

Determination of Histamine in Microdialysis Samples from Guinea Pig Skin by High-Performance Liquid Chromatography with Fluorescence Detection

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Key Words

Histamine • Guinea pig skin • Microdialysis • Allergy • Compound 48/80 • Ovalbumin • High-performance liquid chromatography • Fluorescence derivatization

Abstract

Aim: To develop a sensitive and selective liquid-chromatographic method for the determination of histamine in microdialysis samples from guinea pig skin following allergenic provocation. **Methods:** The novel fluorescence derivatization method is based on an intramolecular excimer-forming reaction between 2 amino moieties of histamine and 2 molecules of 4-(1-pyrene)butanoyl chloride (PBC) yielding the corresponding dipyrene-labeled derivative. **Results:** The PBC derivative of histamine was separated within 20 min, and the detection limit (signal-to-noise ratio = 3) of histamine was 0.6 fmol/20 μ l volume injected. The basal extracellular levels of histamine in guinea pig skin microdialysates were 20.6 ± 1.7 fmol/10 μ l. Subcutaneous administration of histamine liberator compound 48/80 (3 mg/kg) increased the extracellular histamine levels in the skin dialysates by about 860%, whereas ovalbumin challenge (2 mg/kg i.v.) in the sensitized guinea pigs increased the extracellular histamine levels by about 3,030%. **Conclusion:** The novel tech-

nique for histamine determination in microdialysis samples from the guinea pig skin may be utilized in preclinical research of antihistaminergic drugs and evaluation of allergenic properties of various dermal preparations such as transdermal drug delivery systems.

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Introduction

Measurement of histamine levels in the skin is an important clinical parameter in various allergy diseases such as anaphylaxis [1, 2], atopic dermatitis [3, 4], pruritus [5, 6], allergic skin inflammation [7, 8], mastocytosis [9], hives [10, 11] and psoriatic skin [12], as well as in preclinical experimental allergy research [13–18].

Microdialysis carried out in peripheral tissues such as skin and combined with suitable analytical techniques is a widely used technology for in vivo monitoring of molecules identified as surrogate markers of various disease states and investigation of bioavailability and bioequivalence [for a review, see 19]. Radioenzyme or spectrophotometric enzyme immunoassays [20–23], high-performance liquid chromatography (HPLC) [24, 25] and spectrofluorometric assays [26] have been used for analysis of

histamine in animal and human skin microdialysis samples. However, these methods do not provide the sensitivity and selectivity required for the determination of trace levels of extracellular histamine, and/or they are too laborious or unsuitable for routine use in a standard analytical laboratory.

Previously, we have developed a fluorometric HPLC method for the determination of histamine in human urine [27] and rat brain microdialysate [28], based on an intramolecular excimer-forming fluorescence derivatization with 4-(1-pyrene)butyric acid N-hydroxysuccinimide ester. The method was later modified by implementing a new excimer-forming reagent, 4-(1-pyrene)butanoyl chloride (PBC), for the derivatization of polyamines (putrescine, cadaverine, spermidine and spermine) [29]. In this study, we describe the PBC-based derivatization method for the determination of histamine in the microdialysis samples from the skin of the anesthetized guinea pig. The method was applied for the determination of histamine concentrations following local application of compound 48/80 and systemic administration of ovalbumin.

Materials and Methods

Chemicals and Regents

Histamine dihydrochloride, potassium carbonate (K_2CO_3) and acetonitrile were purchased from Wako Pure Chemicals (Osaka, Japan). PBC was purchased from Toronto Research Chemicals Inc. (North York, Ont., Canada) and was used without further purification. Ovalbumin (grade V), compound 48/80 and diamine oxidase (DAO, EC 1.4.3.6.; 1 unit oxidizes 1.0 μ mol of putrescine per hour at pH 7.2 at 37°C) were purchased from Sigma Chemical Co. Ltd. (St. Louis, Mo., USA). All other chemicals and solvents were of the highest purity available and were used as received.

Stock solution of histamine at a concentration of 0.1 mM or lower was stored in polypropylene test tubes at 4°C. Histamine standard solutions were prepared just before the use by diluting 0.1 mM stock solution in deionized water. Histamine standard and all other aqueous solutions were prepared in distilled and deionized water with Milli-Q system (Millipore, Milford, Mass., USA). The solution of 3 mM PBC was prepared in acetonitrile and stored in an amber-colored test tube at -20°C, and the solution of 3 mM K_2CO_3 was prepared in water and stored at 4°C. Histamine stock solution, PBC and K_2CO_3 solutions were stable for at least 1 week when stored under the conditions appropriate for each substance.

Derivatization Procedure

To a 10- μ l portion of a histamine standard solution (or microdialysis sample), 35 μ l of 3 mM PBC and 5 μ l of 3 mM K_2CO_3 were added. The tube was tightly sealed and heated at 75°C for 30 min. Thereafter, the reaction mixture was cooled in iced water and a 20- μ l portion of the reaction mixture was injected onto a chro-

matographic column. For the reagent blank, Krebs-Ringer bicarbonate solution (NaCl, 120 mM; KCl, 5 mM; $MgSO_4$, 1.2 mM; $NaHCO_3$, 25 mM; KH_2PO_4 , 1.2 mM; EDTA-2Na, 0.027 mM; $CaCl_2$, 2.4 mM and glucose, 11 mM) was subjected to the same procedure.

HPLC System and Conditions

A column liquid-chromatographic apparatus was purchased from Hitachi (Tokyo, Japan) and included an L-2100 pump and an L-2485 fluorescence spectromonitor equipped with a 12- μ l flow cell. The fluorescence detector operated at an excitation wavelength of 345 nm and an emission wavelength of 485 nm. The column was Capcell Pak C_{18} (150 $\times$ 2.0 mm i.d., C_{18} silica, particle size: 5 μ m; Shiseido, Tokyo, Japan). The separation of the PBC derivative of histamine was achieved using a mixture of acetonitrile and water (78:22, v/v; mobile phase A). The flow rate was 0.22 ml/min. The column temperature was ambient (25°C). The column was washed by a mixture of acetonitrile and water (93:7, v/v; mobile phase B) at a flow rate of 0.22 ml/min for 10 min after each analysis and then re-equilibrated with the mobile phase A for 10 min before the next sample injection. Data were collected on a PC (Windows operating system; Microsoft) using an EZChrom Elite (Hitachi).

Animals

Male Hartley guinea pigs (310–440 g) were purchased from Charles River, Japan. Guinea pigs were housed at a constant temperature (23 $\pm$ 2°C) at a constant relative humidity (55 $\pm$ 15%) and an automatically controlled 12-hour light-dark cycle (lights on at 7.00 a.m.) and had free access to food and water. The animal experiments were performed in accordance with general recommendations of Japanese animal protection legislation and following the directives of the Care and Use of Animals in the Field of Physiological Science approved by the Council of the Physiological Society of Japan. All animal experiments were approved by the local ethical committee.

Surgery and Skin Microdialysis

Guinea pigs were anesthetized with pentobarbital (50 mg/kg intraperitoneally), and anesthesia was maintained by additional pentobarbital infusions during the experiment. A microdialysis probe was inserted into the dermis of the skin at the upper neck of each guinea pig. Skin microdialysis probes (OP-100-4, membrane length: 4 mm, diameter: 0.26 mm, Eicom Corporation, Japan) with a 23-gauge needle were used in all experiments. The connecting tubing used was Teflon® tubing (JT-10, 0.1 mm i.d., 50 cm length; Eicom Corporation). The inlet tubing of the microdialysis probe was connected to a microinfusion pump (model 55-4143, Harvard Apparatus, USA), and a Krebs-Ringer bicarbonate solution was pumped at a flow rate of 1 μ l/min. The outlet tubing was connected to the refrigerated fraction collector (EFC-82, Eicom Corporation); the length of the outlet tubing was 100 cm, corresponding to the internal volume of 8 μ l. The dialysates were collected every 10 min.

The time delay between the sample collection and the drug injection was corrected for each individual experiment.

Enzymatic Degradation of Histamine in the Microdialysis Sample

Solutions of DAO were freshly prepared by diluting DAO to a volume activity of 4 U/ml with 100 mM phosphate buffer (pH 7.2).

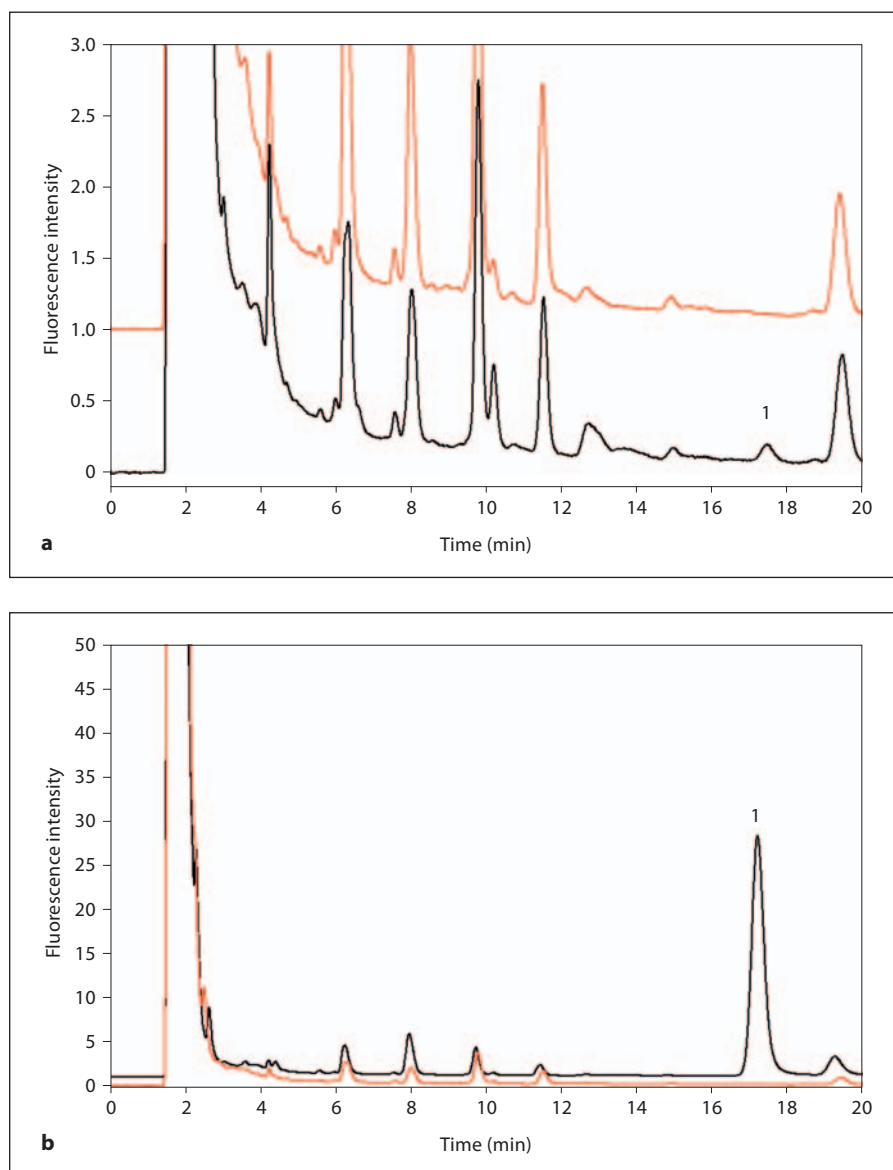


Fig. 1. Chromatograms of histamine standard solution at concentrations of 6.4 fmol/injection (**a**) and 160 fmol/injection (**b**). The histamine-PBC derivative is marked as peak 1. The corresponding blank samples (Krebs-Ringer bicarbonate solution) added as the light-gray/red chromatograms in **a** and **b** show no detectable peak at the retention time of histamine. The detailed chromatographic conditions are described in the Materials and Methods section.

The DAO solution (1 μ l) was added to 10 μ l of microdialysis sample, and the resulting mixtures were incubated at 37°C for 60 min. The incubated mixture was treated as described in the derivatization procedure.

Compound 48/80 Administration

Compound 48/80 (3 mg/kg) was injected subcutaneously 5 mm beside the placement of the microdialysis probe in guinea pigs ($n = 5$).

Sensitization

Guinea pigs ($n = 5$) were actively sensitized 3 times by the intraperitoneal injection of 1 ml of an emulsion made by mixing equal volumes of complete Freund's adjuvant (Difco, Franklin Lakes, N.J., USA) and ovalbumin (5 mg/ml) in normal saline solu-

tion on days 0, 2 and 4, respectively. The sensitized guinea pigs were challenged with single-dose intravenous injections of ovalbumin (2 mg/kg) on days 22–25 to initiate the anaphylactic response [30].

Results and Discussion

HPLC and Derivatization Conditions

Chromatograms in figure 1 show typical separations of histamine standard at concentrations of 1.6 nM (**a**) and 40 nM (**b**) (corresponding to 6.4 and 160 fmol, respectively, per 20 μ l injection) derivatized with PBC, as well

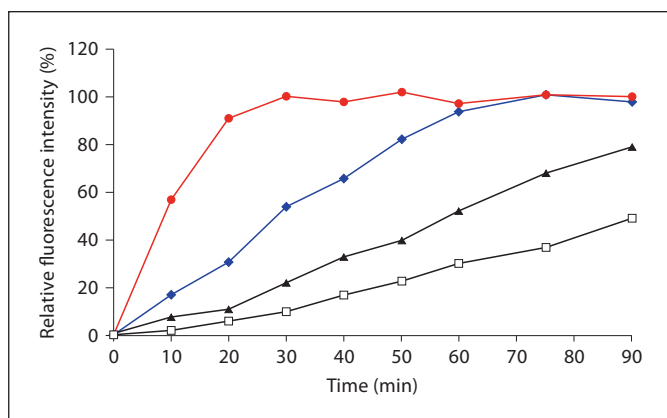


Fig. 2. Effect of reaction time and temperature on the fluorescence reaction of histamine with PBC. Reaction temperature (temperature of heat or cool block): ● = 75°C; ◆ = 50°C; ▲ = 35°C; □ = 20°C. The concentration of histamine standard: 40 nM (160 fmol/injection).

as chromatograms of the reagent blank. As seen, a good separation of the PBC-histamine derivative was achieved within 20 min on a reversed-phase column at given chromatographic conditions. Other biogenic polyamino substances (spermine, spermidine, cadaverine, putrescine, arginine, ornithine, lysine, δ -hydroxylysine and glycyl-L-lysine) also react with PBC under the present conditions. However, the polyamino substances eluted at retention times different from that of histamine.

The maximal fluorescence of the PBC-histamine derivative was achieved at the excitation and emission wavelengths of 345 and 485 nm, respectively. This is in good agreement with emission maxima of dipyrrenylalkanes as reported elsewhere [27]. The concentration of acetonitrile in the reaction mixture affected the fluorescence yield; the strongest fluorescence intensity was achieved at acetonitrile concentrations in the range of 60–85% (v/v) in the derivatization solution, thereby 70% (v/v) acetonitrile in the reagent solution was employed. Similarly, the optimal concentrations of PBC and potassium carbonate were set to 3 mM each (from the range of 1–5 mM tested). The kinetics of the derivatization reaction of histamine with PBC was evaluated within the range of 20–75°C by use of the histamine standard at a concentration of 40 nM (160 fmol per 20 μ l injection; fig. 2). At a reaction temperature of 75°C, the maximum fluorescence intensity was achieved already at 30 min and remained constant for up to 90 min, whereas at 50°C, the reaction time of 75–90 min was necessary to achieve the same level of detec-

tion signal. Further lowering of reaction temperatures caused reduction of fluorescence yields by about 50–60% at the end of the 90-min reaction period. Based on these results, the optimal reaction conditions of 75°C and 30 min were chosen for derivatization of skin microdialysis samples. These conditions are rather mild when compared to the protocol described earlier [27, 28], using derivatization with 4-(1-pyrene)butyric acid N-hydroxysuccinimide ester (100°C, 90 min). Thus, the PBC derivatization is better suited for routine determination of histamine in biological samples such as microdialysis perfusates. The PBC derivative of histamine was stable at least for 24 h when stored at 4°C.

Determination of Basal Histamine Levels in Guinea Pig Skin

Figure 3a shows a typical chromatogram of a microdialysis sample from the guinea pig skin. The peak at 17.2 min (peak 1 in fig. 3a) was identified as the fluorescent derivative of histamine when comparing its retention time with that of the standard and the sample and at varying concentrations of acetonitrile (60–85%) in the mobile phase. The fluorescence excitation (maximum, 345 nm) and emission (maximum, 485 nm) spectra of the eluate of peak 1 in figure 3a were in good agreement with those obtained for pure histamine. When the dialysis sample from the guinea pig skin was replaced with water, no peaks except those for the blank components were observed in the chromatogram as shown in figure 1. In addition, the polyamino substances added to the microdialysis samples eluted at retention times different from that of the histamine peak (data not shown) as discussed for the peaks shown in figure 1. Further identification of histamine in the skin microdialysis samples was performed by employing enzymatic degradation of histamine via DAO. Thus, following pretreatment with DAO, peak 1 (in fig. 3a) identified as histamine in the microdialysis samples ($n = 5$) was completely diminished, as illustrated in figure 3b. This observation supports the conclusion that peak 1 in figure 3a corresponds only to a single component, the PBC derivative of histamine.

Figure 4 shows the time course of changes in histamine levels sampled from guinea pig skin after implantation of the microdialysis probe. As seen, the decline of histamine concentrations to achieve the stable basal levels took about 150 min after the probe implantation. Following the initial stabilization period of 180 min, the microdialysis samples were collected in 10-min intervals. The basal extracellular levels of histamine in the dialysates (10 μ l) were 20.6 ± 1.7 fmol (mean $\pm$ SEM, $n = 8$)

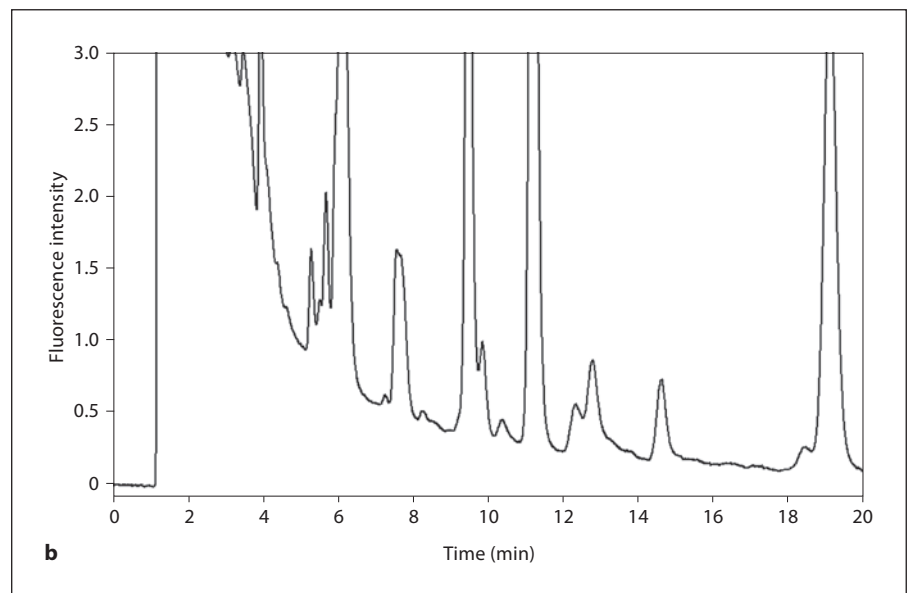
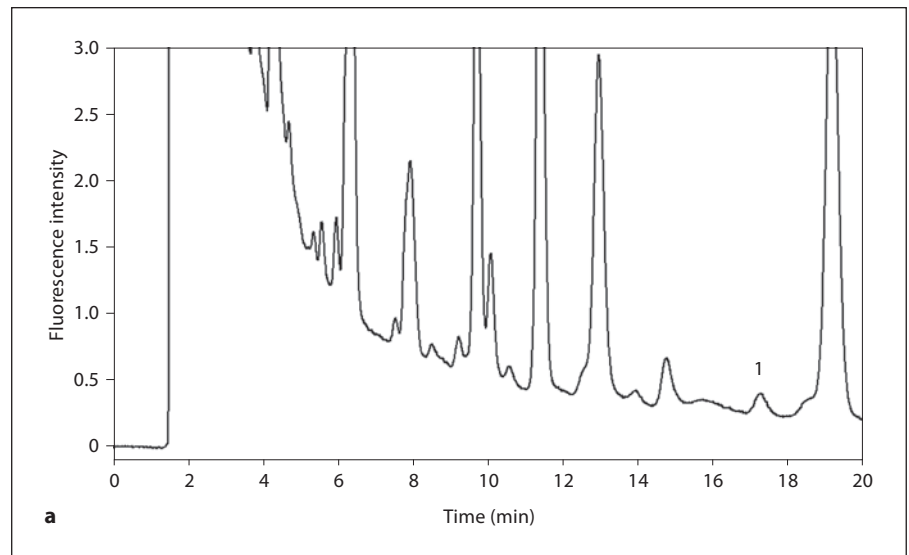


Fig. 3. A chromatogram of 10- μ l microdialysis samples collected at basal conditions from the guinea pig skin (**a**) and a microdialysis sample pretreated with DAO prior to PBC derivatization (**b**). Peak 1 corresponds to the histamine-PBC derivative.

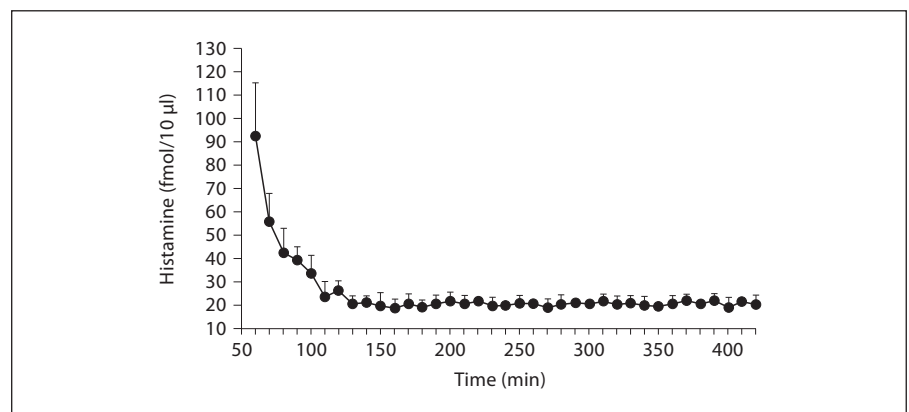


Fig. 4. Stabilization of histamine output 60 min after the implantation of a dialysis probe in the guinea pig skin. Histamine values are expressed in femtomoles per 10 μ l, the levels are not corrected for probe recovery, expressed as means $\pm$ SEM, $n = 8$.

Fig. 5. Chromatogram of the microdialysis sample at 10 min following administration of compound 48/80. Peak 1 corresponds to the histamine-PBC derivative.

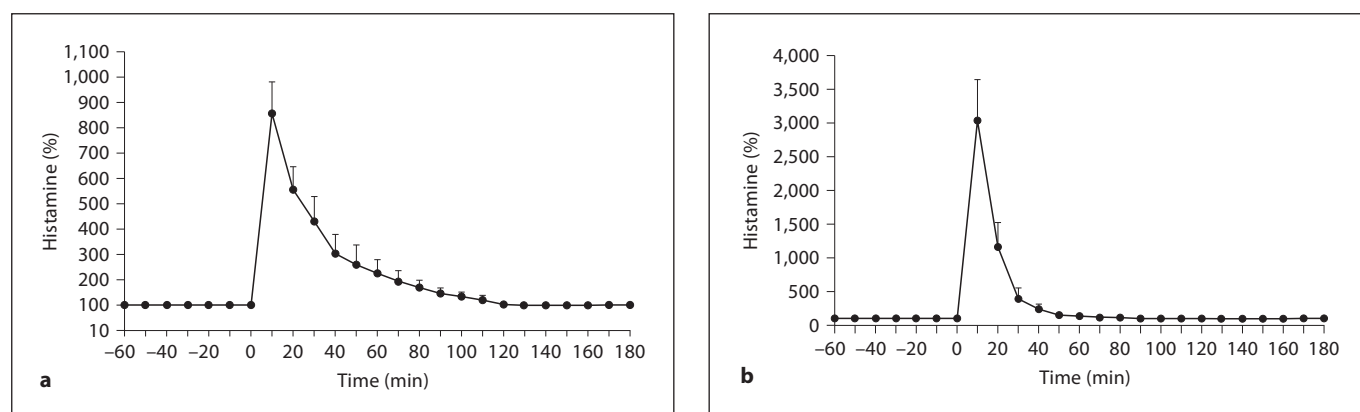
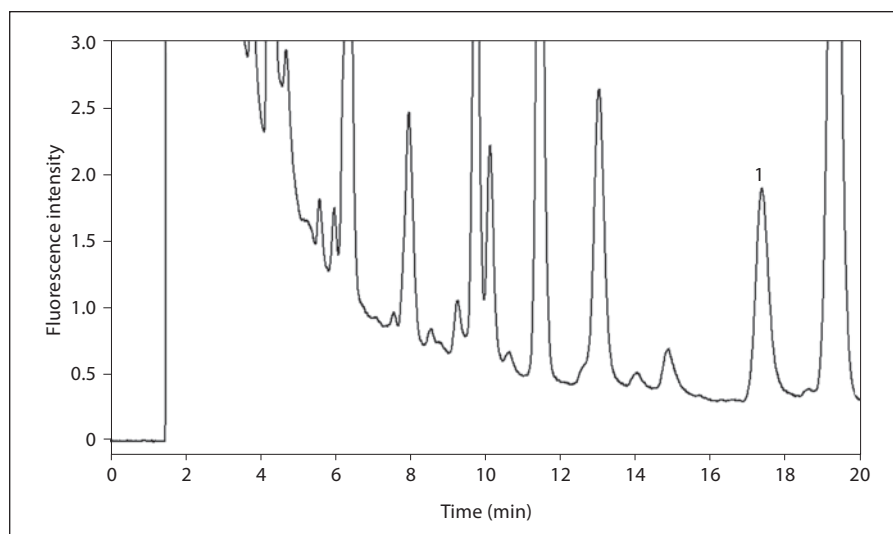


Fig. 6. Effects of compound 48/80 (a) and ovalbumin (b) challenges on histamine release in the guinea pig skin. Histamine values are calculated as percentage of the basal levels and expressed as means $\pm$ SEM, $n = 5$.

from the samples collected between 180 and 420 min after the probe implantation. The recovery of histamine from the probe in the in vitro external solution (Krebs-Ringer bicarbonate solution, 1 ml at 25°C) was $38.6 \pm 1.7\%$ (mean $\pm$ SEM, $n = 3$) at a flow rate of 1 $\mu\text{l}/\text{min}$.

Calibration Graph, Precision, Detection Limit and Recovery

The relationship between the peak heights and amounts of histamine was linear up to at least 2 pmol per 20 μl injected; the linear correlation coefficient was 0.999 ($n = 5$). The precision was estimated by repeated ($n = 10$) analysis of standard solution of histamine (15 fmol/20 μl

injection). The relative standard deviation was 3.1%. The detection limit for histamine (signal-to-noise ratio = 3) was 0.6 fmol in an injection volume of 20 μl . The chromatographic recoveries of histamine standard spiked at two concentrations (2 and 20 nM) where the skin microdialysis samples were 98.8 ± 4.2 and $99.6 \pm 3.2\%$ (mean $\pm$ SD, $n = 3$), respectively.

Monitoring Histamine Levels in the Microdialysates from the Guinea Pig Skin after Administration of Compound 48/80 or Ovalbumin

Administration of the allergenic compound 48/80 is established as a well-known test for activation of the mast

cell degranulation and release of histamine. A challenge with compound 48/80 infused approximately 5 mm beside the dialysis membrane caused a significant increase in extracellular histamine levels as shown in figure 5 for the chromatogram and in figure 6a for the time course. Administration of ovalbumin resulted in immediate and robust release of histamine to about 3,030% of the control levels, which returned to the basal values within 60 min, as shown in figure 6b. The effects of compound 48/80 and ovalbumin are in good agreement with data reported elsewhere [22, 25, 31, 32].

Conclusion

The proposed HPLC method based on the intramolecular excimer-forming derivatization of histamine with PBC label offers a highly sensitive and selective determination of histamine in biological fluids such as the microdialysis samples from the guinea pig skin. The new technique can be utilized in preclinical and clinical research of antihistaminergic drugs and evaluation of allergenic properties of various dermal drug formulations and transdermal drug delivery systems.

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