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THIN-LAYER CHROMATOGRAPHIC ASSAYS OF HISTAMINE AND ITS METABOLITES IN URINE OF MAN AND DOG

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SUMMARY

A method for the extraction and quantitation of histamine and its metabolites from relatively small volumes of urine is described. It employs primarily ion-exchange and thin-layer chromatography and allows for quantitation in the μg range. Studies of atopic man and dog that employed this procedure yielded values comparable to those reported with gas chromatographic methods.

INTRODUCTION

Histamine, a vasoactive amine stored in mast cells, is one of the many and important pharmacologically active agents released in the allergic reaction between an allergen and an IgE sensitized cell. Upon release from their storage sites, histamine along with bradykinin, SRS, catecholamines and acetylcholine evoke reactions in the vasculature and smooth muscle which produce recognizable clinical signs which usually are grouped under the title of allergic disease.

The pharmacodynamics and physiology of histamine in man and dog has been the study of many investigators and excellent reviews of the subjects recently have been written[1,2]. The first major contribution relative to the metabolism of histamine was made by Schayer and Karjala[3] who, by employing an isotope dilution method, demonstrated two major catabolic pathways: methylation of histamine to form methylhistamine (MH) and oxidative deamination to form imidazoleacetic acid (IAA). The former (MH) subsequently may be oxidized to 1,4-methylimidazoleacetic acid (1.4-MIAA) and the latter (IAA) conjugated to riboside-imidazoleacetic acid (IAA-R).

The major metabolic pathway in man, dog and cat follows the methylation route while in rodents and ungulates, the oxidative pathway predominates. When labelled histamine is administered to man, approximately 45% of the radioactivity is found in the excreted 1,4-MIAA, 10% in IAA, 25% in IAA-R, 5% in MH and 1–2% in free histamine. Though isotope studies may not necessarily reflect endogenous metabolism[4], recent quantitative investigations of endogenous metabolites generally have supported Schayer and Karjala's metabolic schemes[4–6].

Two additional compounds may be recovered in urine, *viz.*, acetylhistamine (AH) and 1,5-methylimidazoleacetic acid (1,5-MIAA). However, they are not con-

sidered to be by-products of histamine metabolism but rather are of dietary origin[6]. It is worth mentioning that the amount of IAA recovered in urine is influenced by the dietary intake of histidine; and for this reason, quantitation of this imidazole derivative in urine is not considered a useful measure of histamine metabolism[7]. The consensus at this time is that the quantitative study of 1,4-MIAA excretion is the best parameter of endogenous histamine metabolism[6].

The ability to measure the amount of histamine and its metabolites in urine is the result of the application of ion-exchange chromatography, fluorimetry and gas chromatography. The amounts of histamine and its metabolites recovered in urine of normal man is given in Table I.

TABLE I

QUANTITY OF HISTAMINE AND METABOLITES (μg PER 24 h) RECOVERED IN HUMAN URINE AS REPORTED IN THE LITERATURE

References in parentheses; ch = children.

Histamine (free)	*Histamine (conjugated)*	*1,4-MIAA*	*IAA*	*MH*	*1,5-MIAA*
32.4 (8)	16–727 (8)	900–8900 (12)	300–3400 (7)	140–480 (4)	0–11,400 (5,6)
12.6 (9)	30 (9)	760–4490 (5)			
42 (10)	6–32 (7)	1520–2020 (5) (ch)			
7–41 (11) (ch)	17–1840 (11)				
6–10 (11)	2–56 (11)				
16–53 (4)					
6.4–19 (7)					
15–90 (6)					
6–34 (6) (ch)					

To the authors' knowledge, there are no studies dealing with the quantitative analysis of all of the histamine metabolites in urine. Rather, investigators have reported on the amounts of one or two of urinary metabolites recovered, particularly histamine, histamine and 1,4-MIAA, or histamine and MH[4,6,8–12]. The practice of not quantitating the total metabolites has obvious shortcomings and may lead to incorrect interpretation of the dynamics of histamine metabolism as it relates to health or disease states. Quantitative studies of histamine only cannot be interpreted without knowledge of the methylated or oxidized products to which histamine is normally metabolized. That is to say, a low urinary histamine value may be the result of an increased conversion of histamine to IAA or 1,4-MIAA as well as a decrease in the release of the amine from the mast cells, elimination by other routes or destruction at the site of the reaction. Similarily, studies which deal solely with 1,4-MIAA may be misleading because the metabolic processes may stop at methylation without subsequent oxidation (higher values of MH) or there may be a metabolic shift from methylative pathways to oxidative (higher values of IAA).

For the above reasons and because of long-standing interest in allergy and histamine metabolism in normal and allergic animals and man, our laboratory undertook a method for quantitating histamine and its metabolites in urine. Our first report[13] dealt with a thin-layer chromatographic (TLC) method for quantitating histamine and

its metabolites. The subject of the present report is the extraction of histamine, MH, 1,5- and 1,4-MIAA, and IAA from urine of allergic man and dog together with the application of the TLC method for the quantitative analysis of these compounds in urine.

MATERIALS AND REAGENTS

Materials

TLC Plates silica gel G, 250 μm thick, 20 × 20 cm, scored in 20 × 1 cm lanes (Analtech, Newark, Del., U.S.A.); LQ4D (preadsorbant) plates, silica gel G, 250 μm thick, 20 × 20 cm, pre-scored 15 mm lanes (Quantum Industries, Fairfield, N.J., U.S.A.); Chromoscan densitometer, Joyce Loebl, Model CSC with thin layer attachment (Tech/Ops Instruments, Burlington, Mass., U.S.A.), tungsten halogen light source.

Reagents

Bio-Rex 70, 20–50 mesh, Na^+ (Bio-Rad, Richmond, Calif., U.S.A.); IRA 401-S, 20–50 mesh, Cl^- (Mallinckrodt, St. Louis, Mo., U.S.A.); glacial acetic acid (J. T. Baker, Phillipsburg, N.J., U.S.A.); methanol (J. T. Baker); *n*-butanol (J. T. Baker); hydrochloric acid (J. T. Baker); sodium hydroxide, 50% w/w (Fisher Scientific, Fair Lawn, N.J., U.S.A.); iodine (Merck, Rahway, N.J., U.S.A.), U.S.P., resublimed; histamine dihydrochloride (J. T. Baker); *o*-phthaldialdehyde (Calbiochem, San Diego, Calif., U.S.A.); 1,4-methylhistamine dihydrochloride (Calbiochem), A grade; 1-methyl-4-imidazoleacetic acid hydrochloride * (Calbiochem), A grade; imidazoleacetic acid hydrochloride * (Calbiochem), A grade; activated charcoal, Darco, Type G-60 (A. H. Thomas, Philadelphia, Pa., U.S.A.); 1-methyl-5-imidazoleacetic acid hydrochloride, a generous gift of Dr. Tsung-Min (Eli Lilly, Indianapolis, Ind., U.S.A.).

METHOD

Collection of urine

The initial morning urine voided by an individual was discarded; thereafter for a 24-h period, all urine was collected in a glass jar containing 10 ml of concentrated hydrochloric acid. The urine was kept under refrigeration (4°) during the 24-h collection time. A creatinine determination was performed and the urine divided into 10 equal aliquots, frozen and stored at −10°. In the procedure described below, 1–3 aliquots were utilized for each extraction and quantitation. All samples were run in duplicate.

Extraction and quantitation of basic metabolites

Urine (60–200 ml) was centrifuged to eliminate any precipitate in the sample.

* Both metabolites were converted from the hydrochloride to the acetate form by placing them on 401 S columns prepared in the acetate form and washed to neutrality, and then eluting them with 0.5 *N* acetic acid. The eluate was flash evaporated and the dried metabolite was weighed and dissolved in methanol and a few drops of 1 *N* acetic acid to give a final concentration of 2 μg/μl. From these two stock solutions, combined IAA and 1,4-MIAA standards of 0.2, 0.4, 0.5, 0.6, 0.8 and 1.0 μg/μl were prepared.

The urine was then adjusted to pH 6.5 with 50% (w/w) sodium hydroxide and applied to a BR70 cation-exchange column, prepared as described by Bergstrom and Hansson[14]. The flow-rate was 1 ml/min. The urine sample which passed through the column was saved along with the first of the two 25–50 ml water washes which followed. The combined urine and water was refrigerated, until the elution and quantitation of histamine and methylhistamine was completed.

The BR70 column was then made acidic with 1 *N* hydrochloric acid to elute the basic amines from it. At the point where the pH of the eluate changed rapidly from neutrality to 3, the column was stopped and any 1 *N* hydrochloric acid remaining on top of the column was removed and replaced with 10–20 ml of 0.01 *N* hydrochloric acid. The column flow was then resumed and the subsequent eluate was collected and flash-evaporated at a temperature not in excess of 50°. The dried material was taken up in 4 ml of 0.1 *N* hydrochloric acid. (Internal standards of 0.01, 0.025 and 0.05 μg/ml histamine in 4 ml of 0.1 *N* hydrochloric acid and a reagent blank were prepared simultaneously with the sample for the purpose of plotting a standard curve). To this solution were added 1.5 g sodium chloride, 5 ml butanol, 5 ml chloroform, and 0.5 ml of 5 *N* sodium hydroxide. Immediately after the addition of the latter, the tubes were shaken for 5 min, centrifuged in the cold for 15 min and the contents allowed to settle for an additional few minutes. Nine millilitres of the upper organic layer were removed and added to test-tubes containing 15 ml of heptane and 2.5 ml of 0.1 *N* hydrochloric acid. The tubes were inverted gently 10–15 times for one minute and then the contents were allowed to settle for 15 min in a refrigerated environment (4°).

Two millilitres of the bottom acid layer were removed and divided into two fractions: (A) 0.2 ml for the quantitation of histamine and (B) 1.8 ml for the quantitation of methylhistamine. Fraction A was brought up to a volume of 2.0 ml with 0.1 *N* hydrochloric acid and its histamine content (along with internal standards) were determined fluorimetrically after the addition of *o*-phthaldialdehyde. The quantity of histamine in the 24-h urine sample was determined using the standard curve.

Fraction B was flash evaporated. Three millilitres of methanol and one drop of 0.1 *N* hydrochloric acid were added to the extract and the solution was transferred to a graduated test-tube. The volume was reduced to 350–500 μl by placing the test-tube in a water bath (45°) under a jet of nitrogen. Seventy-five to one hundred μl of the methanol extract were plated on the pre-adsorbant area of four lanes of a LQ4D TLC plate. After drying, 5 μl of methanol were spotted on the pre-adsorbant area of the first lane, and 3, 5 and 10 μg of methylhistamine in 5 μl of methanol in each of the remaining three lanes, respectively. The plate was developed in chloroform–methanol–ammonium hydroxide (70:30:5) in a paper-lined tank to 1 cm from the top, then dried in a hood for 10 min, heated in an oven for 10 min at 70° and immediately exposed to iodine vapors for 30 min[15]. After removal from the iodine vapor tank, the plate was placed horizontally in an exhaust hood for 3–5 min and finally in an oven maintained at 300° for 30 min. The plate was scanned in the densitometer (primary filter 300–400 nm and secondary filter 340 nm)* and the peak areas quantitated by triangul-

* Fluorimetric scanning (primary filter 300–400 nm and secondary filter 490 nm) was employed when increased sensitivity was desired.

ation. A standard curve for methylhistamine was plotted utilizing the peak areas of standards minus the peak area of the methylhistamine in the sample alone (lane 1). The quantity of methylhistamine in the 24-h urine sample was calculated using the standard curve and making the necessary adjustments relative to a 38% procedural loss and the percentages of the sample utilized.

Extraction and quantitation of acid metabolites

The urine and distilled water sample which contained the acid metabolites was adjusted to pH 7 with 1 *N* sodium hydroxide and 0.5% of the volume by weight of charcoal was added. After shaking the suspension for 5 min, it was centrifuged in the cold for 5 min and the urine decanted through filter paper. The sample then was adjusted to pH 2 with concentrated hydrochloric acid and flash evaporated almost to dryness. The "gummy" residue was extracted by the addition of 20 ml of methanol, shaken for 2–3 min, and decanted. This was followed by a 10-ml methanol washing of the flask, which was added to the initial 20-ml methanol extraction. The suspension was shaken for 3 min, centrifuged for 5 min and the supernatant decanted and flash-evaporated to dryness. To obtain further drying of the residue, the flask was placed in a vacuum desiccator overnight and an additional extraction of the dried urinary material was performed with 15–20 ml methanol. This was followed by an absolute alcohol extraction and the final dried extract was dissolved in 10 ml of deionized water.

The aqueous solution was adjusted to pH 10 by the addition of 50% (w/w) sodium hydroxide. The solution which was relatively free of salt and urochrome then was applied to a 401-S anion-exchange column prepared in the acetate form and washed with deionized water to neutrality. A bed volume of 20 ml of ion exchange was provided for each 100 ml of urine. The flow-rate through the column was 1 ml/min, and after complete entry of the urine into the column, the column was washed to neutrality with de-ionized water. Thirty millilitres of 0.5 *N* acetic acid then were applied to the column and the eluate was collected in 2-ml aliquots in test tubes. The acid metabolites eluted from the column between pH 5.4–2.5 (ref. 15), and thus the 2-ml aliquots in this pH range were pooled into a single sample and evaporated. The extract was dissolved in 4 ml of methanol and one drop of 1.0 *N* acetic acid. The resulting suspension was transferred to a centrifuge tube to which was added 2 × 3 ml methanol washings of the flask. The 10-ml sample was centrifuged and the supernatant decanted into a graduated test-tube. The volume of the solution then was reduced to 1 ml and the extract was placed in a freezer (−10°) overnight to allow for the additional elimination of salts. After removal from the freezer, the test-tube was centrifuged, the supernatant decanted and the volume adjusted again to 1 ml by the addition of methanol.

Ten microlitres of the methanol extract then were spotted in six lanes 2 cm from the bottom edge of each of two Analtech silica gel G plates (10 × 20 cm) which previously were kept in a desiccating chamber. After drying, 10 μl of methanol was spotted at the origin in the first lane. 2 μg of IAA and 1,4-MIAA in 10 μl of methanol in lane 2, 4 μg of IAA and 1,4-MIAA in 10 μl of methanol in lane 3, 5 μg of IAA and 1,4-MIAA in 10 μl of methanol in lane 4 and 6 and 8 μg of IAA and 1,4-MIAA in 10 μl of methanol in lanes 5 and 6. The plates were developed in butanol–methanol–glacial acetic acid–water (50:10:36:8) and butanol–glacial acetic acid–

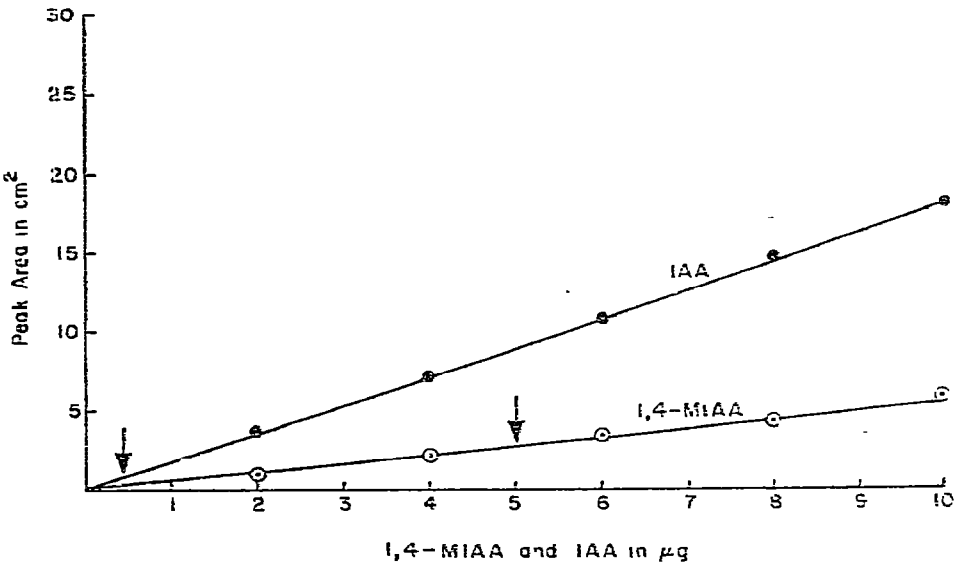

Fig. 1. Standard curves produced as indicated in the text by subtracting peak areas of acid metabolites in samples from those of samples plus standards. The arrows indicate the point on the curve which corresponds to the peak area of the metabolite measured in the sample. The butanol–methanol–acetic acid–water solvent (50:10:36:8) was used for separating 1,4-MIAA, the butanol–acetic acid–water solvent system (60:22:23) for IAA.

water (60:22:23); the subsequent treatment of the plates which allows for quantitation of the acid metabolites followed the procedure already described in the methylhistamine extraction and quantitation. As a 25% loss of IAA and 1,4-MIAA is associated with the extraction procedure, this figure was used in the calculations. Fig. 1 illustrates a typical standard curve produced from a plate developed in each of the two solvent systems.

Studies involving the loss of basic and acid metabolites of histamine which occurs with the extraction procedures, were performed by analyzing two identical urine samples, to one of which varying amounts of authentic metabolites had been added. In the case of MH, eight experiments were performed; for 1,4-MIAA and IAA both ten experiments.

The purity of the individual bands, 1,4-MIAA (solvent system 50:10:36:8), IAA (60:22:23) and MH (70:30:5) was investigated by development of each in a secondary solvent system. In the case of 1,4-MIAA, 10 μl of a sample (methanol extract of 401 S column eluate) were spotted in each of eight lanes; 10 μl of a 1,4-MIAA standard (10 μg) were added to the same sample in the last two lanes. The plate was developed in the butanol–methanol–glacial acetic acid–water (50:10:36:8) and placed in the iodine tank for 15 min in order to visualize the 1,4-MIAA bands. The latter were marked by streaking the silica gel immediately above and below bands and the plate was left in an exhaust hood overnight to allow for the evaporation of the iodine. The bands previously outlined in the first six lanes (sample alone) were scraped from the glass and pooled. The sample plus standard bands also were scraped from the plate and pooled. Each pooled silica sample was placed into tubes to which 4 ml

of methanol were added. The tubes were shaken for 1 h, centrifuged and the supernatant decanted. The volume of the latter was reduced to 50 μl. Twenty microlitres of each extract then were spotted on a silica gel plate in a 10-mm lane, developed in chloroform–methanol–ammonium hydroxide (40:40:15), blown dry, placed in an iodine tank and treated as previously described. A similar procedure was followed for checking the purity of the IAA and MH bands. In the former case, the primary solvent system was butanol–glacial acetic acid–water (60:22:23) and the secondary was chloroform–methanol–ammonium hydroxide (40:40:15); in the latter case, the primary solvent system was chloroform–methanol–ammonium hydroxide (70:30:5) and the secondary system, butanol–glacial acetic acid–water (60:22:23).

RESULTS AND DISCUSSION

It is not the purpose of the present report to interpret the results of the urinary

TABLE II

RECOVERY OF HISTAMINE AND METABOLITES (μg PER 24 h) FROM THE URINE OF ALLERGIC MEN, HYPERSENSITIVE ONLY TO RAGWEED POLLEN

Figures in parentheses represent values obtained from duplicate assays.

Patient	*Histamine (free)*	*MH*	*IAA*	*1,4-MIAA*
S-r				
(5-21-73)*	37 (35)	218 (223)	1,470 (1,434)	4,660 (4,625)
(10-2-73)**	7.8	0 (0)	0	11,466
B-e				
(6-13-73)*	45 (43)	68 (70)	0 (0)	1,470 (1,497)
(7-18-73)***	11.2	49.3	974	1,043
K-m				
(6-13-73)***	24 (25)	25 (25)	1,870 (1,815)	4,191 (4,100)
N-n				
(5-24-73)***	19 (19)	1,913 (1,927)	3,733	7,200
(10-2-73)**	12.83	129	1,230	13,333
T-i				
(7-18-73)*	15 (16)	492 (480)	0 (0)	6,933 (6,714)
(7-25-73)*	15 (13)	91	8,800	9,866
(9-11-73)**	8.4	2,153	967	15,248
R-t				
(7-19-73)	19 (18)	450 (435)	0 (0)	8,000 (7,864)
L-o				
(7-27-73)*	14.58 (11.88)	126	0 (0)	7,066.6 (3,980)
(8-10-73)***	31	308 (294)	3,470	3,733.3
M-i				
(7-27-73)*	20.25	197	0 (0)	15,466
(8-3-73)*	15 (15)	25 (25)	0 (0)	15,330 (14,480)
(8-10-73)***	17 (19)	138 (140)	0 (0)	9,460 (9,775)

* The collection of urine samples and assays were performed from May–July, 1973, during a clinically quiescent period.

** The collection of urine and assays were performed in September and October, during or shortly after a clinically active period.

*** The assays were performed on urine collected one day following a week of steroid therapy.

TABLE III

RECOVERY OF HISTAMINE AND METABOLITES (μg PER 24 h) FROM THE URINE OF ALLERGIC DOGS

All animals were multisensitive. They were housed in an air-conditioned environment and were clinically asymptomatic. The collection of urine samples and assays were performed from February–April, 1973. Figures in parentheses represent values obtained from duplicate assays.

Dog	*Histamine (free)*	*MH*	*IAA*	*1,4-MIAA*
Ti	79 (75)	483 (470)	914 (947)	3,721 (3,690)
Dona	93 (96)	531 (529)	1,126 (1,100)	4,417 (4,400)
SW-5	87 (84)	491 (490)	1,087 (1,110)	5,320 (5,249)
SW-6	120 (124)	597 (582)	2,413 (2,336)	6,229 (6,334)

assays, but rather to discuss the TLC procedure utilized. The effects of steroids on histamine metabolism and the urinary excretion of histamine metabolites relative to active and quiescent stages of allergic disease will be reported elsewhere when more data have been accumulated. However, a few preliminary comments can be made on the results of the limited data in this study. The amounts of histamine and its metabolites recovered from the urine of allergic man and dog are presented in Tables II and III. With the exception of the unusually high recovery of MH from patients N-n (5-24-73) and T-i (9-11-73), the reported values for basic metabolites are in general agreement with previous assays dealing with normal individuals. The values obtained for IAA are consistent with the literature with the exception of T-i (7-25-73); those for 1,4-MIAA in assays of urine from asymptomatic patients (May–July, 1973) fall into the reported ranges with the exception of M-i (7-27-73 and 8-3-73).

In two instances in which assays were performed during both clinically quiescent and active stages of the disease, it should be noted that an increased excretion of 1.4-MIAA occurred in the latter period: S-r, 4.7–11.5 mg; and T-I, 6.9 or 9.9–15.2 mg. Interestingly, the histamine values of the two patients were lower in the active stage of the disease than during a clinically quiescent period. In three cases where pre-steroid and post-steroid collections were assayed, a significant reduction in the amount of 1.4-MIAA was noted in the post-steroid samples: M-i, 15,5 or 15.3–9.5 mg; L-o, 7.0–3.7 mg; and B-e, 1.5–1.0 mg. Unfortunately, it was not possible to control the diets of the patients studied and thus it cannot be said unequivocally that the intake of histamine in the food did not affect the results[16]. There is insufficient literature relative to the urinary excretion of histamine metabolic in the dog to allow for comparison of our results with canine urines.

In studies of the estimation of losses associated with the extraction procedure, the following recoveries were obtained: MH, 62 ± 2.8%; 1,4-MIAA, 75 ± 1.2%; and IAA, 75 ± 2.1%. In studies directed to ascertaining the purity of the bands, the secondary development demonstrated single bands for each metabolite. In the duplicate assays of the human and canine urine samples performed in this study, less than 7.5% variation in values was obtained.

The TLC separation and quantitation of MH was facilitated by the employment of the Quantum pre-adsorbant plate which permits application of large (100–300 μl) volumes of samples to the silica gel. Optimum results in plating the sample were achieved when the latter was divided into 10-μl aliquots and each application

(10 μl) to the pre-adsorbant area was dried under a stream of hot air before the subsequent 10 μl were streaked on the area.

The butanol–methanol–glacial acetic acid–water (50:10:36:8) development system gave a clear separation of the 1,4-MIAA from contaminating substances. Thus, the quantitation of 1,4-MIAA employed scans of plates developed in this system. The 60:22:23 development system produced a clear separation of IAA from other substances and plates developed in this system were used for the quantitation of IAA. 1,5-MIAA migrated in advance of 1,4-MIAA and was clearly separated from it in both development systems. No attempt was made to quantitate the 1,5-MIAA but only six of the sixteen samples showed bands with a R_F value comparable to the 1,5-MIAA standard. As authentic IAA-R could not be obtained, the metabolite was not identified or quantitated.

As our laboratory has not had experience with gas chromatography (GC), a direct comparison of TLC and GC quantitation of histamine metabolites could not be made. The GC methods[5,15] appear to have slightly greater sensitivity and less time or effort need be expended in the extraction procedure.

The most significant problem associated with the present method was the elimination of urochromes from the final methanolic extract from the 401-S column. This is of importance because the urochromes move in the development of the plate to areas where the acid metabolites migrate. The chemical and physical similarities of the histaminic amines and urochromes make it extremely difficult to separate them. In early work, the anion exchanger was prepared in the OH^- form and the metabolites eluted with hydrochloric acid. Though the conjugated histamine was eluted with the de-ionized water wash and the acid metabolites with the change of the eluate from neutrality to acid, the amount of urochrome in the acid eluate along with its tendency to obscure the acid metabolites on the silica gel plates made this approach undesirable. Preparation of the anion exchanger in the acetate form resolved the problem to a great degree, as most of the urochromes remained bound to the anion-exchange column during the acetic acid elution.

Another problem which was encountered was related to the development of the sample on silica gel G plates when the ambient relative humidity was high. This resulted in smearing and inadequate separation of the bands. To avoid this problem the TLC procedures were performed only when the ambient relative humidity was less than 50% and plates always were desiccated before use and developed in tanks placed in an incubator room where the temperature was 37° and the relative humidity less than 25%.

The availability of GC and TLC methods which allow for the quantitation of all the metabolites of histamine in the urine now should stimulate investigators to study again the metabolism of histamine in disease states.

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