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Development of an assay for histamine using automated high-performance liquid chromatography with electrochemical detection

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Abstract

Previous studies have measured histamine by derivatization with *o*-phthaldialdehyde (OPA) and mercaptoethanol (ME), followed by reversed-phase HPLC separation and electrochemical detection. The derivatization product, however, was very unstable. In the present study, inclusion of less polar solvents (e.g., acetonitrile or tetrahydrofuran) in the OPA/ME derivatization reaction produced an OPA/ME-histamine product that was stable for many hours. Changes of the HPLC mobile phase (increasing its ionic strength and pH and including triethylamine) dramatically improved the chromatography and reduced the histamine detection limit to <0.1 pmol. The modified assay was suitable for batchwise manual derivatization of histamine samples followed by their automated analysis by HPLC with an automatic injector.

1. Introduction

Many analytical procedures have been developed for qualitative and quantitative measurements of histamine. They include, radioenzymatic [1], fluorimetric [2–4] or electrochemical assays [5–8]. The addition of chromatographic separations such as thin-layer chromatography, gas chromatography, low-pressure liquid chromatography and high-performance liquid chromatography (HPLC) [4] have allowed the separation of histamine from its methylated derivatives and other biogenic amines. Most of these techniques, however, suffer from some disadvantages such as low specificity and sensitivity, the possible appearance of interfering substances, critical enzyme preparation, time-consuming sample preparation, or expensive instrumentation.

In 1971, Roth [3] showed that primary amines reacted with *o*-phthaldialdehyde (OPA) in the presence of 2-mercaptoethanol (ME) in aqueous alkaline medium yield a product with intense fluorescence. The OPA/ME-amine reaction gives an 1-alkylthio-2-alkyl-substituted isoindole [9,10] and the reaction is a general property of primary amines [9]. Following the introduction of electrochemical detection in conjunction with HPLC (HPLC–ED) by Kissinger et al. [11], it was reported that the fluorescent OPA/ME-

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amine product of some primary amines, histamine included, could be oxidized electrochemically at moderate potentials [5,12]. Tsuruta et al. [13] showed that OPA derivatives of histamine were well retained and resolved on reversed-phase columns, and in combination with electrochemical detection a very sensitive method for histamine determination was achieved [6–8].

In all the assay methods where the OPA/ME-amine reaction is used, a major disadvantage hitherto has been the instability of the product [6,7,14]. This meant that each sample had to be individually derivatized and immediately analysed by HPLC–ED, before the next sample could be processed. Such assays were either very labour-intensive or required expensive automated sample derivatization equipment coupled to the automatic injector of the HPLC system.

In this paper the following will be described. (i) Stabilization of the OPA/ME-histamine product for more than 5 h with non-polar solvents in the derivatization buffer. The improved stability of the reaction product allows for batchwise manual derivatization of samples followed by their automated analysis on a standard HPLC–ED system, without expensive premixing instrumentation. (ii) Improved chromatographic analysis of the OPA/ME-histamine reaction product, resulting in an assay with a high sensitivity. (iii) Successful application of these improved derivatization and HPLC–ED assay methods to the determination of histamine release from rat mast cells.

2. Experimental

2.1. Chemicals

Histamine dihydrochloride, *o*-phthaldialdehyde (OPA) powder, 2-mercaptoethanol (ME) and bovine serum albumin (BSA) were from Sigma (St. Louis, MO, USA); Percoll was from Pharmacia Fine Chemicals (Uppsala, Sweden); HEPES was from Calbiochem-Novobiochem (La Jolla, CA, USA); triethylamine was from BDH (Kilsyth, Australia); methanol (HPLC grade) was from Ajax (Sydney, Australia); acetonitrile (HPLC solvent grade) was from FSE (Homebush, Australia); and tetrahydrofuran (ChromAR HPLC grade) was from Mallinckrodt Specialty Chemicals Australia (Meadowbank, Australia) and redistilled. All salts for the buffers where from BDH. The water was redistilled deionised water (Millipore MilliQ-grade water was tried, but did not give any detectable improvement).

2.2. Chromatography system

Separations were performed on a Bioanalytical Systems (West Lafayette, IN, USA) Phase-II ODS 3 μm, 100 × 3.2 mm I.D., reversed-phase cartridge column. The outlet of the column was connected to a Bioanalytical Systems Model LC4B Amperometric Detector with an LC17A thin-layer electrochemical cell. The latter had a glassy carbon working electrode at +0.5 V with respect to a Ag/AgCl reference electrode. Signals (oxidation currents) were recorded with a BBC Model 120 chart recorder. Samples were injected using a Waters WISP Model 712B automatic sample injector. Chromatography was performed in the isocratic mode with a Waters Model M510 solvent delivery system at a flow-rate of 0.6 ml/min at ambient temperature (20–22°C). Four different mobile-phase buffers were used: (1) 0.07 *M* Na_2HPO_4 with 1 m*M* Na_2EDTA adjusted to pH 4.5 with 0.07 *M* citric acid containing 1 m*M* Na_2EDTA. (2) 0.07 *M* Na_2HPO_4 with 1 m*M* Na_2EDTA adjusted to pH 6.4 with 0.07 *M* NaH_2PO_4 containing 1 m*M* Na_2EDTA. (3) 0.1 *M* Na_2HPO_4 with 1 m*M* Na_2EDTA adjusted to pH 6.4 with 0.1 *M* NaH_2PO_4 containing 1 m*M* Na_2EDTA. (4) 0.1 *M* NaH_2PO_4 containing 1 m*M* Na_2EDTA and 0.4% triethylamine (v/v) gives a pH of 6.4. To all four mobile-phase buffers, methanol and acetonitrile were added to give 16% (v/v) and 14% (v/v), respectively.

2.3. Derivatization procedure

For the derivatization of histamine a derivatization buffer, made of 0.4 *M* boric acid (H_3BO_3) adjusted to pH 9.5 with 1 *M* sodium

hydroxide, was used, since aqueous borate buffers have been found most useful for the OPA/ME reaction [3,15,16]. In some cases, ethanol, tetrahydrofuran (THF) or different concentrations of acetonitrile were added to the borate buffer before the derivatization step. For development of the methodology, histamine standards (histamine dihydrochloride) were prepared directly in the derivatization buffer. For preparation of histamine standard curves and for measurement of histamine in mast cell extracts, histamine standards were prepared in mast cell incubation buffer and processed as described below for the mast cell samples.

The dried samples were reconstituted in 100 μl of derivatization buffer. After dissolving the dried samples we added 20 μl of a 1:1 mixture of OPA (3.8 m*M* in methanol, freshly prepared) and ME (2.5 ml/l in methanol) at room temperature. It was found to be important to mix OPA with ME before adding to the samples containing histamine, because OPA is capable of reacting directly with primary amines in the absence of ME to produce products that are not electrochemically active [3,7]. After adding the derivatization reagents to the samples, the mixture was vortex-mixed and left for 5 min at room temperature (or for periods up to 5 h, as indicated). Aliquots of the reaction mixture (typically 50 μl) were then injected into the HPLC.

One major source of variability was found to be the instability of the derivatization reagents. We found that it was necessary to prepare fresh derivatization reagents from crystalline OPA, ME, methanol and borate buffer each day, because the stability of pre-prepared commercial OPA/ME reagents have been found to vary from <1 day to 6 months [17].

2.4. Extraction of histamine from mast cells

Peritoneal mast cells were prepared from anaesthetized male Sprague-Dawley rats (250–300 g) by the method of Penner et al. [18]. After isolation, the cells were suspended in an incubation buffer consisting of (in m*M*): NaCl, 150; KCl, 4.0; $CaCl_2$, 1; $MgSO_4$, 1.2; Na_2HPO_4, 2.46; KH_2PO_4, 0.615; glucose, 5.6; HEPES, 10; and 0.1% BSA (pH 7.4). The suspension was divided into 0.5-ml samples of equal cell density. The number of cells per sample varied between experiments from 40 000 to 216 000 cells/sample. Cells were incubated for 45 min at 37°C, and the incubations stopped by adding 1.5 ml of ice-cold incubation buffer without BSA, to avoid adding unnecessary additional protein. The cells were sedimented by centrifugation (250 *g*, 10 min, 4°C), the supernatant collected and 2.0 ml of incubation buffer without BSA was added to the cell pellet. The pellet (cell extract sample) and supernatant (release sample) were boiled for 3 min, placed on ice, centrifuged (500 *g*, 10 min, 4°C) and the supernatant collected. Aliquots of the cell extract sample (50 μl) and the release sample (200 μl) were dried in a vacuum centrifuge (Dynavac Model DC40) and redissolved in 100 μl derivatization buffer, prior to derivatization with OPA/ME.

2.5. Preparation of standard curves and quantitation of histamine

Known amounts of histamine standard (histamine dihydrochloride) were dissolved and diluted in mast cell incubation buffer (see above). Aliquots of 100 μl of these standard solutions were boiled for 3 min, dried down in a vacuum centrifuge, redissolved in 100 μl derivatization buffer, derivatized and injected into the HPLC system. Four histamine standard curves were constructed in order to cover the range of 0.1 pmol to 5000 pmol.

The amount of histamine in samples was quantified by the height of the oxidation current peaks in the chromatograms. These peak heights were compared with the peak heights of known quantities of histamine standards prepared as described above. Histamine standards were always prepared, incubated and derivatized in parallel with the mast cell samples. A histamine standard was included for every five mast cell samples, to identify and correct for any drift in the chromatographic conditions. In some cases, histamine standards were added to samples of mast cell release samples and extracts before

they were evaporated to dryness, reconstituted and derivatized.

2.6. Final assay conditions for measurement of histamine from mast cells with HPLC–ED

The derivatization buffer was modified to consist of 0.1 *M* sodium tetraborate buffer, pH 9.5, containing 30% tetrahydrofuran (v/v). The dried samples were reconstituted in 100 μl of derivatization buffer. After dissolving the dried samples we added 20 μl of a 1:1 mixture of OPA (3.8 m*M* in methanol, freshly prepared) and ME (2.5 ml/l in methanol) at room temperature. The chromatographic separation was performed under isocratic conditions with a mobile phase comprising 0.1 *M* sodium phosphate buffer with 0.4% triethylamine, pH 6.4, with 16% methanol (v/v), 14% acetonitrile (v/v) and 1.0 m*M* Na_2EDTA.

3. Results

The sensitivity of OPA/ME assay depends on the amount of the OPA/ME-histamine product registered by the electrochemical detector. This yield depends on the synthetic yield, the stability of the product in the derivatization buffer and the stability of the product during the chromatographic separation and detection. We have investigated conditions for improving the OPA/ME derivatization, for stabilising the OPA/ME-histamine product and for improving the chromatographic separation and detection of this product.

3.1. The electrochemical properties of OPA/ME-histamine product

For characterization of the electrochemical properties of OPA/ME-histamine product, chromatographically assisted hydrodynamic voltammograms were generated. Electrochemical oxidation of the OPA/ME-histamine product was performed in a potential range of +0.3–0.7 V (Fig. 1A). An oxidation potential of +0.6 V

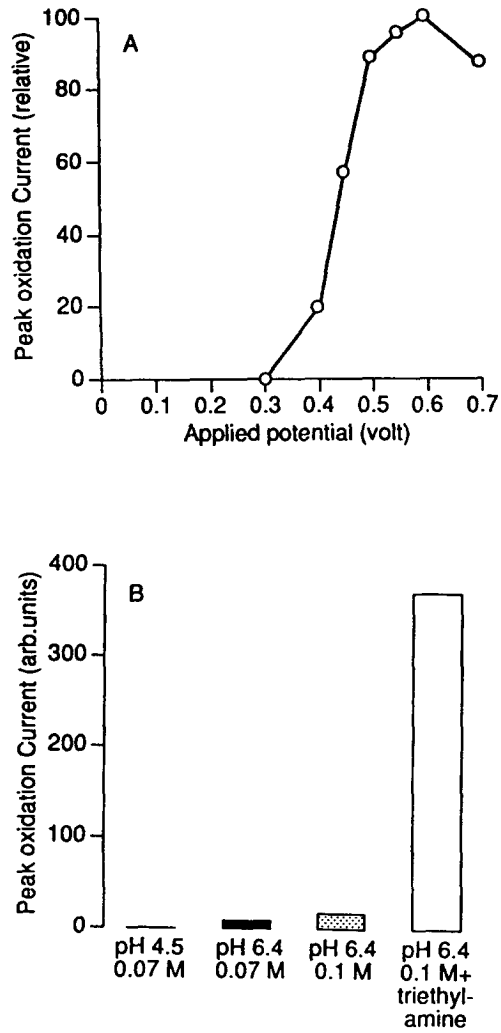

Fig. 1. (A) Hydrodynamic voltammogram for the OPA/ME-histamine product in a mobile phase consisting of 0.1 *M* sodium phosphate buffer containing 0.4% triethylamine (v/v) (pH 6.4) with 16% methanol (v/v) and 14% acetonitrile (v/v) and 1 m*M* Na_2EDTA. Abscissa: applied oxidation potential; ordinate: ratio of peak oxidation current at a particular potential to the current obtained at +0.6 V. (B) Effect of pH and ionic strength of the mobile phase on the sensitivity. The following four different mobile phases were used. Column 1: 0.07 *M* (pH 4.5) phosphate-citric acid buffer. Column 2: 0.07 *M* (pH 6.4) phosphate buffer. Column 3: 0.1 *M* (pH 6.4) phosphate buffer. Column 4: 0.1 *M* (pH 6.4) phosphate buffer containing 0.4% triethylamine (v/v). All four buffers contained 16% methanol (v/v), 14% acetonitrile (v/v) and 1 m*M* Na_2EDTA. The detector response is shown as the peak oxidation current (arbitrary units).

gave maximum oxidation peak height. As shown in Fig. 1A, the response observed at +0.5 V was very similar to that at +0.6 V; however, the background current was undesirably high at +0.6 V compared to that at +0.5 V. Therefore all subsequent determinations were carried out at +0.5 V.

3.2. Chromatographic conditions: effect of the mobile-phase pH, ionic strength and presence of triethylamine

The best separation, the sharpest electrochemical oxidation peak for the OPA/ME-histamine product and a suitable retention time of around 10 min were obtained when the mobile phase contained 16% methanol and 14% acetonitrile. The results obtained with either solvent on its own were not as good.

The stability and retention of the OPA/ME-histamine product on the reversed-phase column were examined with different mobile-phase buffers. In all cases, the mobile phases contained 16% methanol, 14% acetonitrile and 1 m*M* Na_2EDTA. The effects of pH was assessed by performing isocratic runs with a mobile phase containing 0.07 *M* phosphate buffer at either pH 4.5 or at pH 6.4. The peak oxidation current of the OPA/ME-histamine product was increased by 150% when the pH was increased to 6.4 (Fig. 1B, compare first two columns). When the ionic strength of the pH 6.4 mobile phase was increased from 0.07 *M* to 0.1 *M* sodium phosphate, the response was further increased by 100% (Fig. 1B, compare second and third columns). The chromatograms of the OPA/ME-histamine product under these two conditions showed that the peak width was smaller in the 0.1 *M* phosphate buffer (not shown).

When 0.4% triethylamine was added to the mobile phase comprising 0.1 *M* phosphate buffer pH 6.4, the peak oxidation current response was increased 20-fold (Fig. 1B, last column). The chromatogram clearly showed that this significant increase in sensitivity was due to dramatically improved peak sharpness for the OPA/ME-histamine product.

3.3. Stability of the OPA/ME-histamine reaction product in the derivatization buffer

The stability of the OPA/ME-histamine reaction product in the derivatization buffer before its injection into the HPLC column was determined by varying the time between adding the OPA/ME derivatization reagents and injecting the reaction mixture into the HPLC system. Using borate buffer alone as the derivatization buffer, the peak height declined rapidly with time (Fig. 2): with a 1 h delay between derivatization and injection into the HPLC system only 65% of the histamine product was still detectable. Inclusion of increasing amounts of acetonitrile in the sodium borate derivatization buffer progressively improved the stability of the reaction product (Fig. 2). In the presence of 30% acetonitrile the derivatized product was stable for at least 4 h. The stability was increased further, to 5 h, by using 30% tetrahydrofuran

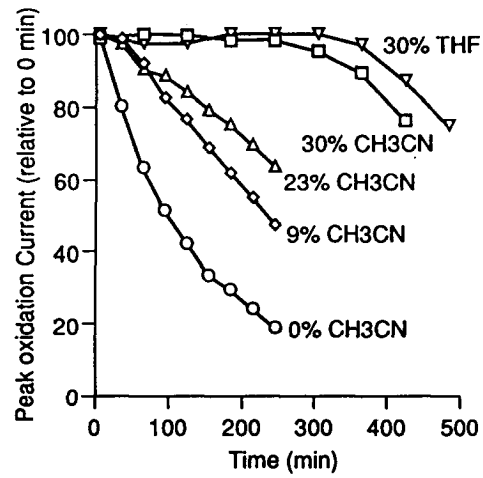

Fig. 2. Stability of the OPA/ME product of histamine in derivatization buffer (borate buffer, pH 9.5) containing increasing amounts of acetonitrile or 30% THF. Peak oxidation currents are presented for samples injected into the HPLC system at the indicated time intervals after the addition of derivatization reagents. The peak oxidation currents are given relative to the response obtained when the reaction products were injected directly into the HPLC without delay. The mobile phase consisted of 0.1 *M* sodium phosphate buffer containing 0.4% triethylamine (v/v) (pH 6.4) with 16% methanol (v/v) and 14% acetonitrile (v/v) and 1 m*M* Na_2EDTA.

(THF) in place of acetonitrile (Fig. 2). THF is more non-polar than acetonitrile but still miscible with water.

3.4. Measurement of histamine release from rat mast cells

From the above studies, the derivatization and chromatographic conditions were modified to give optimum conditions (see Sect. 2.6). We have applied these improved conditions to the measurement of histamine release from rat mast cells. A chromatogram of the detection of derivatized standard histamine is shown in Fig. 3 (left), followed by a chromatogram showing the basal release from rat mast cells (middle), and a chromatogram of histamine in a mast cell extract (right). In the absence of histamine standard or mast cell sample (i.e., only with mast cell incubation buffer), several large peaks were observed in the break-through region of the chromatogram, before the histamine peak. These early peaks were "reagent-specific" contaminants as described by Lee and Dreschet [19], due to unavoidable trace contamination of primary amines in the reagent grade chemicals used in the preparation of the incubation and derivatization buffers.

The basal release of histamine from rat mast cells was found to be 1–4% of the cell content over 45 min. The cellular histamine content was between 5 and 30 pg histamine/mast cell.

The OPA/ME-histamine product produced from standard histamine, derivatized either alone in mast cell incubation buffer or by spiking mast cell samples prior to derivatization, co-eluted in the HPLC–ED chromatogram, indicating that the presence of small amounts of incubation buffer salts does not interfere in the assay.

3.5. Recovery

The overall recovery of histamine was assessed ($n = 5$) by comparing the peak height after 22 pmol standard histamine have been processed in the same way as the mast cell samples with the peak obtained from direct injection of equivalent

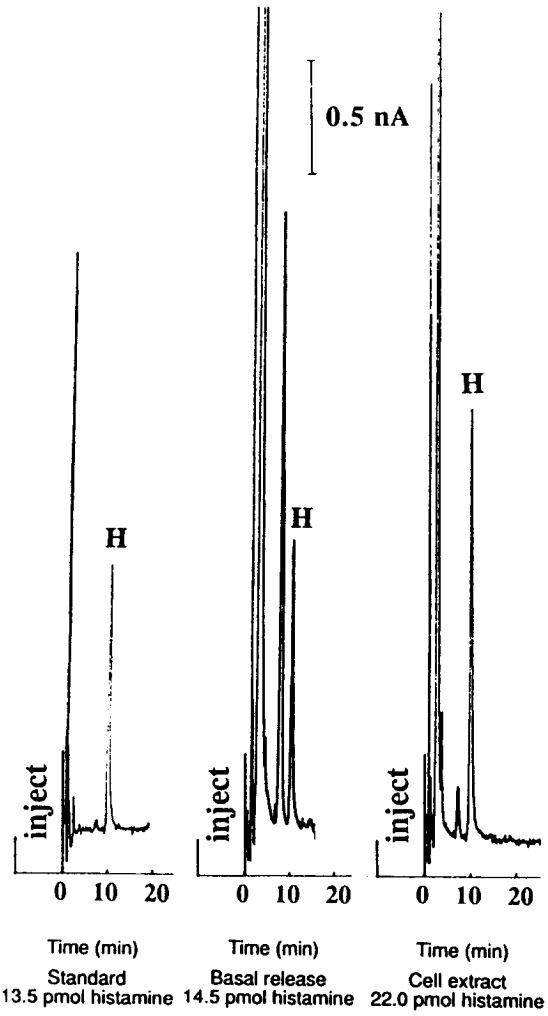

Fig. 3. Reversed-phase chromatogram of an isocratic separation of the OPA/ME product of: a histamine standard (in pmol), histamine originating from basal release from rat peritoneal mast cells, and histamine originating from mast cell extracts. Ordinate: the oxidation current, which is proportional to the concentration of the components being oxidized; abscissa: the time needed for the components to come off the column after injection. The mobile phase consisted of 0.1 *M* sodium phosphate buffer containing 0.4% triethylamine (v/v) (pH 6.4) with 16% methanol (v/v) and 14% acetonitrile (v/v) and 1 m*M* Na_2EDTA. The dried samples were reconstituted in 100 μl of derivatization buffer comprising borate buffer, pH 9.5, containing 30% tetrahydrofuran, before the addition of the OPA/ME derivatization reagents (see methods).

quantities of pure derivatized histamine standard. The overall recovery was 99–100%.

3.6. Linearity of the assay and detection limit

Standard curves for histamine prepared in mast cell incubation buffer and carried through the mast cell sample extraction procedure were found to be linear in the range of 0.1 pmol to 5000 pmol, when the oxidation current peak heights of the derivatized histamine were plotted against the amounts of histamine product injected.

The high efficiency of the HPLC separation combined with the enhanced sensitivity of the electrochemical detection under the conditions given allowed an extremely low detection limit of less than 0.1 pmol of histamine.

3.7. Repeatability

The intra-day repeatability of the derivatization of histamine was assessed by analysis at three concentrations: 23, 90, and 180 pmol standard histamine ($n = 5$). The coefficients of variation were 2.9, 3.3, and 2.0%, respectively.

3.8. Reproducibility

Inter-day reproducibility was assessed for 5 days by analysing a 23-pmol standard histamine solution. The coefficient of variation was 15.7%.

4. Discussion

To the best of our knowledge this is the first reported assay for histamine from rat mast cells by use of HPLC with electrochemical detection.

4.1. The electrochemical properties of the OPA/ME-histamine product

Histamine may be directly oxidised electrochemically at + 1.05 V, but it gives an unacceptably high background current [7]. We found that using + 0.5 V gave optimal conditions for detection of OPA/ME-histamine, which are a low background current and a high efficiency. It is important to note that the product of histamine and OPA alone is not oxidized at + 0.5 V [6,7]. Therefore this product is not detected in the chromatogram.

4.2. Effect of the mobile-phase pH

The chromatographic separation is the result of hydrophobic interactions between the C_{18} stationary phase and the hydrophobic moiety of the solute molecules. The OPA/ME-histamine product is a base. By increasing the pH the OPA/ME-histamine product is deprotonated and hence the strength of its hydrophobic interaction with the C_{18} solid-phase is increased. Over the pH interval 4.5 to 6.4 the oxidation efficiency is not very dependent on pH [6,7], but the indole product is unstable at lower pH [9]. This can explain why the oxidation response was found to be 150% greater at pH 6.4 than at pH 4.5. We have not tried to increase the pH above 6.4 because the silica-based column material is not resistant to eluents having pH values higher than 7.

4.3. Effect of the mobile-phase ionic strength

Phosphate buffer afforded sharp peaks for OPA/ME-amine products [5]. We found that by increasing the concentration from 0.07 *M* to 0.1 *M* the peak width was reduced and hence improved the sensitivity by increasing peak height. It is possible that higher phosphate concentrations than 0.1 *M* can improve the sensitivity further, but higher phosphate concentrations occasionally give rise to precipitation problems [13,20].

4.4. Effect of including triethylamine in the mobile phase on the peak shape

A common problem experienced with three of the mobile-phase buffers used in Fig. 1B (columns 1, 2 and 3) was that the peak shape of the OPA/ME-histamine product exhibited significant tailing. This tailing is due to adsorption of the histamine product to the remaining free silanol

groups of the stationary phase [4]. Triethylamine competitively inhibited this adsorption [4] and prevented the histamine peak from tailing. The sensitivity was greatly increased due to the sharper peaks, since the amount detected was measured by the peak height. Similar improvements could probably be obtained by using suitably end-capped silica-based columns.

4.5. Stability of the OPA/ME-histamine product

A number of studies have emphasized the labile nature of the OPA/ME-histamine reaction product [2–8]. The instability of the OPA/ME-amine product in the derivatization buffer depends on the primary amine involved, the pH, and the composition of the derivatization buffer. The two first parameters have been characterized for the derivatization of histamine [3]. The present study has shown that the use of a mixed solvent system such as THF–aqueous buffer increases the stability of the OPA/ME-histamine product up to at least 4 h, compared to a derivatization buffer made of aqueous buffer alone.

Stobaugh et al. [21] found that the destabilising effect was due to excess OPA, and not excess ME as suggested by Simons and Johnson [9,10,16,22,23]. These disagreements seem to be due to the differences in the analytical procedure. Where Simons and Johnson's studies [9,10,16,22,23] were carried out under conditions of equimolar quantities of OPA and primary amines, Stobaugh et al. [21] have made the derivatization, as in this study, with excess OPA and ME.

OPA undergoes extensive hydration to a reactive product which attacks the amine isoindole product [21]. This may partly explain why non-polar substances can reduce the degradation of the OPA/ME-amine product, by solvating the OPA and reducing its reaction with water. This mechanism does not fully explain the differences in efficiency between acetonitrile and THF at stabilising the OPA/ME-histamine product, and why ethanol is not effective in this assay. Cowgill [24] suggested that the environment around the isoindole product was altered by the non-polar solvent dimethylsulfoxide (DMSO). He used this mechanism to explain the loss of quenching of isoindole fluorescence in DMSO. The degree of non-polarity increases in the order: ethanol < acetonitrile <THF. We found that the most non-polar substance (THF) provides a longer protection from degradation of the OPA/ME-histamine product, and this finding is most likely explained by such environmental changes as described by Cowgill [24].

Higher concentrations of non-polar solvents in the derivatization buffer, will probably increase the stability of the product [10,14,16,22,23,25]. This approach is not useful for histamine measurements using reversed-phase HPLC, because the histamine product will not stick to the C_{18} column.

It has been suggested that the use of other SH-containing compounds, for example ethanethiol, *t*-butylmercaptan and 3-mercapto-1-propanol, instead of ME, can improve the stability of fluorescent products [5,10,16,21–23,25]. We tried ethanethiol instead of ME, as reported [5,25]. The OPA–ethanethiol-histamine product, however, could not be detected in our HPLC–ED system.

4.6. Mast cell histamine release

The mast cell histamine content and basal release of histamine were in good accordance with the amounts of histamine measured with another method [26].

In conclusion, although a number of previous reports had been published on similar assays for histamine [6–8], the assay described in the present study showed a number of improvements. (i) The histamine derivatization product was stable for many hours allowing batchwise manual preparation of derivatized samples followed by automated processing of the samples on an HPLC system with a standard automatic injector. This obviates the need for expensive automated equipment for the derivatization step prior to automated HPLC analysis. (ii) HPLC of the histamine product on reversed-phase columns was improved by complex but subtle modification to the HPLC mobile-phase system. This

greatly increased the assay sensitivity. (iii) The assay was sensitive with a detection limit of 0.1 pmol histamine, which is sufficient for many biological applications. As a result, the modified assay was sensitive, specific and simple. It allows the rapid determination of low quantities of histamine, and provides a valuable new technique for medical research on histamine release from mast cells during allergic, inflammatory and other medical conditions.

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