

Thin-Layer Chromatographic Separation of Pineal Gland Derivatives of Serotonin-¹⁴C

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Metabolism of serotonin (5HT) by the pineal gland and liver results in the formation of *N*-acetylserotonin (NAS), hydroxyindoleacetic acid (HIAA), and hydroxytryptophol (HTOH) (1,2). In addition to these compounds, several others have been reported to be formed by pineal gland extracts by the unique enzyme hydroxyindole-*O*-methyl transferase (2,3). This enzyme *O*-methylates the above compounds to form methoxy *N*-acetylserotonin (melatonin) (MEL), methoxyindoleacetic acid (MIAA), methoxytryptophol (MTOH), and methoxyserotonin (5MT) (2). Although a variety of chromatographic techniques are available for the separation of some of these compounds, a single system has not been found satisfactory. Previous methods have involved preparative organic extractions followed by chromatographic separations using a variety of combinations of TLC adsorbants, paper, and different solvents in order to separate NAS from HTOH, MIAA from HIAA, and MTOH from MEL and MIAA (4-7).

A two-dimensional thin-layer chromatography system which accomplishes these separations has been developed. This chromatographic method is used to separate trace amounts of ¹⁴C-indoles (1 picomole/10 μ l) formed by pineal glands incubated with serotonin-¹⁴C in organ culture.

MATERIALS AND METHODS

Thin-layer glass plates precoated with Merck-type silica gel, 0.25 mm layer (Brinkman Instrument Co.) have provided reproducible results. The Brinkman plates are activated by drying for 60-90 minutes at 100-115°C; plates are cooled in a desiccator. Routine chromatography is performed on a 10 \times 20 cm plate; acceptable separations can be obtained with a 5 \times 20 cm plate.

Standard compounds (Regis Chemical Co.) are dissolved in a solution of ethanol: 1% (w/v) ascorbic acid in 0.1 N HCl (1:1). The ascorbic acid/HCl solution is prepared every 2-3 weeks and stored at 4°. Fresh standards are prepared weekly in 10 mM stock solutions and stored at -10°. A millimolar standard mixture is made up each day from the 10 mM stock solutions. For chromatography, a sample of 10 μ l of incubation medium from an incubation of two to six pineal glands in 0.5 ml of medium is used. Final concentration of serotonin-¹⁴C (46 mc/mmole, Nuclear-Chicago) is 0.05-0.125 mM. The aliquot containing serotonin-¹⁴C and metabolites of serotonin-¹⁴C is diluted with an equal volume (10 μ l) of the standard carrier mixture containing 5HT, NAS, MEL, HTOH, HIAA, MTOH, MIAA, and 5MT (approximately 2 μ g of each). This 20 μ l sample-standard mixture is applied to the gel under a continual gentle stream of nitrogen to form a 4-5 mm spot. Plates cannot be stored and exposure to room air is kept to a minimum to prevent oxidation of the sensitive indole compounds.

Chromatographs are developed in darkness in solvent A (chloroform/methanol/glacial acetic acid, 93.7:1) along the long dimension. Plates are rapidly dried with nitrogen and immediately placed again in the darkened developing tank for a second development with solvent A. The plates are dried with nitrogen and immediately placed in solvent B (ethyl acetate) for development in darkness in the second direction. The entire procedure takes three to four hours.

The plates are sprayed with methanol/12.5 M HCl (1:1) for fluorescent visualization of the hydroxyindole and methoxyindole compounds using ultraviolet light. Upon exposure to air, chromatographed standards will become oxidized as evidenced by the appearance of colored products in the areas identified by fluorescence.

The separated metabolites of serotonin are carefully scraped from the glass plates, pulverized to a fine powder between glass, and placed in counting vials with 0.6 ml of NCS (Amersham Searle). Then 10 ml of scintillation fluid is added and the sample counted by standard liquid scintillation techniques. Quenching is uniform and results in efficiency of 50-55%.

RESULTS AND DISCUSSION

A tracing of a typical chromatogram appears in Figure 1. Examination of the tracing reveals excellent separation of (1) MIAA from HIAA, (2) MEL from MIAA and MTOH, and (3) NAS from

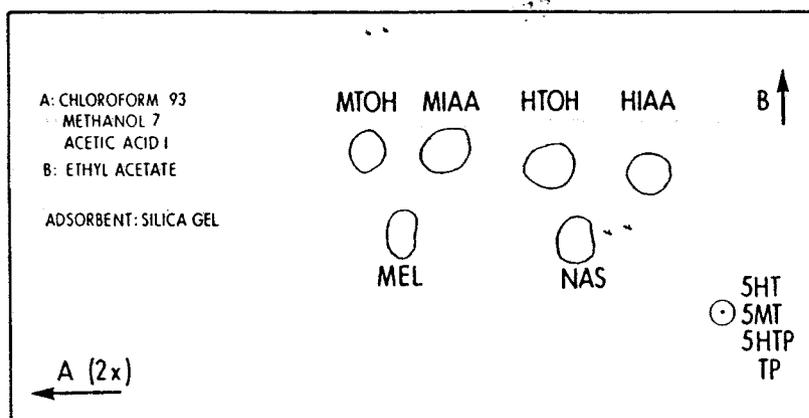


FIG. 1. Tracing of chromatographic separation of pineal indole compounds. Tryptophan (TP), hydroxytryptophan (5HTP), serotonin (5-hydroxytryptamine) (5HT), hydroxyindoleacetic acid (HIAA), hydroxytryptophol (HTOH), methoxytryptophol (MTOH), methoxyindoleacetic acid (MIAA), *N*-acetylserotonin (NAS), methoxy *N*-acetylserotonin (melatonin) (MEL), methoxyserotonin (5MT). Actual size of chromatographic plate is 10 × 20 cm.

HTOH. Solvent A is a modification of a solvent system described by Wurtman *et al.* (6). The addition of one part glacial acetic acid results in complete separation of MIAA from HIAA using silica gel. Without this modification, these compounds did not separate. Solvent B described by Tyce *et al.* (4) for similar use separates HTOH from NAS and improves the separation of MEL from MIAA and

TABLE 1

Separation of Labeled Metabolites of Serotonin-¹⁴C Formed by Pineal Glands during 24-Hour Organ Culture^a

| Chromatographic region | cpm in chromatographically separated areas |
|------------------------|--|
| Origin (5HT,5MT) | 40000 ± 2000 |
| NAS | Not detectable |
| MEL | 50 ± 4 |
| HIAA | 2000 ± 350 |
| HTOH | 630 ± 13 |
| MIAA | 30 ± 5 |
| MTOH | 100 ± 20 |

^aFour individual organ cultures containing two glands each were used in this experiment. Each value above represents the mean ± S. E. of four determinations of the radioactivity associated with standards separated by the thin-layer chromatographic technique described. All values are corrected for the background activity of unchromatographed silica gel prepared as detailed above. In this study, a total of 43000 ± 2000 cpm was applied. Serotonin-¹⁴C was 5 μc/ml (46 mc/mmmole).

MTOH. The small amount of acid which remains on the gel from the first solvent development enhances the effective separation by solvent B. The 5HT and precursors of 5HT, tryptophan and hydroxytryptophan, all remain at the origin as does 5MT.

When unincubated medium containing serotonin-¹⁴C is chromatographed, 98–100% of the activity is recovered from the plate. It is difficult to determine the recovery of metabolites of serotonin-¹⁴C since the serotonin metabolites are not available as radioactive compounds. However, 98–100% of the radioactivity in medium incubated with pineal glands is recovered in the areas associated with added standard serotonin and serotonin metabolites (Table 1). When the remaining gel is scraped off the plate no radioactivity is found. This chromatographic system should provide an important tool to investigators interested in indole metabolism by the pineal gland.

SUMMARY

A two-dimensional TLC method has been developed which separates *N*-acetylserotonin, hydroxytryptophol, hydroxyindoleacetic acid, methoxyindoleacetic acid, methoxytryptophol, and melatonin.

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