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Note

Thin-layer chromatographic separation of imidazole compounds and its application to radiotracing studies of brain histidine metabolism

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Recently, there has been great interest in various imidazole compounds found in the nervous system. The separation of imidazoles by paper chromatography[1–3] and thin-layer chromatography[4–9] has been well documented. However, no report has yet appeared that is concerned with the separation of histidine and its six important metabolites, *e.g.*, 3-methylhistidine, histamine, imidazolepyruvic acid, imidazolelactic acid, imidazoleacetic acid and urocanic acid, which represent N-methylation, decarboxylation, transamination and deamination pathways of histidine metabolism, on a single chromatogram.

Two-dimensional thin-layer chromatography combined with a multiple *o*-phthalaldehyde spray method for the separation of the above compounds and its application to the study of brain histidine metabolism is presented in this paper.

MATERIALS AND METHODS

Chemicals and equipment

The following compounds and equipment were obtained from the sources indicated; histamine free base (Hm), imidazoleacetic acid HCl (IAA), urocanic acid (UA), imidazole (Im), *o*-phthalaldehyde (OPT), ninhydrin and cellulose MN 300 were obtained from Sigma (St. Louis, Mo., U.S.A.); L-histidine free base (Hd), L-3-methylhistidine (3-Me-Hd), imidazolepyruvic acid HCl (IPA) and imidazolelactic acid monohydrate (ILA), from Calbiochem (San Diego, Calif., U.S.A.); sulfanilic acid, formic acid, *n*-butanol and ethyl acetate, from J. T. Baker (Phillipsburg, N.J., U.S.A.); iron(III) chloride, isopropanol and glacial acetic acid, from Wako (Osaka, Japan); uniformly labelled [^{14}C]-L-histidine, from New England Nuclear (Boston, Mass., U.S.A.); all solvents were of analytical-reagent grade; the thin-layer chromatographic equipment and glass tanks were supplied by Scientific Manufacturing Industries (Emeryville, Calif., U.S.A.).

Preparation of cellulose thin layers

Cellulose powder MN 300 (12 g) was suspended in 72 ml of distilled water and homogenized for 3 min in an electric mixer. The slurry was spread over six plates

(20 × 20 cm) at an initial thickness of 250 μm, and the coated plates were allowed to dry overnight before use.

Development of two-dimensional chromatograms

Mixtures of imidazole standards, 1 μl containing 20 nmoles each (except IPA, 30 nmoles), were placed on the thin-layer with a 1-μl micropipet, at a distance of 1.5 cm from the two edges of the layer in the bottom left-hand corner of the plate. The following solvents were used for development. For the first direction: isopropanol–formic acid–water (41:2:9), the development distance being 16 cm and the time taken approx. 3 h; for the second direction: *n*-butanol–ethyl acetate–glacial acetic acid–water (1:1:1:1), the development distance being 15 cm and the time taken approx. 2.5 h.

The tank atmosphere was saturated with the vapor of both freshly prepared solvents for 20–30 min prior to development. After development, the plates were transferred to a fume-hood and the solvents were evaporated off the layer with a stream of air from an electric fan for about 30 min. An undulating yellow-colored band formed along the solvent front during the development in the first dimension. To prevent it from interfering in the second-dimension development, it was necessary to scribe a parallel line just below the yellow band, as described by Jones and Heathcote[10].

Detection reagents

The following detection reagents were used. A 0.2% solution of ninhydrin in acetone and a 0.02% solution of *o*-phthalaldehyde (OPT) in acetone. Pauly's reagent was prepared as described by Von Arx and Neher[11]. Multiple spray procedures using ninhydrin and Pauly's reagent for detecting imidazoles were according to that used by Smith[3].

However, for application to radiotracing studies, a multiple OPT spray procedure without quenching was developed as follows. After two-dimensional chromatographic separation, the plates were transferred to the fume-hood and the electric fan was used for only 5–10 min so that the plates should remain acidic. UA could be scribed first with a pencil under short-wave UV light and then, after spraying with OPT, IAA, ILA and 3-Me-Hd, which were viewed under UV light. The remaining Hd, Hm and IPA could be indicated after spraying with alkaline methanol (2 drops of 10 *N* NaOH per 10 ml) followed again by OPT solution. In none of the above steps was heat treatment needed.

Extraction of tissue imidazoles

Male Sprague-Dawley rats (105–135 g) were killed by decapitation, the right cerebral hemisphere being quickly removed and cut into slices (0.4 mm thick). The slices of the mid-anterior portion, weighing between 95 and 105 mg, were placed in 2 ml of Krebs-Ringer hydrogen carbonate medium[12] containing 0.25 μCi [^{14}C]histidine and which was 0.5 m*M* in histidine, incubated and equilibrated with 95% oxygen–5% carbon dioxide in a Dubnoff metabolic shaking incubator at 37° for 60 min. Finally, brain slices were separated from the medium by filtration with suction, washed twice with distilled water and samples from two rats were pooled in 2 ml of ice-cold acidic methanol (95% methanol–5% 0.1 *N* HCl), then subjected to sonifier

disruption. The homogenates were centrifuged and the volume of the supernatant fraction was reduced by evaporation to 0.4 ml. After recentrifugation, 0.2 ml of the final supernatant, which showed no interference on the chromatograms, was taken for further analysis.

RESULTS AND DISCUSSION

Two-dimensional chromatography on thin layers

We compared many solvent systems for imidazoles on the paper, cellulose and silica gel thin layers reported by other workers[1–9], and found that in general the quality of separation and the effectiveness of detection on a cellulose thin layer were superior to those on a silica gel layer.

The solvent isopropanol–formic acid–water was introduced by Neufeld and Chayen[6] to enable some imidazoles to be separated. Although the separation was generally good, IPA, ILA and Hm as well as 3-Me-Hd and Hd overlapped each other to a certain extent (Fig. 1).

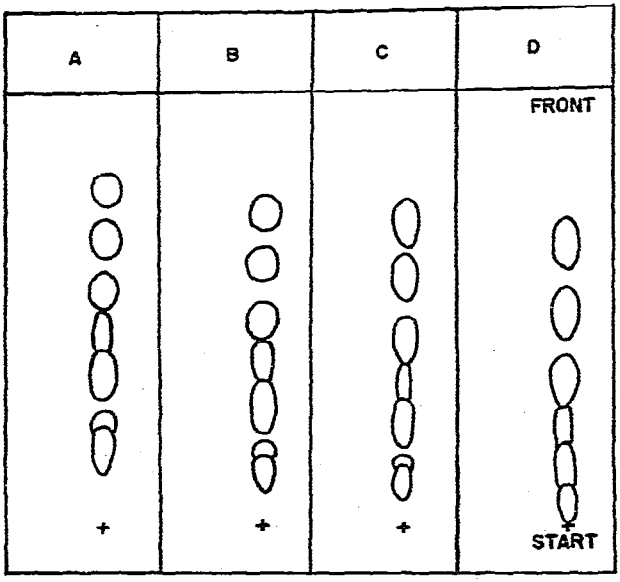

Fig. 1. Chromatography of imidazoles on cellulose thin layer. Solvent: isopropanol–formic acid–water; A, 40:2:10; B, 41:2:9; C, 42:2:8; D, 44:2:6. Development distance 16 cm. Spray reagents, ninhydrin followed by Pauly's reagent. Imidazole standard mixtures containing 10 nmoles each (except IPA, 15 nmoles) were used. The spots from top to bottom in order were UA, IAA, ILA, IPA, Hm, 3-Me-Hd and Hd.

To avoid edge-phenomenon interference[13] by UA in the second direction of development, modifications of the original volume ratio of the solvent isopropanol–formic acid–water (40:2:10) were tried as follows, 41:2:9, 42:2:8 and 44:2:6. Decreasing R_F values for all spots occurred (Fig. 1), and with the last two solvents some tailing appeared. However, with the solvent that had a ratio of 41:2:9, it was possible

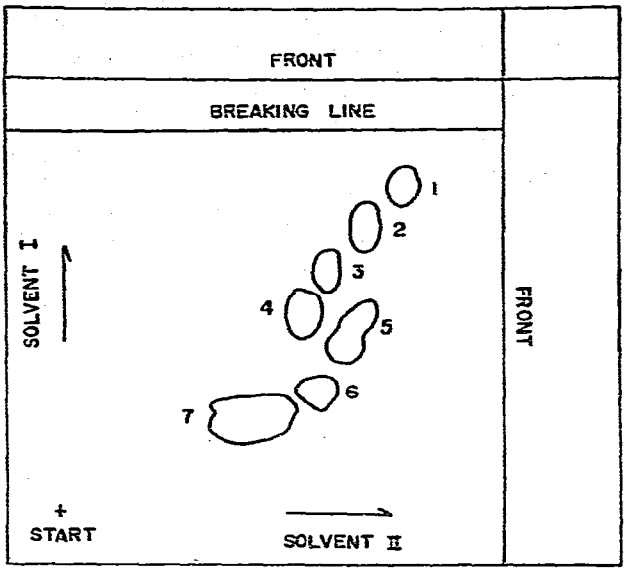

Fig. 2. Two-dimensional chromatography of imidazoles on cellulose thin layer. Solvents, 1st dimension: isopropanol–formic acid–water (41:2:9); 2nd dimension: *n*-butanol–ethyl acetate–glacial acetic acid–water (1:1:1:1). Development distance, 16 cm for 1st dimension and 15 cm for 2nd dimension. Spray reagents as for Fig. 1. Spots 1 to 7 were UA, IAA, ILA, IPA, Hm, 3-Me-Hd and Hd in that order.

to maintain good separation and also to reduce the development time by about 30 min. Therefore, this volume ratio was chosen for the development in the first direction.

Use of the modified isopropanol solvent combined with the *n*-butanol solvent enabled Hd and its six metabolites to be completely separated (Fig. 2). Both Figs. 1 and 2 are xerox copies of the original chromatograms and show the actual sizes of the spots. To our knowledge, this is the first report to appear in which it is shown that these seven imidazoles could be separated on a single thin-layer chromatoplate. Carnosine and 1-Me-Hd partly overlapped with Hd on the chromatogram, but 1-Me-Hd was found to be clearly separated from other imidazoles by using the modified isopropanol solvent and *p*-dioxan-*n*-butanol–water–formic acid (40:40:15:5). The modified isopropanol solvent could be used repeatedly, but the *n*-butanol solvent should be freshly prepared or streaking of Hm will occur.

OPT had previously been used for detecting Hm and some imidazoles was used before by other workers[5,14,15]. In our modified OPT spray procedure, based mainly on the fluorescence intensity, depending on the pH, seven imidazole compounds could quickly and accurately be located, which was confirmed by continuing to spray with ninhydrin and Pauly's reagent[15]. The fluorescent spots were orange–yellow, except for Hd and Hm, which were blue. The fluorescence of IPA with OPT was relatively weak, but it could be intensified by pre-alkalinization of the chromatoplates and increasing the amount in standard mixtures. Trace amounts of secondary components with lower R_F values were observed when using the *n*-butanol solvent for Hm and IPA.

In our preliminary tests, solvents containing ammonia or *p*-dioxan, especially the latter, interfered with the OPT fluorogram. Our preliminary studies also showed

that R_{Im} values of imidazoles were more constant than values expressed by R_F for both solvents. The multiple OPT spraying did not cause quenching in a following radiometric assay.

Biological application

The metabolism of ^{14}C-Hd in rat-brain slices is shown in Table I. Most of the ^{14}C-Hd taken up by tissue was unmetabolized (93.8%), IAA was next (3.8%) and then other metabolites (less than 1%). There was no significant radioactivity of 3-Me-Hd. In rat or mouse brain, only a very small proportion of the added labelled amino acids was converted into carbon dioxide and incorporated into protein[16,17], and could be neglected in our studies.

In the heat-treatment control (brain slices were boiled for 10 min prior to incubation), only ^{14}C-Hd could significantly be detected. Its radioactivity was much less than found in native brain slices and showed that there was active uptake of Hd by native brain slices. It is of interest to note that IAA is the most abundant metabolite of Hd (Table I), which suggests that IAA may be the only stable and end metabolic product of Hd in rat brain. This finding seems to confirm that in rat brain a large pool of endogenous IAA may exist arising from Hd, as proposed by Snyder and coworkers[18,19]. Details of these radiometric assays for Hd metabolism and their implications will be presented elsewhere.

The two-dimensional chromatography on cellulose thin layers described above is simple and has good reproducibility. The multiple OPT spray method is rapid and sensitive compared with the method of detection by conventional autoradiography. Therefore, the methods presented are useful for the study of imidazole compounds and histidine metabolism.

TABLE I

CONCENTRATION AND DISTRIBUTION OF RADIOACTIVITY OF LABELLED HISTIDINE AND ITS METABOLITES IN RAT-BRAIN SLICES

Rat-brain slices (95–105 mg) were incubated in Krebs-Ringer hydrogen carbonate medium (pH 7.4) containing 0.25 μCi of ^{14}C-Hd and which was 0.5 mM in Hd at 37° for 60 min. The tissue was extracted with acidic methanol. Final concentrated aliquots, 4 μl containing about 1700 cpm, were co-chromatographed with seven imidazole standards. The spots were detected by multiple OPT spraying, scraped off and suspended in 0.2 ml of distilled water, then counted in 10 ml of modified Bray's fluor solution[9] by liquid scintillation spectrometer. The counts were corrected for background and losses, as determined by measuring the recovery of known amounts of labelled histidine subjected to the analytic procedures. The recovery was 75.4 ± 1.5% for six determinations.

Imidazole	*Radioactivity** *(dpm per 100 mg)*	*Total radioactivity** (%)*
Hd	108,473 ± 3520	93.76 ± 0.68
Hm	844 ± 89	0.73 ± 0.11
IPA	705 ± 102	0.64 ± 0.08
ILA	436 ± 74	0.43 ± 0.07
IAA	4192 ± 553	3.76 ± 0.47
UA	888 ± 81	0.84 ± 0.08

* Results are given for fresh brain tissue; values represent mean ± S.E. of eight determinations. In heat-treatment control, the radioactivity of Hd was 11,455 ± 309 dpm per 100 mg of fresh tissue for four determinations, no other metabolites could significantly be detected.

** Values are the mean ± S.E.

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