HISTAMINE CHROMATOGRAPHY AND ELECTROPHORESIS

THE *o*-PHTHALALDEHYDE FLUOROGRAM*

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Although histamine has long been biologically assayed by its effect on smooth muscle, the best modern quantitation technics are based on spectrofluorometry[1–3]. Nevertheless, both approaches remain time consuming and require meticulous attention to detail. With a view to simplifying and accelerating the quantitative estimation of histamine we have turned to the new worlds of chromatography and electrophoresis.

Youthful and vigorous, the field of chromatography has attracted many investigators as five comprehensive review volumes will attest[4–8]. Of the several branches available for study, we chose paper and thin-layer partition chromatography. The other popular branch, gas chromatography, is rapid and gives remarkable separation of compounds in an ultra micro sample. Indeed, it has been used in the determination of histamine[9] but it requires specimens with far higher concentrations of histamine than exist biologically, except in cell depots.

Paper and thin-layer chromatography allow separation and identification of compounds by virtue of their partition coefficients in carefully selected solvents moving over paper or a thin layer of a supportive solid. In practical use for over twenty years, chromatographic observations have been made on an unbelievable number of substances. These have included histamine, but the majority of this literature relates to histamine as an incidental member of a series of compounds, *e.g.* the amino acids. Table I summarizes the salient composite data from the previous reports[10–20]. Nearly all of these values relate to paper chromatography. The newer thin-layer techniques have been recently tried[7,8,19], and appear to offer the advantages of speed and sensitivity.

Reviewing Table I, it can be concluded that alkalinization greatly accelerates the migration of histamine. Thus almost any degree of movement can be achieved by using the proper solvent. Colorimetric evocation of the histamine spot has been achieved either by employing the relatively insensitive non-specific ninhydrin or by the more sensitive Pauly reagent, an azo dye (diazotized sulfanilic acid or *p*-nitroaniline). A surprising degree of sensitivity is achieved in certain laboratories, some detecting as little as 0.05 γ on silica gel thin-layer plates. The general average appears to be of the order of magnitude of 1 γ. All of the paper chromatographic methods are time consuming requiring hours, whereas the thin-layer techniques are much more rapid.

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The application of an electrical field to paper thin-layer strips[4] and more particularly to gels[21] has been another way of separating compounds. Here, the ion mobilities are the critical determinant rather than partition coefficients in carefully selected solvents. As a small highly charged molecule, histamine would appear particularly suited to such iontophoresis or electrophoresis. Studies on histamine have not appeared in the gel electrophoresis literature, but there is one report concerned with thin-layer electrophoretic systems[22].

TABLE I

REPRESENTATIVE DATA FROM PREVIOUS CHROMATOGRAPHIC METHODS OF DETECTING HISTAMINE
Whatman No. 1 paper, ascending.

Approx. R_F*	*Solvent set (ratios)*	*Visualization spray*	*Sensitivity (μg)*
0.0	Isobutyric acid–water (8:2)		
0.1	*n*-Butanol–acetic acid–water (4:1:5)		
0.2	*n*-Butanol–acetic acid–water (8:2:2)	Ninhydrin 0.2 % in water saturated *n*-butanol	1
0.3	2,6-Lutidine–water (65:35)	or	
0.4	2,6-Lutidine–collidine–water (1:1:1)	Diazotized sulfanilic acid (Pauly reagent)	
0.5	2,6-Lutidine (water satd.)–collidine (3:1)		
0.6	Propanol–0.2 *N* ammonia (3:1)		
0.7	Pyridine–water (65:35)		
0.8	*n*-Butanol–95 % ethanol–conc. ammonium hydroxide (8:1:3)	*p*-nitroaniline	0.3
0.9	Phenol–water (ammonia atmosphere) (10:2)		

* $R_F = \frac{\text{distance histamine carried}}{\text{distance solvent front moved}}$.

The above techniques may be intermingled with the result that some workers have employed two dimensional chromatography (two solvent sets), others, paper chromatography followed by electrophoresis, and a few, chromatography coupled with elution and subsequent biologic or spectrophotometric assay.

Our survey of the literature indicated that histamine has been but a stepchild in chromatographic studies. It has received scant primary attention. Accordingly, the present investigation was undertaken to determine directly the most sensitive, specific, rapid method of separating and detecting histamine using the latest chromatographic techniques. This study of histamine has thus included comparative observations on both paper and thin-layer chromatography as well as paper, thin-layer and gel electrophoresis. Particular emphasis has been laid on developing a more sensitive and specific means of staining for histamine.

OBSERVATIONS AND RESULTS

Initial studies centered on spray reagents for detecting the histamine spot in as sensitive and specific a manner as possible. None of the previously employed visualization techniques seemed to offer as much as might be achieved by employing a new compound, *o*-phthalaldehyde (OPT). This compound readily forms a fluorescent condensate with histamine in alkaline solution and this reaction serves as the basis for the most popular and sensitive fluorometric test for histamine today[2,3]. Although

OPT had previously been employed by PATTON AND FOREMAN[23] as a simple colorimetric agent for the detection of glycine, histidine and tryptophan in paper chromatograms, it has never been used in detecting histamine chromatographically.

Preliminary tests showed that unless the histamine were in aqueous solution, *i.e.* ionized, it did not form a fluorochrome with OPT. It also became apparent that for ultimate sensitivity the OPT would have to be applied in a solvent in which neither histamine nor the histamine-OPT condensate would dissolve. Indeed the following requisites proved desirable for the OPT spray solvent:

(1) Dissolves OPT, and provides stable non-reactive milieu.
(2) Immiscible with water.
(3) Does not dissolve histamine.
(4) Releases OPT to aqueous phase.
(5) Does not interfere with histamine-OPT condensate formation.
(6) Does not dissolve condensate.
(7) Neither fluoresces nor quenches fluorescence.

Over 125 solvents were screened by these seven criteria, and it was found that xylene, *p*-xylene and diethyl ether were the best. They met all of the requirements. α-Chloronaphthalene and dibutyl carbitol were two other satisfactory solvents, but xylene and *p*-xylene proved to be best in general usage. Spraying solutions of OPT in any of these solvents on an alkaline spot of dilute histamine on Whatman No. 1 paper was regularly followed by the appearance of a blue color when viewed under ultraviolet light (3600 Å). This color was faint and evanescent at the greatest dilutions, apparently due to the photosensitive nature of the condensate formed. It should be noted that unlike histamine the condensate is thermolabile.

Next, it was shown that OPT in a 0.2–1% (w/v) concentration gave the optimal sensitivity. Samples from different companies showed variations, but the best and most consistent results were obtained by using OPT from California Biochemical Corporation. Immediately preceding the application of the OPT spray, the chromatogram was rendered alkaline to promote OPT-histamine condensate formation. For this, the best spray proved to be 0.2 *N* sodium hydroxide (fluorescent grade, Hartman-Leddon Company, Inc.), although a pH 11 buffer was also satisfactory. Under exceptional instances, *e.g.* when using the magnesium silicate plates, this alkaline spray step could be eliminated.

Using the 0.2 *N* NaOH–1% OPT *p*-xylene sprays, sixty papers and thin-layer plates were screened to see which afforded the greatest degree of spot test sensitivity (Table II). Magnesium silicate thin layer proved to be the most satisfactory of all the supportive media studied. Although similar sensitivity could be achieved with other paper and plates, magnesium silicate gave the most vivid and lasting spot reaction. Viewing was best done under an intense Wood light.

Having established the technique of *o*-phthalaldehyde fluorescent spot testing for histamine, we turned to examine its specificity (Table III). Previous authors had reported that OPT formed fluorescent condensates with certain biologic compounds. Under the conditions of testing in our laboratory, only histidine and glutathione (reduced) were found to interfere significantly. It was found that these two compounds could readily be separated from histamine by either chromatographic or electrophoretic means. With this background we turned to the application of the OPT technique to chromatography and electrophoresis.

TABLE II

SPOT TEST FOR HISTAMINE HYDROCHLORIDE. COMPARISON OF PAPERS AND THIN-LAYER PLATES

Factors: sample, 0.01 *N* NaOH, 1% OPT in *p*-xylene (0.01 ml each); U.V. light viewing: blue spot.

Paper	*Limit of detection* (*μg*)
Whatman No. 1	0.02
Whatman No. 2	0.02
Sepraphore III (Gelman)	0.02
Schleicher & Schüll 576	0.05
Schleicher & Schüll 2045	0.05
Thin-layer plates	*Limit of detection* (*μg*)
Magnesium silicate*	0.01
Alumina Basic*	0.02
Absorbasil	0.02
Cellulose MN 300	0.03
Silica H	0.05

* No buffer added. Read at once.

The following papers were less satisfactory or not suitable: Whatman Nos. 3 MM, 4, 5, 7, 11, 20, 31, 40, 42, 44, 50, 52, 54, 541. Schleicher & Schüll 507, 589 (red, white, blue, orange, green, black), 2041, 602, 2040 A, 2043 A, 2045 B, 2043 B, 598-4 D, 598, 470 A, 470. Cellogel (Colab), Millipore UF, RA, HAB, OH, HA, Duralon. Oxoid Electrophoresis Strips.

The following plates were less satisfactory or not suitable: Neutral Alumina, Acidic Alumina, Alumina G, Alumina GF, Cellulose ECTEOLA, Cellulose DEAE, Talc, Kieselguhr, Silica G, Polyamide, Eastman Silica Paper.

TABLE III

BIOLOGIC SPECIFICITY OF *o*-PHTHALALDEHYDE SPOT TEST REACTION

Whatman No. 1 paper, 0.01 *N* NaOH, 1% OPT in *p*-xylene, immediate reading of blue color under U.V. light.

Reactive biologic compounds	*Minimal amount detectable* (*μg*)	*Color*
Histamine	0.02	Blue
Histidine	0.1	Blue
Glutathione (reduced)	1.0	Blue
Citrulline	10.0	Blue

See Fig. 1 for thin-gel data.

Non-reactive compounds (100 *μg* or less) include: acetophenetidin, *l*-alanine, ammonia, amphetamine, arginine, asparagine, *dl*-aspartic acid, creatine, creatinine, cysteine, cystine, dopa, dopamine, *dl*-ethionine, galactosamine, glucosamine, glutamine, glycine, glycogen (rabbit liver), glycogen (shellfish), glucose 6-phosphate, guanidine, heparin, homocitrullin, *dl*-homocystine, hyaluronic acid, 5-hydroxy-3-indoleacetic acid, hydroxyproline, imidazole, indole, isoleucine, *l*-leucine, *dl*-lysine, methionine, *d*-*β*-naphthol, nor-epinephrine, norleucine, *dl*-norvaline, *dl*-ornithine·HCl, *l*-proline, procaine, pyribenzamine, sarcosine, serotonin-creatinine sulfate, *dl*-serine, spermidine, spermine, streptomycin, sulfanilamide, taurine, thiamine, thiourea, threonine, tryptophan, tryptamine, tyramine·HCl, *l*-tyrosine, urea, uric acid, urocanic acid, *l*-valine.

1. Paper chromatography

Despite its technical simplicity, paper chromatography is ordinarily a slow process requiring many hours. Hence only limited studies were done in this area since both thin-layer chromatography and electrophoresis gave answers within minutes.

Ascending chromatograms could be satisfactorily prepared using Whatman No. 1 paper and a solvent system of propanol–0.2 *N* ammonia (3:1). The ammonia had to be removed by heating (150°, 10 min) before OPT was added to prevent non-specific staining of ammonia by the OPT. It should be noted that, although histamine is heat stable, it is not stable in alkaline solutions so that prolonged periods in such a solvent may lower the yield and makes this approach unsatisfactory for detecting trace amounts. Thus, amounts less than 1 μg could not be visualized well.

2. Thin-layer chromatography

In this procedure, 0.01 ml test solution is applied to carrier plates coated with a uniformly thin layer (250 μ) of powdered adsorbent. The commercially prepared plates (Mann Research Laboratories, Inc.) given in Table IV were used.

Ascending chromatograms were made using propanol–0.2 *N* ammonia (3:1). Varying degrees of separation and sensitivity can be achieved but for general work, the Cellulose 300 MN plates proved to be the best.

TABLE IV

PLATES FOR THIN-LAYER CHROMATOGRAPHY

No.	*Commercial name*	*Absorbent*	*Binder*	*Reaction*
1	Absorbasil	Silica	Calcium sulfate	Neutral
2	Alumina-Acidic	Aluminum oxide	None	Acidic
3	Alumina-Basic	Aluminum oxide	None	Basic
4	Alumina-Neutral	Aluminum oxide	None	Neutral
5	Alumina G	Aluminum oxide	Calcium sulfate	Basic
6	Alumina GF	Same as 5 with zinc fluorescent agent added		
7	Cellulose MN 300	Cellulose powder	None	Neutral
8	Cellulose MN 300 ECTEOLA	Epichlorohydrin-triethanolamine cellulose (anion exchange resin)	None	Neutral
9	Cellulose MN 300 DEAE	Diethylaminoethyl cellulose (ion exchange resin)	None	Basic
10	Hy-Flo Talc	Magnesium silicate	None	Neutral
11	Kieselguhr	Diatomaceous earth	Calcium sulfate	Neutral
12	Magnesium silicate	Magnesium silicate	Calcium sulfate	Basic
13	Polyamide	Polyamide	None	Neutral
14	Silica G	Silica gel	Calcium sulfate	Acidic
15	Silica GF	Same as 14 with zinc fluorescent agent added		
16	Silica H	Silica gel	Hydrated silica	Acidic

3. Electrophoresis

The addition of an electrical potential to the basic chromatographic techniques greatly accelerated and simplified separation techniques. Using horizontal strip electrophoresis apparatus (Arthur H. Thomas Model or Turner Model 310), it was possible to increase sensitivity and shorten separation time to as little as 10 min. Although these rapid clear separations were achieved with paper, thin-layer and gel techniques, the thin-gel procedure proved to be the best.

(*a*) *Paper electrophoresis.* Using either Whatman No. 1 paper or Gelman Sepraphore III, excellent separation of histamine, histidine and glutathione was attained. The following general technique was employed:

Add 0.01 ml sample to 2 × 8 in. strip and dry.

Spray lightly with pH 4.6 buffer (potassium acid phthalate–sodium phosphate).

Place face up in apparatus rack.

Run 10–30 min (Thomas unit, 300 V, 5 mA/8 in. strip) with pH 4.6 buffer in side wells.

Dry paper in oven at 150° for 5 min.

Spray lightly with 0.2 *N* NaOH.

Spray lightly with 1 % OPT in *p*-xylene.

Read blue spots under Wood light immediately.

Within 30 min, 0.050 μg of histamine in aqueous solution may be separated and detected.

(*b*) *Thin-layer electrochromatography.* Thin-layer plates as described above were all tested and Cellulose 300 MN proved to be the most satisfactory. Thus, using this plate with the same technique outlined for paper electrophoresis, with the exception that the plates are not dried in the oven, histamine moves 8 cm toward the cathode in 30 min. Histidine moves about half as far and glutathione is stationary, with a resultant excellent separation. It is possible to detect a 1/600,000 dilution of histamine hydrochloride. Histamine 1/100,000 added to plasma can be separated and identified by this means. Alumina G and Absorbasil plates were also useful, but gave a lower order of sensitivity. The other plates were distinctly less satisfactory or completely unusable.

(*c*) *Gel electrophoresis.* This proved to be the most sensitive and generally satisfactory technique for detecting, identifying, separating and quantitating trace amounts of histamine in fluids, blood and tissue extracts. It is possible to separate and detect as little as 2 ng (0.002 μg) of the free base of histamine under optimal conditions, using the following OPT–thin-layer method:

Alcohol cleansed glass plates, 4 × 3 ¼ in., are covered with 5 ml of hot 1 % agar (Ionagar® No. 2) in pH 4.6 potassium acid phthalate, sodium phosphate buffer. Plate is kept at absolute level during cooling.

Seven 1 mm wells are punched out in a line.

Add 0.004 ml samples to each well.

Plate placed face down in Turner Model 310 with pH 4.6 buffer in side wells.

Run for 5–10 min., 200 V.

Remove and spray lightly with 0.2 *N* NaOH.

Spray lightly with 1 % OPT in *p*-xylene.

Read instantly in intense transmitted U.V. light. Direct Wood light is much less satisfactory. All data recorded is based on reading with the transmitted light of a microscope mercury vapor lamp (Osram HBO 200 W, Filter UG 1).

Thick gels are employed in disc electrophoresis (Canalco®) and vertical gel electrophoresis (E. C. Apparatus®) were extensively tried but proved of no value for histamine detection. Both polyacrylamide and agar gels were tried in varying concentration. It should be noted that the tris and riboflavin commonly added to these

media had to be eliminated due to fluorescence. Polyacrylamide could, however, be used as a gel in the thin-gel procedure described above (5 % in a pH 4.6 buffer). The high voltage (10,000 V) electrophoretic apparatus was not tried since separation of histamine from plasma could be made in 5 to 10 min with the present method.

Unfortunately at this stage the fluorescence cannot be recorded quantitatively since routine densitometers and reflectance fluorometers have not proved adequate.

Two dimensional thin-gel electrophoresis may be undertaken by making a second run employing a 9.6 buffer, thus eliminating all OPT reactive substances with an isoelectric point between 4.6 and 9.6.

Histamine, histidine and glutathione can be sharply separated electrophoretically by virtue of their electrical charge in solutions of varying pH. The distance separating histamine and histidine remains approximately the same throughout the range pH 2.6 to 11.0. However, both move to the cathode if the pH is less than 8. At its isoelectric point (pH 8) histidine remains stationary whereas histamine moves still to the cathode. Between pH 8 and 10 histidine moves toward the anode whereas histamine is still moving toward the cathode. Finally, at pH 11 histamine is stationary, whereas histidine migrates even more toward the anode. In view of the fact that separation distance is essentially the same, we elected to work at an acid pH 4.6 since here histamine has maximal stability, and glutathione never interferes inasmuch as it is moving toward the opposite pole.

Extraction tests with this thin-gel–OPT method revealed histamine in the cases given in Table V.

TABLE V

DETECTION OF HISTAMINE BY THIN-GEL ELECTROPHORESIS AND OPT

Source	*Extraction technique*
Rat mast cells	Freeze-thaw
Rabbit platelets	Freeze-thaw
Rat tongue	Butanol–benzene
Rat skin	Butanol–benzene
Human stratum corneum	Trichloracetic acid
Human stratum normal	Trichloracetic acid
Human stratum psoriatic	Trichloracetic acid
Human stomach	Trichloracetic acid
Guinea pig lung	Trichloracetic acid
Rat skin	Trichloracetic acid
Rat tongue	Trichloracetic acid

DISCUSSION

We have found the *o*-phthalaldehyde fluorogram a simple, rapid and specific means of analyzing for trace amounts of histamine. The procedure is based on the application of the OPT reagent in an organic solvent which dissolves neither the histamine nor the OPT-histamine fluorescent condensate. This permits precise chromatographic localization of very small amounts of histamine.

The OPT reaction is not rigidly specific for histamine. Hence the spot test is of limited value, although if an extraction method has been employed which excludes

interfering biologic compounds (Table III), it is possible to detect rapidly traces of histamine in an aqueous medium. With the employment of chromatography of varying types, it has been possible to isolate and identify histamine with precision. In our experience, neither paper chromatography nor thin-layer chromatography was ideal, since sensitivity was relatively low. With the addition of an electrical field, these techniques became more satisfactory. However, in all of our studies the singular and best approach was thin-gel electrophoresis. This had all of the advantages of reproducibility, speed and sensitivity (Table VI and Fig. 1). The electrical field resulted in marked separation of all of the interfering substances which give fluorescent condensates with OPT. Thus in our laboratories this technique has been employed regularly with success.

TABLE VI

SUMMARY OF RECOMMENDED TECHNIQUES FOR FLUOROGRAM DETECTION OF AQUEOUS HISTAMINE HYDROCHLORIDE

Alkaline spray followed by 1% OPT in *p*-xylene. Read in U.V. light.

Technique	*Absorbent system*	*Solvent*	*Time*	*Detection limit (μg)*
Spot test: thin-layer plate	Magnesium silicate	None	Instantaneous	0.01
Paper chromatography	Whatman No. 1	Propanol–0.2 *N* ammonia (3:1)	3 h	1.0
Thin-layer chromatography	Cellulose MN 300	Propanol–0.2 *N* ammonia (3:1)	30 min	0.1
Paper electrophoresis	Whatman No. 1	Buffer pH 4.6	30 min	0.05
Thin-layer electrophoresis	Cellulose MN 300	Buffer pH 4.6	30 min	0.02
Thin-gel electrophoresis	Ionagar 1%	Buffer pH 4.6	10 min	0.005

Quantitation of the fluorogram has proved to be a difficult problem. None of the equipment tested by us proved satisfactory, but this is still under study. It is possible, however, to elute the histamine spot in the chromatogram and reap the elution photo-fluorometrically.

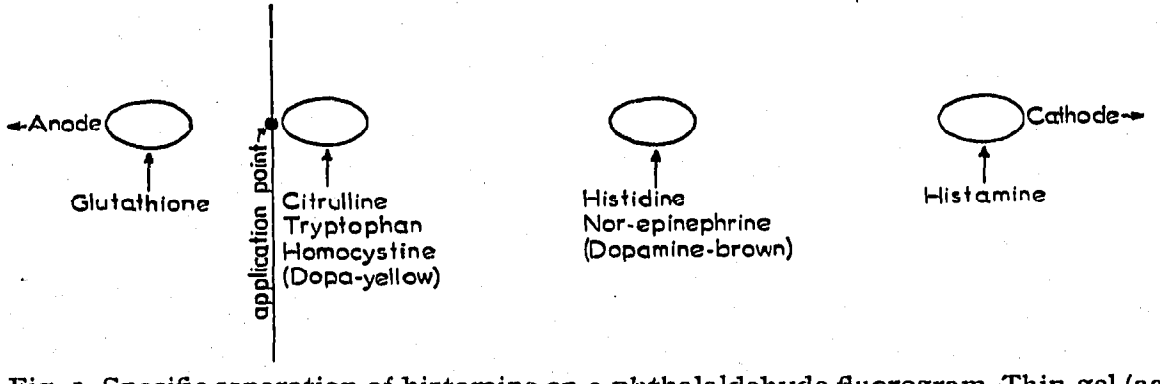

Fig. 1. Specific separation of histamine on *o*-phthalaldehyde fluorogram. Thin-gel (agar) electrophoresis (10 min, 200 V, 0.004 ml sample, pH 4.6 buffer). Localization and size of spot drawn to scale. Dilution limits for identification in 0.004 ml sample: 10^{-6} histamine, histidine; 10^{-5} nor-epinephrine, citrulline, glutathione; 10^{-4} tryptophan, dopamine, dopa; homocystine (saturated solution). Over sixty other biologic compounds gave no fluorescence at dilutions of 10^{-3} or greater.

SUMMARY

Rapid new sensitive chromatographic techniques are described for the isolation and detection of trace amounts of histamine. These are all based on the fact that in an alkaline medium, *o*-phthalaldehyde (OPT) and histamine immediately form a relatively specific and highly fluorescent condensate. Such OPT fluorograms may be made employing spot tests, paper and thin-layer chromatography, as well as paper, thin-layer and gel electrophoresis. With spot tests, thin-layer magnesium silicate plates proved to be the most sensitive, allowing the detection of less than 0.01 μg of histamine. For separation and more definitive identification of histamine, agar thin-gel electrophoresis was found to be the best method. Employing this, it was possible within 10 minutes to isolate and identify free histamine base in quantities as small as 0.002 μg.

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