SEPARATION OF CATECHOLAMINES ON THE PHOSPHO-CELLULOSE COLUMN

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Abstract: Metanephrine, adrenaline (together with tyramine and normetanephrine) and noradrenaline (together with dopamine) were separated by a simple chromatographic procedure on a phospho-cellulose column. The elution was carried out with 0.025M phosphate buffer (pH 6.2) containing EDTA. The method was successfully applied to neutralized perchloric acid extract of biological material. Separations of other amines (tryptamine, serotonin, histamine, agmatine, putrescine, cadaverine, spermidine and spermine) and basic amino acids (lysine, histidine and arginine) also were achieved utilizing the same column.

The authors previously reported that 5-HT*, H, Cad (with Put), Spd and Spm were separated from each other by a P-cellulose column chromatography (1). In the chromatography, catecholamines, TrA and basic amino acids were found to be eluted together in the same fraction. The present study was undertaken to separate these substances. Separation was possible in some cases, in particular, A from NA on a longer column.

MATERIALS AND METHODS

Materials (P-cellulose, reagents, amines and amino acids) and the procedure of extraction of amines from tissues were the same as described previously (1). P-cellulose column (1 x 22 cm) was equilibrated with 0.01 M phosphate buffer (pH 6.2) containing 0.25 mM EDTA. The elution buffers were exchanged stepwise as follows;
1) 0.01 M phosphate buffer (pH 6.2) containing 0.25 mM EDTA,
2) 0.025 M phosphate buffer (pH 6.2) containing 0.25 mM EDTA,
3) 0.1 M phosphate buffer (pH 6.2) containing 0.25 mM EDTA,
4) 0.1 M phosphate buffer (pH 7.5),
5) 0.1 M borate buffer (pH 9.0) containing 0.3 M NaCl,
6) 0.1 M borate buffer (pH 9.0) containing 0.6 M NaCl.

The methods for assay of amines and amino acids used in the present study were: a) Measurements of native fluorescence for catecholamines and indole amines, b) Trihydroxyindole (THI)-method by Häggendal (2) for A and NA, c) THI-method by Anton

* Abbreviations: phospho-cellulose (P-cellulose), adrenaline (A), noradrenaline (NA), metanephrine (M), normetanephrine (NM), tyrosine (Tyr), tyramine (TyA), dopa (D), dopamine (DA), tryptophan (Trp), tryptamine (TrA), serotonin (5-HT), histamine (H), agmatine (Agm), putrescine (Put), cadaverine (Cad), spermidine (Spd), spermine (Spm), lysine (lys), histidine (His), arginine (Arg).
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TABLE 1. Sensitivities of assays for amines.

<table>
<thead>
<tr>
<th>Amine</th>
<th>Concentration* of reaction mixture</th>
<th>F.I.** of reaction (blank)</th>
<th>Method for assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.25 nmoles/3 ml</td>
<td>100</td>
<td>THI-method</td>
</tr>
<tr>
<td>NA</td>
<td>0.25 nmoles/3 ml</td>
<td>100</td>
<td>THI-method</td>
</tr>
<tr>
<td>DA</td>
<td>0.25 nmoles/3 ml</td>
<td>70</td>
<td>THI-method</td>
</tr>
<tr>
<td>5-HT</td>
<td>0.5 nmoles/3 ml</td>
<td>85</td>
<td>native fluorescence</td>
</tr>
<tr>
<td>H</td>
<td>0.5 nmoles/3 ml</td>
<td>80</td>
<td>OPT-method</td>
</tr>
<tr>
<td>Spd</td>
<td>25 nmoles/3.5 ml</td>
<td>0.40</td>
<td>TNBS-method</td>
</tr>
<tr>
<td>Spm</td>
<td>25 nmoles/3.5 ml</td>
<td>0.25</td>
<td>TNBS-method</td>
</tr>
</tbody>
</table>

* Each concentration is in the range of linear relationship with fluorescence intensity or absorbance.
** fluorescence intensity

The mixture of Tyr, M, TyA, A, NA and DA was subjected to a P-cellulose column chromatography. Amines in the eluate were detected by the native fluorescence (Fig. 1-a). TyA and DA were eluted together with A and NA, respectively. The aliquots of eluate of Fig. 1-a were subjected to specific THI-reactions for A, NA and DA (Fig. 1-b). The results showed A and NA to be completely separated. A, NA and others did not interfere with the assay of DA (1, 3). Fig. 1-c shows the elution patterns of D, M and NM.

Fig. 2-a and 2-b show the elution patterns of catecholamines in the extracts of rat adrenal gland and brain, respectively. In several experiments, the amounts of A and NA in the adrenal glands of rats (approx. three months old, females) were 2.3–3.5 μmoles/g tissue and 0.33–0.38 μmoles/g tissue, respectively. The value of A content was in good agreement with that estimated from the native fluorescence. The low content of DA (0.01–0.06 μmoles/g tissue) also was detectable in a single tissue. Similar values for A (2.6 μmoles/g) and DA (0.03 μmoles/g) were reported by A. Cession-Fassion and R. Vandermeulen (6), but the value for NA (1.6 μmoles/g) as stated by these authors appears very high. The chromatographic patterns shows that the ratio of A to NA is approx. 10 to 1. In rat brain, A was not detected. Other amines (NA, DA, 5-HT, H, Spd and Spm) that were determined by the methods outlined in the legend of Fig. 3 were on the same levels as described previously (1).
The mixture of each 0.05 ml of standard solution of amines (1 μmole/ml of 0.01 M acetate buffer, pH 5.0) was added to 3 ml of buffer-1, then the mixture was applied to a P-cellulose column (1 x 22 cm) equilibrated with buffer-1. The elution was carried out with buffer-1 (from fraction 1 to 10) and buffer-2 (from fraction 11 to 40). The flow rate was 1 ml per min and fractions of 3 ml per tube were collected.

a) and c) Elution patterns obtained by the native fluorescence of fractions (285,315 nm, not corrected).

b) Elution patterns obtained by THI-method. Aliquots (0.1 ml) of fractions of Fig. 1-a were added to 2 ml of buffer-2 and subjected to both THI-reactions, (● ●): A and NA, (× ×): DA.

* F.I.: Fluorescence intensity

Fig. 3 shows separation of many other amines and basic amino acids on a P-cellulose column to be the same size as in Figs. 1 and 2. Results obtained by a short column (1 x 11 cm) (1) have been reported. His, Lys, Arg and TrA, that were eluted together in the same fraction by the short column, were separable by a longer column. 5-HT and H
were eluted stepwise with buffers of high ionic strength and pH. Agm, Put (together with Cad), Spd and Spm were eluted separately with borate buffers of different NaCl concentrations.

Fig. 2. Separation of catecholamines in rat adrenal glands and brain.

Elution procedure was the same as described in the legend of Fig. 1.

a) A single adrenal gland (approx. 20 mg) was homogenized in 2 ml of 0.4 N HClO₄ containing 2 mM EDTA. After centrifugation of the homogenate, the neutralized supernatant (2 ml) was applied to a P-cellulose column (1 x 22 cm). Aliquots (0.2 ml) of fractions were added to 2 ml of buffer-2 and then subjected to the THI-reaction for A and NA (○—○). Aliquots (2 ml) of fractions were subjected to the THI-reaction for DA (×—×).

b) The neutralized perchloric acid extract (4 ml) from a single rat brain was applied to a P-cellulose column (1 x 22 cm). Aliquots (2 ml) of fractions were subjected to the THI-reaction for A and NA (○—○). Aliquots (1 ml) of fractions were added to 1 ml of buffer-2 and then subjected to the THI-reaction for DA (×—×).

In Fig. 2-a and b, ○—○ shows the native fluorescence (303/335 nm) for 5-HT. F.I.: fluorescence intensity
FIG. 3. Elution patterns of several authentic amines and amino acids except for catecholamines.

A mixture (3.2 ml) of standard solutions of amines and amino acids in 0.01 M acetate buffer (pH 5.0) was applied to P-cellulose column (1 x 22 cm) equilibrated with buffer-1. The amounts of amines and amino acids applied to the column and the methods for detection were as follows: Trp, TrA, 5-HT : 5 nmoles, native fluorescence; His, Lys, Arg, Put, Cad : 0.3 µmoles, TNBS-method; H : 2.5 nmoles, OPT-method; Agm, Spd, Spm : 0.15 µmoles, TNBS-method. In TNBS-method, His, Lys, Arg and Agm (1 ml of fractions) were assayed after adjustment of pH (9.0) by addition of 2 ml of 0.2 M borate buffer (pH 9.0) and Put, Cad, Spd and Spm (3 ml of fractions) were assayed without pH adjustment. In the assay of H, 1 ml of each fraction was added to 2 ml of buffer-4 and subjected to the OPT-method. The values obtained converted to those for 3 ml of fractions. Flow rate : 1 fraction = 3 ml/3 min. F.I. : fluorescence intensity (••••). A420 : absorbance at 420 nm in TNBS-method (○○○○).

DISCUSSION

It has always been difficult to assay A and NA in the extract containing both amines, as they have similar fluorescence in the fluorometric assay (2) and separation is difficult.

Strong acidic cation exchange resins such as Amberlite CG-120 (7, 8) and Dowex-50 (9) have been used for the separation of catecholamines. Häggendal reported the separation of A, NA, NM, M and DA by Amberlite CG-120 column (7). The method of separating A from NA required much time, however. Usually, the estimation of A and NA in the sample has been carried out by differential fluorometry (2, 10), paper chromatography of the acetylated derivatives (11) after some purification by ion-exchange resins or alumina etc.

P-cellulose made possible separation of many amines by a simple chromatographic procedure. The authors simplified the procedures for assay of some amines (1). In our method, Agm and Spd that interfere with the assay of H in the fluorometry (12), were separated from H. These successful results obtained from experiments on rat brains and adrenals suggest a wider application of the method to amines of biological interest.
REFERENCES

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