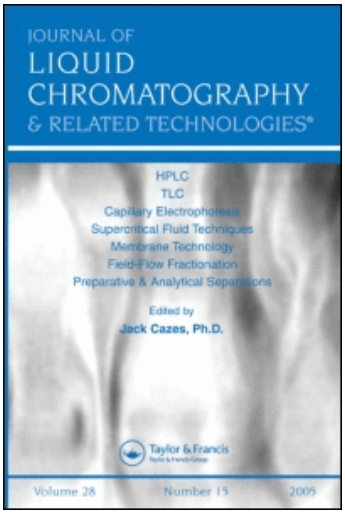

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A FACILE HPTLC METHOD FOR DETERMINATION OF HISTAMINE RELEASED FROM RAT SEROSAL MAST CELLS

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ABSTRACT

THIS PAPER DESCRIBES DEVELOPMENT OF A QUANTITATIVE HPTLC ASSAY FOR DETERMINATION OF HISTAMINE RELEASED FROM THE RAT SEROSAL MAST CELLS. BRIEFLY, HISTAMINE IS QUANTIFIED BY DERIVATIZATION WITH DANSYL CHLORIDE, THEN SEPARATED BY HPTLC AND DETECTED BY DENSITOMETRIC SCANNING UNDER FLUOROMETRIC MODE. THE METHOD USES R-PHENYLEPHRINE AS AN INTERNAL STANDARD, AND SHOWS AN EXCELLENT LINEARITY OVER THE RANGE OF 50 TO 300 NG/ML. THE CHROMATOGRAPHIC CONDITIONS ALLOW RESOLUTION OF THE DANSYLATED HISTAMINE AND INTERNAL STANDARD FROM THE OTHER DANSYLATED CO-EXTRACTABLE POLYAMINES.

INTRODUCTION

The modulation of histamine release from isolated rat mast cells is widely used as one of the screening assays for antihistamine activity. A major

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component of these assays is the measurement of histamine released from the mast cells. Due to physicochemical instability of histamine and concomitant release of 5-HT and cationic polyamines from the mast cells–speed, sensitivity and specificity are absolute requirements for these assays.

Pharmacological bioassay (1) and Shore's classical spectrofluorometric method (2-4) are still the most widely used technique for the measurement of the histamine released from the mast cells. In general, these methods involve elaborate sample preparation and may be subject to interferences from 5-HT or cationic polyamines (5). Some of the recent applications of these methods are listed in references 6 through 9. Gas chromatographic (10), LC (11, 12) and GS-MS (13) assays are the alternate methods discussed in the literature. These assays elegantly address the issues on specificity and sensitivity. However, inherently they have slower sample throughputs. Thus, radioimmunoassays or the radioenzymatic assays may have an advantage over these methods, but may suffer from poor specificity due to cross-reactivity of the biological probes (14).

High sample throughput has been a definite advantage of TLC methods. Recent advancements in TLC sampling and scanning instrumentation, and availability of high-performance plates have further improved the overall specificity and sensitivity of the technique. In this paper we have described the development of a facile HPTLC method for quantitative measurement of histamine. Rapid throughput, specificity and comparative sensitivity are the merits of the method.

METHOD

Reagents

Histamine dihydrochloride, R-phenylephrine hydrochloride (internal standard), spermidine trihydrochloride and spermine tetrahydrochloride were purchased from Aldrich (Milwaukee WI) and used as such. The compound 48/80 (histamine releasing agent–HRA) was purchased from Sigma (St. Loius MO). The derivatizing reagent dansyl cholride and the silconization fluid Aquasil ™ was obtained from Pierce (Rockford IL). Analytical grade sodium bicarbonate and triethylamine; and HPLC grades of methanol, chlorofrom, acetone and benzene were supplied by Merck (Bombay, India). The double distilled deionised water was generated on a Buchi Fontavapor 285 system (Flawil, Switzerland).

Chromatographic Equipments and Conditions

The chromatographic instruments and accessories used in the method were purchased from Camag (Muttenz, Switzerland) . They consisted of a sampling device–Linomat IV, a twin trough chamber with SS lid for chromatoplate development, and the CAMAG TLC II Scanner. On-line acquisition and data evaluation was done on a IBM compatible PC using the Camag Cats 3.03 software package.

The commercially available precoated 10 x 20 HPTLC plates were also purchased from Camag (cat. no. 034.5641). The plates were prepared by eluting with equal mixture of methanol and choroform, and baking for 30 minutes at 80^{o}C. The mobile phase used for the method was benzene: triethylamine (7.5:2.5; elution volume, 10 ml; and migration distance, 8 cm). The developed plates, after air drying, were dipped in a 20% parrafin solution in hexane to enhance the luminiscence of the dansylated analyte spots.

Glassware Siliconization.

The glasswares were siliconized daily using the procedure recommended by the supplier.

Stock Solutions

Standard stock solutions of histamine dihydrochloride (1.07 mg/ml), R-phenylephrine hydrochloride (1.2 mg/ml) and the compound 48/80 (1 mg/ml) were prepared individually in tyrode buffer. Working standards were prepared daily after appropriate dilution of the standard stock solutions. Concentration of the working standard of histamine was 1 μg/ml, that of phenylephrine hydrochloride was 3 μg/ml, and that of compound 48/80 was 50 μg/ml. Stock solution of dansyl chloride (1 mg/ml) was prepared daily in acetone.

Preparation of the Calibration Curve Standards in Tyrode Buffer Matrix

A five point calibration curve was prepared by serial transfer of 50 through 300 μl aliquots of working histamine standard solution in separate test tubes. This corresponded to concentration levels of 50, 75, 100, 200, and 300 ng/ml of histamine (as a free base). The volume in the tubes were then adjusted to 1 ml with tyrode buffer. Subsequently the tubes were spiked with R-phenylephrine

hydrochloride working standard solution (100 μl) and the HRA, compound 48/80, working standard solution (100 μl).

Prepration of the Calibration Curve Standards in Rat Serosal Mast Cell Matrix

A five point calibration curve, as described above, was prepared in rat peritoneal fluid matrix. The concentration range covered by this curve was same as the tyrode buffer matrix curve.

Derivatization

The derivatization involved a three step process. In the initial step, tubes were spiked with 100 μl of the dansyl chloride solution and then incubated at room temperature for 10 min. This was followed by addition of 0.5 ml of freshly saturated sodium bicarbonate solution. Then the tubes were stoppered under nitrogen and heated at 45°C for 20 minutes. In the final step, the tubes were unstoppered and heated for additional five minutes.

Extraction

The dansylated derivatives were directly extracted into 1 ml benzene by reciprocal mixing (10 min). The organic layer was separated by centrifugation and transferred to a separate tube. The organic layer was concentrated to total dryness under vacuum using Speed Vac™ concentrator (Savant Instruments Inc. Farmingdale NY). The dry residue is reconstituted in 100 μl benzene and a 40 μl portion of the solution was spotted in a band form on a previously conditioned plate.

Rat Serosal Mast Cell Preparation

The rat mast cell incubates were prepared from the peritoneal fluid using a established procedure (15). The population of mast cells in the suspensions used in the studies was 2×10^6 cells/ml.

Sample Preparation and Analysis

Histamine in the samples was analysed using 0.75 ml double diluted supernanatant portions of the incubates. Tyrode buffer was used for dilutions

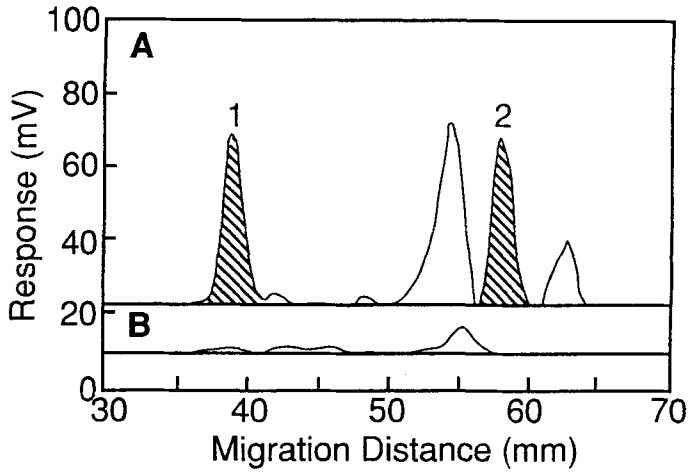

FIGURE 1: HPTLC chromatograms of: (A) Dansylated histamine and internal standard. Peak identification: (1) Histamine; and (2) Internal standard. (B) Blank tyrode buffer solution taken through identical sample processing and derivatization step. Chromatographic conditions as described in the text.

Subsequently the volume was made to 1 ml with tyrode buffer. The volume adjusted samples were then spiked with the internal standard and taken for derivatization and extraction.

RESULTS & DISCUSSION

A representative chromatogram obtained from a histamine spiked tyrode buffer (containing compound 48/80–HRA) is shown in Figure 1 . The histamine retention factor (Rf) is 40 mm and that of the internal standard is 58 mm.

Selection of the Blank Matrix

An unusual problem was encountered during the development of the method. That was the non-availability of a histamine free rat peritoneal fluid. The problem was due to interference from the spontaneously released histamine in the HRA free rat peritoneal fluid. Figures 1 and 2 demonstrate the differences in

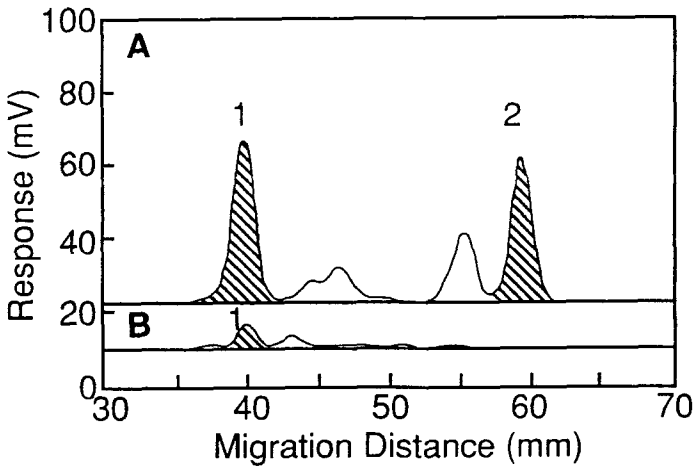

FIGURE 2: Representative HPTLC chromatograms obtained after processing and derivatizing a mast cell incubate sample with (A) and without (B) HRA. Peak identification: (1) Histamine; and (2) Internal standard.

the chromatograms obtained from tyrode buffer spiked standards and HRA free rat peritoneal fluid. The chromatogram obtained from HRA and histamine free rat peritoneal fluid consistently showed the presence of spontaneously released histamine (Figure 2A). This peak was attributed to be histamine as its Rf values (under various mobile phases) and the UV absorbance spectrum (Figure 3) were identical to that of standard histamine. Thus, the calibration curve standards for the method were prepared using compound 48/80 spiked tyrode buffer. The choice of matrix should not affect the estimation of histamine in samples as: (a) The mast cell preparation, including washing, harvesting and suspension is done was in tyrode buffer. (b) The supernatant is used for analysis; and (c) Prior to analysis the supernantant is double diluted with tyrode buffer to further minimize the matrix bias.

Optimization of Derivatization Conditions

The derivatization condition was optimized for time and temperature of the dansylation reaction. The effect of time and temperature on histamine dansylation is shown in Figures 4 and 5. The three step derivatization was essential to drive the reaction to completion. The excess of acetone was removed when the tubes were heated unstoppered in the last step.

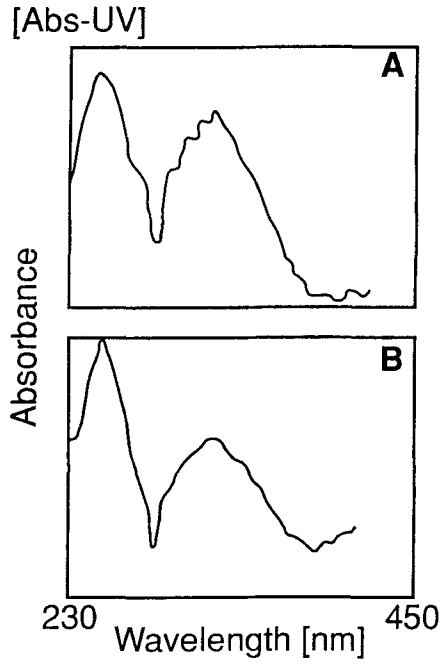

FIGURE 3: Time resolved UV spectrum of the standard histamine band (A) and that of the spontaneously released histamine band (B) after chromatographic resolution. The spectrum was recorded by the densitometer directly off the plate, under scan mode and between the wavelenghts 230 nm and 450 nm.

Screening of Rat Peritoneal Matrix for Interference

Appropriate rat peritoneal matrices were screened for endogenous interference in the region of internal standard; and exogenous interference due to HRA. The chromatograms in Figure 6 were obtained after derivatization of the actual rat peritoneal fluid. The chromatogram in Figure 6A demonstrates absence of endogenous peak in the region of the internal standard. The chromatogram in Figure 6B was obtained after spiking the rat peritoneal matrix with HRA. The HRA was spiked in the mixture after isolating the mast cells. The chromatogram shows a minor peak corresponding to the spontaneuosly released histamine (vide-supra), otherwise it is free of any interference due to HRA.

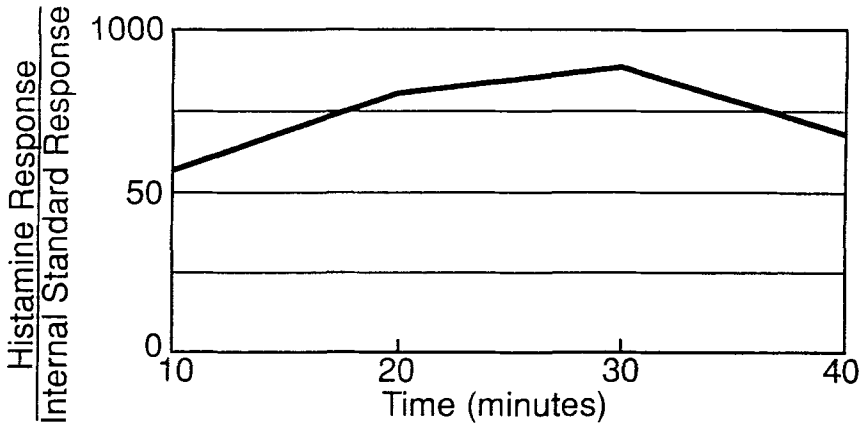

FIGURE 4: Optimization of derivatization conditions–Effect of time. The reactions were carried out at 45°C and the histamine peak response was measured against pre-dansylated internal standard spiked into the reconstituted mixtures.

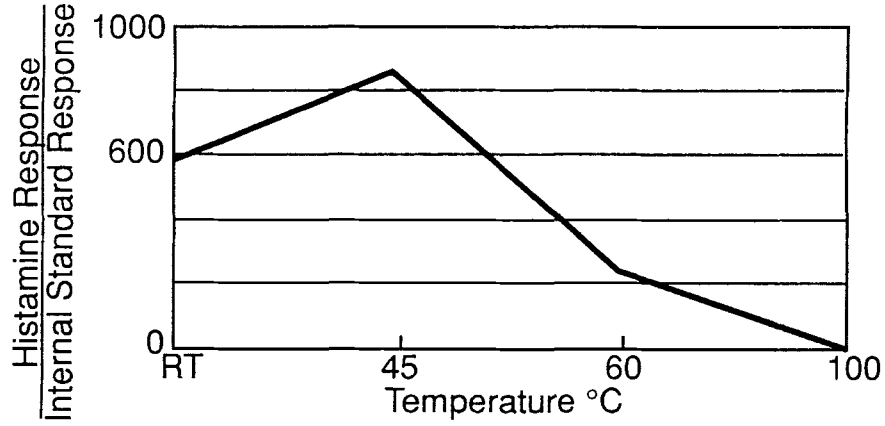

FIGURE 5: Optimization of derivatization conditions–Effect of temperature. The reactions were carried out for 20 minutes each and the histamine peak response measured against pre-dansylated internal standard spiked into the reconstituted mixture.

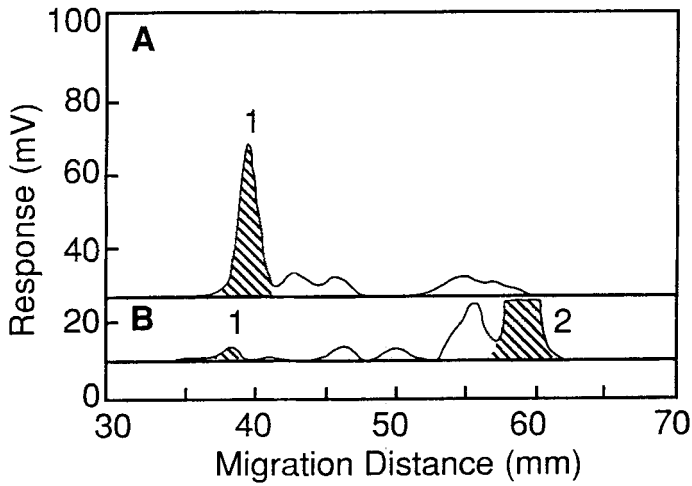

FIGURE 6: HPTLC chromatograms of processed and derivatized mast cell incubates without the internal stanadard (A) and without the compound 48/80 (B). Chromatogram in A demonstrates the absence of endogenous peaks in the internal standard region. The chromatogram in B demonstrates lack of interference from compound 48/80. The minor peak 1 in B represents the spontaneously released histamine.

Calibration Curves

Calibration curves obtained from tyrode buffer and the HRA free rat peritoneal fluid were compared for linearity, and interference (Y-intercept). As expected, the calibration curves obtained using the HRA free rat peritoneal fluid showed high Y intercept values. Otherwise, calibration curves from the either matrix were linear. The coefficient correlation for the curves were typically 0.988 or greater. The accuracy (calculated as the % error of the actual value) and precision of the method were within 10 % (Table 1). Either peak area or peak height mode can be used for data reduction.

Application

The method can be used for routine estimation of histamine in in-vitro mast cell allergenic models. Typical concentrations of histamine released *from rat*

TABLE 1

Accuracy and Precision Calculations for the HPTLC Assay of Histamine (n = 5)

Histamine Concn	Intra-day Precision (%CV)	Intra-day Precision (%CV)	Inter-day Precision (%CV)	Inter-day Precision (%CV)	Accuracy (% Error of Actual	Accuracy (%Error of Actual)
ng/ml	Area	Height	Area	Height	Area	Height
50	3.2	8.32	5.05	10.6	3.3	3.1
75	8.4	7.86	9.75	9.6	9.7	5.6
100	4.2	6.80	10.70	8.0	8.5	8.4
200	0.9	8.44	1.67	10.3	7.5	5.0
300	8.8	10.2	8.49	8.8	2.5	1.3

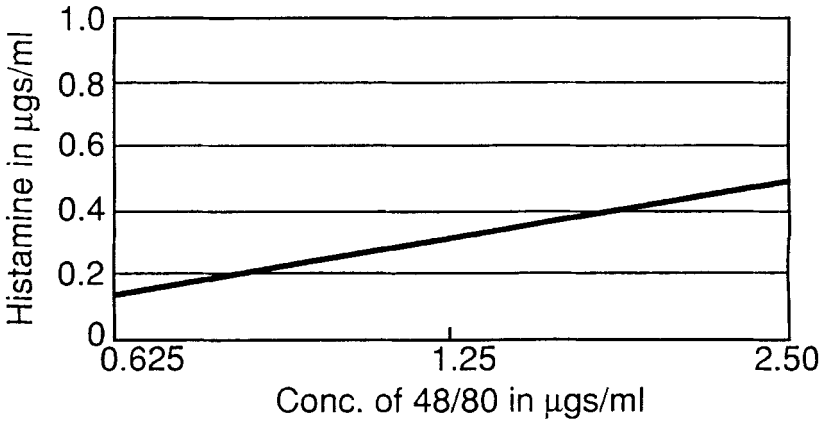

FIGURE 7: Compound 48/80 concentration dependent release of histamine from rat serosal mast cell incubates.

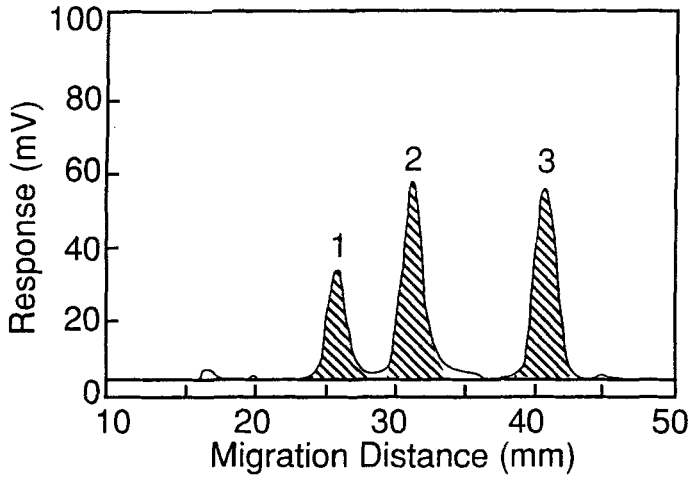

FIGURE 8: HPTLC chromatogram showing resolved dansylated peaks of spermine (1), spermidine (2), and histamine (3).

peritoneal mast cells is shown in Figure 7. These experiments were done to establish the linearity of histamine release response to incremental concentrations of the compound 48/80. The method can also be used for determination of biological polyamines such as spermine and spermidine. Spermine and spermidine are well resolved from histamine under similar chromatographic conditions (Figure 8). Thus, this method can also be used for simultaneous assay of polyamines

CONCLUSION

A facile HPTLC method has been developed for routine estimation of histamine released for rat serosal mast cells. The method is as sensitive and specific as any other chromatographic technique. The method benefits for high sample throughput capabilities of TLC. The method can be routinely used for estimation of histamine and other polyamines.

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