggest advantage of the present method is that direct detection of histamine in rat peritoneal mast cells is achieved by incorporating SDS in the run buffer as a cell-lysing reagent, and that this method induces automatic cell lysis, the labeling of histamine with a fluorescence reagent, electromigration, and detection following the direct injection of cell suspension into the ICD-HPCE separation capillary. Although we were not able to show a clear example of the determination in the case of a single mast cell, the authors hope to be able to do so in future work using a microscope equipped with a micromanipulator.

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