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SEPARATION OF RADIOACTIVE HISTAMINE AND SOME OF ITS METABOLITES BY ONE-DIMENSIONAL PAPER CHROMATOGRAPHY

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SUMMARY

A simple one-dimensional paper chromatography technique is described for the separation of radioactive histamine and some of its metabolites in blood and tissues. After removal of protein, the supernatant fluid (200 μl) is subjected to two-stage one-dimensional paper chromatography. This enables large numbers of samples to be chromatographed simultaneously. Using this technique in conjunction with liquid scintillation counting, it was possible to monitor the small amounts (0.02 nCi) of radioactive histamine and its metabolites in serial blood samples.

INTRODUCTION

Numerous methods exist for the separation and estimation of histamine and its metabolites. Most notable of these is the isotope dilution technique pioneered by Schayer and Cooper[1]. Unfortunately, it is time-consuming and unsuited to large numbers of samples; moreover, its reliability for measuring some of the histamine metabolites is in question[2,3]. Other techniques such as ion-exchange chromatography, alone[3] or followed by paper chromatography[4], are also unsuited to large numbers of samples. One-dimensional chromatography on cellulose phosphate has been advocated[5], although the authors noted that 5- to 50-fold more carrier was needed than with conventional cellulose papers. Two-dimensional chromatography has been successfully applied to the resolution of histamine and its metabolites[3,6,7]. With this technique, one solvent (alkaline based) separates the acid metabolites imidazoleacetic acid, its riboside and *tele*methylimidazoleacetic acid (1-methylimidazole-4-acetic acid[8]) and a second (acid based) separates histamine, *tele*methylhistamine and acetyl histamine. This nomenclature where *tele* designates the ring imidazole nitrogen further from the side chain and *pros* the nearer, was introduced by Black and Ganellin[8] to rationalize the nomenclature of histamine derivatives. Since our interest was to determine serial changes of [^{14}C]histamine and its metabolites in up to 45 samples per experiment, none of the above techniques appeared practicable. A one-dimensional chromatograph method retaining the resolution capability of a two-solvent system was therefore devised.

MATERIALS AND METHODS

Chemicals

Histamine dihydrochloride, *p*-nitroaniline, (BDH, Poole, Great Britain), *tele*-methylhistamine dihydrochloride, *tele*methylimidazoleacetic acid HCl and N-acetyl-histamine (Calbiochem, Bishops Stortford, Great Britain), imidazoleacetic acid HCl (Sigma London, Kingston-on-Thames, Great Britain), N^{α}-methylhistamine and N^{α},N^{α}-dimethylhistamine were generous gifts from Prof. J. Black (University College, London) and Dr. K. Bunce (Smith, Kline & French Labs., Welwyn Garden City, Great Britain). The use of N^{α}-nomenclature[8] is preferred since it distinguishes the side chain nitrogen from the ring nitrogens. Histamine (ring-2-^{14}C) was obtained from The Radiochemical Centre, Amersham, Great Britain. Solvents and other reagents were of the highest purity commercially available.

Blood samples (0.6 ml) containing radioactive metabolites of histamine were treated with concentrated perchloric acid[9] to yield a 0.4 *M* solution and vortexed for 10 sec. Tissue samples were homogenized twice for 10-sec periods at 20,000 rpm in two volumes of 0.6 *M* perchloric acid with an Ultra Turrax, Type TP 18/2 (Janke & Kunkel, Staufen i.Br., G.F.R.). All samples were then centrifuged at 3000 *g* for 20 min at 0–4° (MSE Mistral 6L), the resultant supernatant fluid being used without further preparation.

Chromogenic reagents

Diazotized *p*-nitroaniline was prepared for spraying as described by Fleming and Clark[10]. One volume of sodium nitrite (0.2%) was mixed with an equal volume of *p*-nitroaniline (0.1 g in 2 ml HCl, made up to 100 ml) and kept on ice for 10 min. Immediately before use this solution was mixed with two volumes of potassium carbonate (10%). This reagent produced a colour with histamine, acetylhistamine, N^{α}-methylhistamine, N^{α},N^{α}-dimethylhistamine and imidazoleacetic acid, and a faint but distinct colour with *tele*methylhistamine. Ethanolic iodine, a 5% (w/v) solution of iodine in ethanol, was used to locate *tele*methylimidazoleacetic acid. The brown condensation product produced with this spray was decomposed by spraying with hydrogen peroxide (5.9%, w/v). The specificity of the condensation product formed between *tele*methylimidazoleacetic acid and the ethanolic iodine spray was confirmed with *p*-nitrobenzene diazonium fluoroborate[5]. The reaction with *p*-nitrobenzene diazonium fluoroborate is more sensitive than that with *p*-nitroaniline, but it could not be used routinely other than with reference compounds, since a non-specific colour reaction occurred with samples of acidified supernatant fluid. To produce the diazo compound, the paper was immersed in a 1% (w/v) solution in acetone of *p*-nitro-benzene diazonium fluoroborate (Eastman No. P7078) before being sprayed with 0.1 *N* potassium hydroxide in ethanol. Silver nitrate–sodium hydroxide reagent[11] was used to detect imidazoleacetic acid-riboside, the paper being immersed in silver nitrate solution (0.1 ml saturated solution in 20 ml acetone and water added until silver nitrate redissolved) dried and then sprayed with a 0.5 *M* sodium hydroxide in ethanol solution. A black colour rapidly developed where the riboside was located. Since this compound markedly quenched radioactivity measurements, this reagent was used infrequently. With samples containing large amounts of radioactivity

(> 0.5 nCi) it was possible to obtain radioactivity profiles with a radiochromatogram scanner (Panax Nucleonics, Redhill, Great Britain).

The supernatant fluid (200 μl) and reference compounds [histamine, acetylhistamine, imidazoleacetic acid (each 5 μg), *tele*methylhistamine (100 μg) and *tele*methylimidazoleacetic acid (200 μg)] were applied for descending chromatography in 1.5-cm bands, 12 cm from the top of a 57 $\times$ 44 cm Whatman 3MM paper with a maximum of twelve spots per paper. The paper was then placed in a commercial chromatographic chamber (Shandon 500 Chromatank), the solvent system benzene–chloroform–methanol–ammonia (15:15:17:2, v/v/v/v) added and developed at room temperature (20–22°). After the solvent front had passed 30 cm, the paper was removed, cool-air dried and the paper replaced in the chromatographic chamber and the process repeated with fresh solvent. On completion, a vertical strip of paper on which a deproteinized blood sample and reference compounds had been chromatographed was cut from the edge of the paper, dried and sprayed with ethanolic iodine to locate *tele*methylimidazoleactic acid. The strip of paper was then decolourized with hydrogen peroxide and dried before being sprayed with diazotized *p*-nitroaniline. This procedure revealed the positions of the unresolved amines and imidazoleacetic acid. The strip of paper was then aligned with the main chromatography paper, so that the latter could be divided horizontally between the *tele*methylimidazoleacetic acid spot and the unresolved amines on the upper portion of the paper. The distal portion was then developed in the same direction by ascending chromatography with the solvent ethanol–2 *M* HCl (3:1, v/v) to separate the amines.

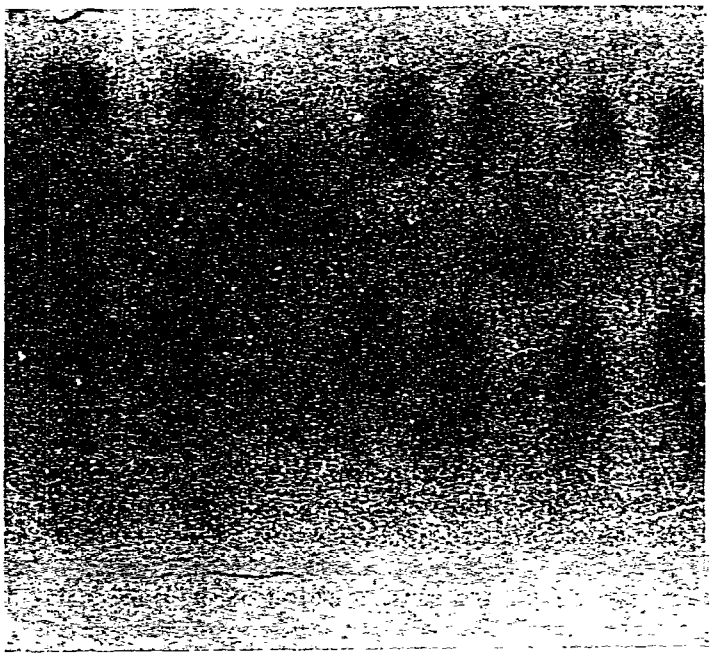
The positions of *tele*methylhistamine, histamine, acetylhistamine, imidazoleacetic acid and *tele*methylimidazoleacetic acid were identified with the two sprays described above. In addition, silver nitrate–sodium hydroxide and nitrobenzene diazonium fluoroborate were used to confirm the positions of the reference compounds.

Radioactive metabolites were quantitated by direct counting[12] of the appropriate paper areas. The areas were cut out, folded and immersed in screened vials containing 10 ml of NE233 scintillation fluid (Nuclear Enterprises, Edinburgh, Great Britain), radioactivity being measured in an NE8312 scintillation spectrometer. Papers were placed horizontally in the vials, the radiolabelled compound remaining on the solid phase.

Linearity between the radioactivity associated with resolved metabolites and the radioactive metabolites in blood was established from serial dilutions of a sample of cat blood. To obtain sufficient quantities of [^{14}C]histamine metabolites in this sample, [^{14}C]histamine was instilled into the duodenum at zero time followed by a 20-min portal vein infusion at 2 h; the blood sample was taken at 2.5 h. This sample was serially diluted with a sample of blood taken at zero time and treated as described above.

RESULTS AND DISCUSSION

As shown in Fig. 1, this procedure separated histamine, *tele*methylhistamine, acetylhistamine, imidazoleacetic acid and *tele*methylimidazoleacetic acid. The R_F values and the colours of the imidazole derivatives with their respective chromogenic reagents are shown in Table I. Fig. 2 is radioactivity profile obtained with a Panax



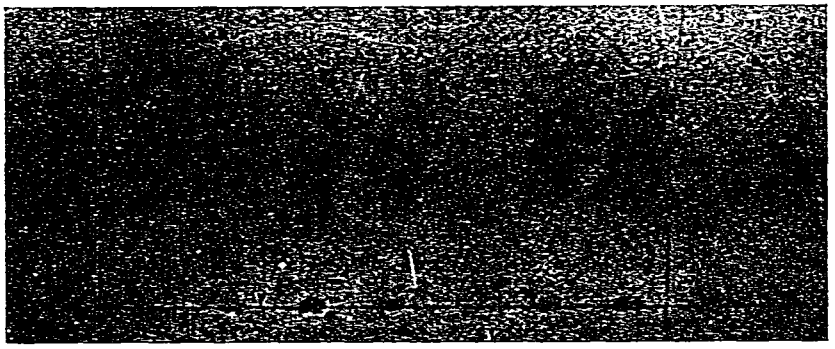


Fig. 1. One-dimensional chromatogram of deproteinized blood samples. The upper paper shows the separation of histamine (His) from its basic metabolites, *tele*methylhistamine (MeHis) and acetylhistamine (AcHis). The lower paper shows the separation of the acidic metabolites imidazoleacetic acid (ImAA) and *tele*methylimidazoleacetic acid (IMemAA). The latter is indicated by broken lines. O denotes the origin.

Nucleonics radiochromatogram scanner of a specimen of acidified (0.4 *M* perchloric acid) cat urine chromatographed as described above. This specimen was collected 1 h after [^{14}C]histamine (5 μCi; specific activity 1 μCi/μmole) had been instilled into the proximal duodenum–jejenum. This confirmed that peaks of radioactivity were isographic with the reference compounds.

TABLE I

R_F VALUES OF HISTAMINE AND SOME OF ITS METABOLITES

Results obtained after chromatography with two passes of solvent 1 (benzene–methanol–chloroform–ammonia, 15:17:15:2) and then with solvent 2 (ethanol–2 *M* HCl, 3:1) on Whatman 3MM paper. For details of procedure see text.

Imidazole compound	*R_F value*		*Colour*	*Chromogenic agent*
	Solvent 1	*Solvent 2*		
Imidazoleacetic acid–riboside	0.0 –0.05	—	White/blue	Periodate reagent
Imidazoleacetic acid	0.1 –0.2	—	Purple/brown	H_2O_2, dried, diazotized *p*-nitroaniline
*Tele*methylimidazoleacetic acid	0.24–0.36	—	Brown	Ethanolic iodine
Paper sectioned at	0.42			
Unresolved amines	0.62–0.82			
Histamine		0.41–0.59	Orange	Diazotized *p*-nitroaniline
N^{α},N^{α}-dimethylhistamine		0.61–0.65	Purple	
*Tele*methylhistamine		0.63–0.74	Faint purple	
N^{α}-methylhistamine		0.72–0.78	Purple	
Acetylhistamine		0.78–0.86	Orange red	

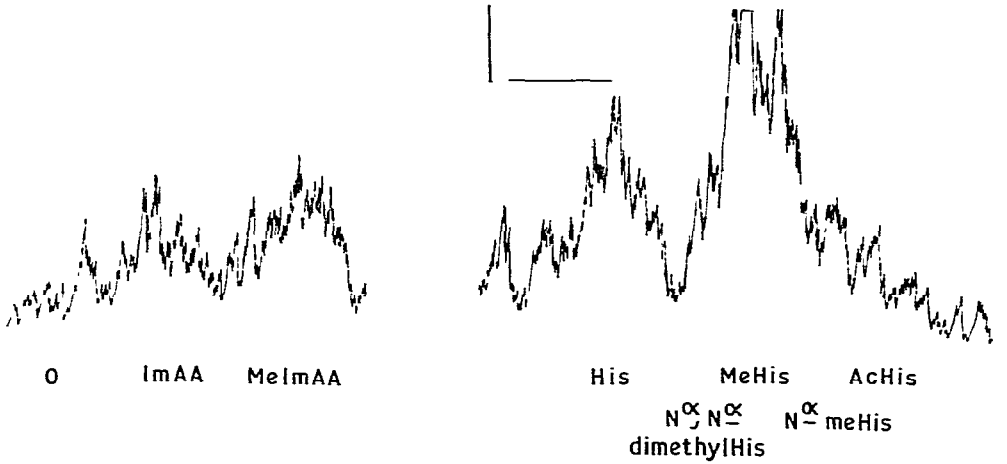


Fig. 2. Radiochromatogram obtained on a Panax Nucleonics radiochromatogram scanner of a specimen of urine taken from a cat in which 5 μCi of [^{14}C]histamine had been intraduodenally instilled. Indicated are the positions of reference compounds, histamine (His), *tele*methylhistamine (MeHis), acetylhistamine (AcHis), imidazoleacetic acid (ImAA), *tele*methylimidazoleacetic acid (MeImAA), N^{α}-methylhistamine (N^{α}-meHis) and N^{α},N^{α}-dimethylhistamine (N^{α},N^{α}-dimethylHis). The origin of the sample is indicated by O. The horizontal bar represents 5 cm, and the vertical bar 5 arbitrary units of radioactivity.

It is evident from the R_F values of N^{α}-methylhistamine and N^{α},N^{α}-dimethylhistamine and from Fig. 2 that these compounds overlap with *tele*methylhistamine and therefore care should be exercised in the interpretation of data. Consequently when 15% or more of the total radioactivity is present in the *tele*methylhistamine position, as might be expected in studies with dog gastric mucosa[13], the amines should

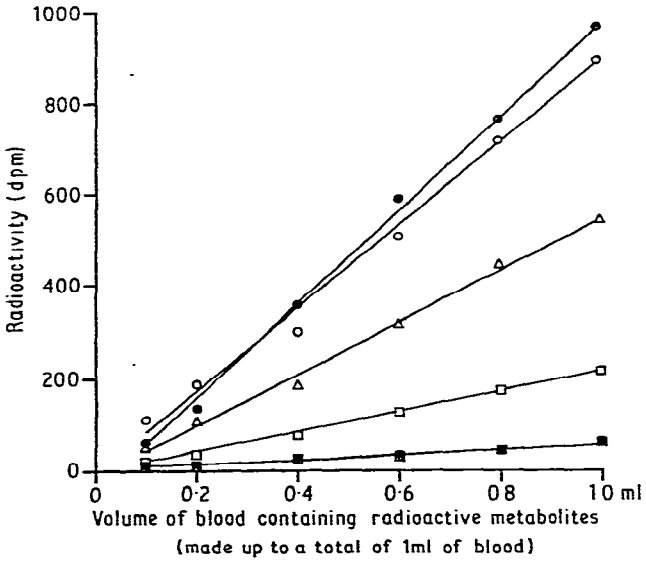


Fig. 3 Relation between radioactivity and concentration of radioactive metabolites. A sample of cat blood taken following the instillation of 2.5 μCi[^{14}C]histamine (0.4 μCi/μmole) into the proximal duodenum–jejenum and the infusion of 2.5 μCi [^{14}C]histamine (2.8 μCi/μmole) into the portal vein was diluted with blood removed prior to the administration of histamine, to give the range of concentrations. ●, Radioactivity in perchloric acid extract for chromatography; ○, total radioactivity recovered after chromatography; △, radioactivity recovered as *tele*methylimidazoleacetic acid (MeImAA); □, radioactivity recovered as imidazoleacetic acid (ImAA); ■, radioactivity recovered as *tele*methylhistamine (MeHis). Slopes were obtained using the method of least squares.

be derivatized with dansyl chloride and then further resolved by chromatography on silica gel (see ref. 14).

As shown in Fig. 3, radioactivity in perchloric acid extracts of cat blood and total radioactivity recovered after chromatography of the same specimens were linearly related to the serial dilutions of blood as was the predominant histamine metabolites in cats, *viz. tele*methylimidazoleacetic acid and imidazoleacetic acid and a third, minor metabolite, *tele*methylhistamine (Fig. 3).

We have been unable to investigate all the very minor metabolites of histamine so that the possibility has not been excluded that some of these metabolites may be isographic with the reference compounds.

The two solvent systems used in this study were introduced because previously described solvent systems[7,13] did not adequately separate histamine and its metabolites. This may, at least in part, have been due to the nature of the sample, *i.e.* the presence of perchloric acid and salts. Among the solvents tested, one of those advocated by Snyder *et al.*[15] (ethanol–0.1 *N* hydrochloric acid 95:5) led to the development of the solvent for separating the basic metabolites. The phenol-based solvent system used by Eliassen[7] appeared most promising for separating the acidic metabolites, but unfortunately phenol reacts with both diazotization reagents. Consequently, phenol was replaced by benzene and the polarity of the solvent restored by replacing ethanol by methanol.

Using the above technique, the small amounts of [^{14}C]histamine metabolites appearing in portal blood following instillation of the labelled histamine into the stomach and small intestine of cats are being examined.

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