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Short communication

Simultaneous determination of histamine and serotonin in mast cells by high-performance liquid chromatography

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Abstract

A sensitive and rapid high-performance liquid chromatographic method for the simultaneous determination of histamine and 5-hydroxytryptamine (serotonin) in supernatants and cellular extracts from mouse bone marrow-derived mast cells was developed. The described method is based on a precolumn derivatization of histamine and serotonin with *o*-phthaldialdehyde (OPA) and subsequent separation of the amine–OPA adducts with an analytical reversed-phase C_{18} column combined with fluorometric detection (excitation wavelength 360 nm, emission at 455 nm). The mobile phase was 0.02 *M* sodiumacetate buffer (pH 8) including 5 m*M* 1-octanesulfonic acid and 50% methanol. The detection limits for histamine and serotonin were 3.3 pmol and 6.9 pmol, respectively (signal-to-noise ratio 2:1). The variations of peak areas of repeatedly injected low or high amounts of amine standards were 23.3% or 6.9% (for 0.5 ng or 12.5 ng histamine) and 26.4% or 4.0% (for 5.0 ng or 250 ng serotonin) during an experimental period of 4 weeks. The variation of retention times over 2 days was 1.1% for histamine and 1.7% for serotonin. A perfect linear relationship of amine concentrations and peak areas was documented by correlation coefficients of $r > 0.999$ for both histamine (0.1–125 ng) and serotonin (0.5–250 ng).

1. Introduction

Rodent mast cells synthesize and store in cytoplasmatic granules both 5-hydroxytryptamine (serotonin) and histamine which are rapidly released upon activation in vitro [1,2]. In vivo these mast cell mediators are important components of the acute biological responses following allergen challenge in sensitized individuals [2]. Mouse bone marrow-derived mast cells (BMMC) and BMMC-derived permanent cell lines are widely used model systems to study various aspects of mast cell biology including the regulation and pharmacological modulation of amine synthesis and release [1–3].

Previously published reversed-phase high-performance liquid chromatographic (HPLC) methods for the detection of histamine and/or serotonin in biological fluids or cell extracts are based on the measurement of serotonin autofluorescence or utilize the fluorescence of adducts formed between the amines and *o*-phthaldialdehyde (OPA) [4–10]. However, these methods are time-consuming [4], require extensive pretreatment protocols of test samples [4–7], or do not allow the simultaneous detection of both amines [6–8,10–13].

In the present study we describe a rapid and

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sensitive HPLC method for the simultaneous quantitation of both histamine and serotonin in supernatants and extracts of cultured mouse BMMC. In experiments aimed to study the regulation and pharmacological modulation of amine synthesis and release this method may prove valuable, especially in the light of a previous report demonstrating that serotonin and histamine are differentially released from mast cells [13].

2. Experimental

2.1. Chemicals

Commercial reagents were obtained from the sources indicated: Tyrode's buffer (Gibco, Eggenstein, Germany); gelatin, *o*-phthaldialdehyde, 1-octanesulfonic acid, 5-hydroxytryptamine hydrochloride (serotonin), 2-(4-imidazolyl)ethylamine (histamine), sodium tetraborate (Sigma, St. Louis, MO, USA); sodium acetate (Fluka, Neu-Ulm, Germany) methanol, HPLC grade (Merck, Darmstadt, Germany); 2-mercaptoethanol (Carl Roth, Karlsruhe, Germany).

2.2. Cells and cytokines

Primary populations of BMMC were generated from femoral bone marrow suspension cultures of normal mice (usually of the inbred strain BALB/c) as described previously [14] but using recombinant (r) murine (mu) IL-3 instead of pokeweed mitogen-stimulated spleen cell-conditioned medium as a source of mast cell growth factor. At day 14, the non-adherent BMMC were cultured for a further two weeks at $(1–3)\cdot 10^5$ cells ml^{-1} in fresh medium [commercially available RPMI (Rosewell Park Memorial Institute) 1640 medium supplemented with 20% fetal calf serum, 2 m*M* L-glutamine, 10^{-5} *M* α-thioglycerol, and 100 U ml^{-1} penicillin/streptomycin] in the presence of different mast cell growth factors [IL-3 plus IL-4 or kit ligand (KL) plus IL-4]. Supernatants from X63Ag8-653 myeloma cells transfected with retroviral vectors carrying the mouse IL-3 gene [15] and NIH/3T3 fibroblasts transfected with the mouse IL-4 gene (kindly provided by Dr. E. Schmitt, Institut für Immunologie, Mainz, Germany) were used as sources of rmuIL-3 and rmuIL-4, respectively. The expression in *Escherichia coli* and subsequent purification by affinity chromatography of a rmu KL (hisKL) has been described recently [16]. Endothelin-1 (ET-1) was obtained from Bachem Biochemica (Heidelberg, Germany). Peritoneal cells (containing 2–3% serosal mast cells) from BALB/c mice were obtained after peritoneal lavage with 10 U/ml heparin and 0.1% bovine serum albumin in 20 m*M* phosphate buffer (pH 7.4).

2.3. Histamine and serotonin release assays

BMMC (in vitro age: 4 to 5 weeks) or freshly isolated peritoneal cells were washed twice with modified Tyrode's buffer (mTB, supplemented with 0.05% gelatin). Aliquots of 0.2 ml with $1\cdot 10^6$ BMMC or $3\cdot 10^6$ peritoneal cells were incubated with 10^{-6} *M* ET-1 or buffer in a shaking water bath at 37°C. The release reaction was stopped after 20 min by placing the tubes in ice-cold water. Cells were separated from supernatants by centrifugation (300 *g*, 10 min, 4°C), resuspended in mTB and sonicated with a Branson Sonifier B-12 (Soest, Netherlands) (3 pulses, 20 s/pulse; setting 3). After centrifugation (15 000 *g*, 5 min, 4°C), 200-μl aliquots were evaporated to dryness and kept in 0.5 ml methanol–water (1:1, v/v) at −20°C overnight. Subsequently, the samples were centrifuged (15 000 *g*, 5 min, 4°C) and 150-μl aliquots were dried by evaporation and kept at −20°C until HPLC determination.

2.4. Determination of histamine and serotonin by high-performance liquid chromatography

The HPLC system consisted of a system controller and programmer (Altex Model 420, Beckman, Munich, Germany), an integrator Shimadzu C-R3A, an injection valve (Organizer 340, Beckman), a solvent delivery module 114M (Beckman) and a Shimadzu RF-530 spectro-

fluorimeter. Both amines were separated simultaneously by reversed-phase HPLC on a C_{18} spherisorb ODS column, 250 × 4.6 mm I.D., 5 μm (Beckman), and a precolumn (C_{18} spherisorb ODS), 20 × 4 mm I.D., 5 μm (Bischoff, Leonberg, Germany) with a mobile phase of methanol–0.02 *M* sodiumacetate (1:1, v/v) supplemented with 5 m*M* 1-octanesulfonic acid (flow-rate: 1 ml/min) after precolumn derivatization with OPA. A 40-μl volume of OPA-reagent consisting of 50% methanol, 19 m*M* OPA, 45 m*M* sodium tetraborate pH 9.5 and 2 m*M* 2-mercaptoethanol were added to dry samples and stirred vigorously for 30 s at room temperature and injected onto the HPLC system exactly 1 min after the reaction was started. The amine–OPA adducts were detected fluorimetrically (excitation 360 nm, emission 455 nm).

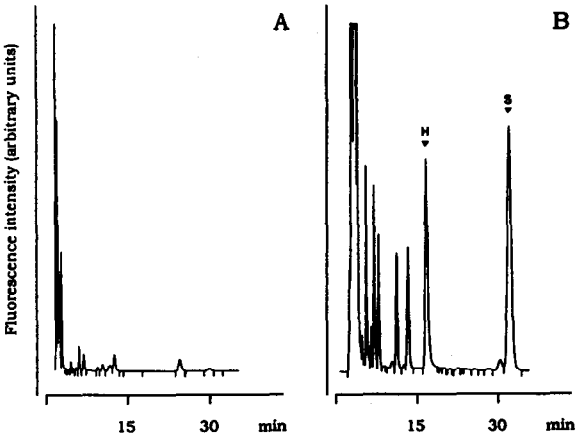

Fig. 1. Identification of histamine and serotonin in extracts of cultured mast cells. (A) Tyrode's buffer alone, (B) mast cell extract containing 45 ng/20 ml histamine (H) and 158.4 ng/20 ml serotonin (S).

3. Results and discussion

A number of experiments have been conducted to optimize the relative concentrations of methanol and 1-octanesulfonic acid and the pH of the elution buffer as well as the OPA concentration and the derivatization time, aimed to simultaneously and specifically detect histamine and serotonin in cellular extracts and supernatants from cultured mast cells. In Fig. 1 the identification of both histamine and serotonin in a mast cell extract under optimized assay conditions is shown. The retention time for histamine was about 17 min and for serotonin 32 min. Total analysis time was about 35 min. The histamine and serotonin peaks were identified by cochromatography of samples with standards. A perfect linear relationship between peak areas and standard concentrations was observed in the range 0.1–125 ng for histamine and 0.5–250 ng for serotonin with correlation coefficients $r >$ 0.999.

Determination of the accuracy as calculated from four measurements of histamine and serotonin standards showed no bias when added to mast cell derived samples. The recovery of standard amounts of histamine and serotinin following the protocol for preparation of mast cell extracts was higher than 95%.

The limits of detection were 122 pg (1.1 pmol) for histamine and 488 pg (2.3 pmol) for serotonin per 20 μl of injected sample at a signal-to-noise ratio of 2:1.

Low and high standard concentrations were injected repeatedly over a period of 4 weeks giving the following coefficients of variation (C.V.) of the peak areas: histamine: 0.5 ng, C.V. = 23.3% ($n = 6$), and 12.5 ng, C.V. = 6.9% ($n = 5$); serotonin: 5 ng, C.V. = 26.4% ($n = 6$), and 250 ng, C.V. = 4.0% ($n = 5$). Retention times measured over a 2-day period had a C.V. of 1.1% ($n = 24$) for histamine and a C.V. of 1.7% ($n = 20$) for serotonin.

We have also compared 4 different protocols for the preparation of extracts from mast cells: (i) sonication in Tyrode's buffer, (ii) sonication in Tyrode's buffer supplemented with 20% $HClO_4$, (iii) sonication in methanol, and (iv) heat treatment (100°C, 10 min) in Tyrode s buffer. Similar amounts of histamine could be detected in all these cell extracts corresponding to 10^6 mast cells in 200 μl. However, due to the known instability of serotonin at 100°C and in acid solution the serotonin peak was completely absent following protocol ii or reduced to about 50% following protocols iii or iv as compared to

our standard conditions (protocol i) (data not shown).

The HPLC method described in this communication is well suited for the simultaneous measurement of histamine and serotonin in mast cell extracts and supernatants under isocratic elution conditions within about 35 min. Pretreatment protocols like ion-exchange chromatography proved to be unnecessary, as the addition of 1-octanesulfonic acid (5 m*M*) and inclusion of methanol (50%) in the elution buffer warranted a clear separation of histamine and serotonin from potentially overlapping substances. Furthermore, this method may be used with 1-methylhistamine as an internal standard, which eluted with a retention time of about 21 min (data not shown). Remarkably, more than 2000 analysis have been performed with the same column without any signs of attrition. Furthermore, stabilization of the amine–OPA adducts through acidification of the derivatization mixture as suggested in Refs. [6,15,17] proved to be unnecessary [9,10]. Our limits for detection of histamine (3.3 pmol/20 ml) and serotonin (6.9 pmol/20 ml) were in accordance with those reported by Davis et al. [4] and were sufficiently low to measure both of these amines in the supernatants and extracts of cultured mast cells as exemplified for the endothelin-1 induced amine release (Table 1 and Ref. [18]). It remains to be established whether it is possible to simultaneously determine histamine and serotonin in other biological samples (e.g. blood, organ tissue extracts) by employing the method described here.

Table 1
Endothelin-1 (ET-1)-induced amine release in cultured mast cells

Cytokines used in cultures	Amine release (%)			
	Histamine		Serotonin	
	− ET-1	+ ET-1	− ET-1	+ ET-1
IL-3 + IL-4	2.9 ± 1.4	15.6 ± 3.0	2.4 ± 1.7	11.3 ± 1.6
hisKL + IL-4	3.4 ± 0.9	40.5 ± 4.0	2.4 ± 0.6	24.4 ± 5.0

% Amine release was based on the total amounts of amine/10^6 mast cells as indicated in brackets for IL-3/IL-4-grown mast cells (135 ng histamine, 3006 ng serotonin) or hisKL/IL-4-grown mast cells (501 ng histamine, 1716 ng serotonin).

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