A CHROMATOGRAPHIC SEPARATION OF THE DIFFERENT FORMS OF VITAMIN B₆

HANS-GÖRAN TISELIUS

Department of Physiological Chemistry, Chemical Centre, University of Lund, Lund (Sweden)
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SUMMARY

A procedure is described in which pyridoxine, pyridoxal, pyridoxamine, pyridoxine 5'-phosphate, pyridoxal 5'-phosphate, pyridoxamine 5'-phosphate, and 4-pyridoxic acid are separated on a column of Dowex AG 50 W-X 8. The column is eluted first with a 0.01 M ammonium formate buffer, pH 3.2, followed by a combined pH-concentration gradient. The method has been used for the separation of vitamin B₆ in perchloric acid extracts of liver, and in a micro modification for the separation of vitamin B₆ in rat cerebrospinal fluid.

INTRODUCTION

In the course of a study on the metabolism of vitamin B₆ in animals and humans we needed a chromatographic procedure for the complete separation of the different forms of the vitamin. The present communication describes a procedure for chromatography on Dowex AG 50 W-X 8 with ammonium formate buffers for elution, which fulfills these requirements.

MATERIALS AND METHODS

Chemicals

Pyridoxamine hydrochloride, pyridoxal hydrochloride, and pyridoxamine 5'-phosphate* were gifts from Merck Sharp and Dohme, Rahway, N.J. Pyridoxine 5'-phosphate was purchased from Calbiochem, Luzern, Switzerland. Pyridoxine hydrochloride and pyridoxal 5'-phosphate were purchased from E. Merck AG, Darmstadt, Germany and 4-pyridoxic acid from Sigma Chemical Company, Missouri. [3H₆]Pyridoxine (1 Ci/g) was obtained from The Radiochemical Centre, Amersham, Bucks, Eng-

* Nomenclature of vitamin B₆ is that recommended by the IUPAC-IUB Commission on Biochemical Nomenclature.
The synthesis of [3H]pyridoxine 5'-phosphate (3.6 Ci/g) will be described elsewhere (Tiselius, in preparation). Dowex AG 50 W-X 8 (hydrogen form) was obtained from BioRad Laboratories Inc., Richmond, California.

**Chromatographic procedures**

A chromatographic glass tube with an internal diameter of 9 mm was filled to a height of 400 mm with Dowex AG 50 W-X 8 (200-400 mesh) in 0.01 M ammonium formate, pH 3.2. The sample to be chromatographed was dissolved in 1-10 ml of water or in 0.01 M ammonium formate, pH 3.2 and carefully applied onto the column. The sample was rinsed into the column and elution started with 200 ml of 0.01 M ammonium formate, pH 3.2. The column was then connected to a 500-ml flask containing 100 ml of 0.05 M ammonium formate, pH 4.25. This solution was stirred with a magnetic stirrer. A 0.5 M ammonium formate buffer, pH 7.5, was added at a rate of 0.5 ml/min through a piece of polyethylene tubing drawn through the stopper. Fractions of 5-ml volume were collected at the same flow-rate.

For work in micro-scale, the following procedure was used. A glass tube with an internal diameter of 4 mm and a height of about 250 mm was connected at one end to a polyethylene tubing PE 160 (Clay Adams, Inc., New York, N.Y.) with a diameter of 0.045 inches. The flow rate could be adjusted by slight bending of this tubing. The glass tube was filled with Dowex AG 50 W-X 8 (200-400 mesh) in 0.01 M ammonium formate, pH 3.2 to a height of 200 mm. The sample, in a volume of less than 0.5 ml, was applied onto the column which was then eluted with 10 ml of 0.01 M ammonium formate, pH 3.2, after which the gradient was started. In this case the first flask had a volume of 100 ml and contained 10 ml of 0.05 M ammonium formate, pH 4.25, and 0.5 M ammonium formate at pH 7.5 was added at a rate of about 0.05 ml/min.

Regeneration of both types of columns was performed with 0.01 M ammonium formate, pH 3.2, until pH in the eluate was 3.2.

**Analytical procedures**

Conductivity was determined with a Philoscope type PR 9500/01 (Philips, Eindhoven, Holland). Absorbance was measured in a Zeiss spectrophotometer PMQ II at a wavelength of 295 nm. Fluorescence of 4-pyridoxic acid was determined in a Bearn spectrophotofluorometer (Jobin & Yvon, Arceuil, France) at 425 nm, with an activating wavelength of 325 nm. Isotope determinations were performed in a Packard Tri-Carb liquid scintillation spectrometer, model 3375 (Packard Instrument Company, Inc., La Grange, Ill.). pH was measured in a pH-meter 22 (Radiometer A/S, Copenhagen, Denmark).

**Preparation of tissue extracts**

Perchloric acid extracts of mouse liver were prepared as described in detail elsewhere. Rat cerebrospinal fluid was obtained by puncture of the posterior atlanto-occipital membrane.

**RESULTS**

The chromatogram obtained when 1 mg of pyridoxine, pyridoxine-\(P\), pyridoxal, pyridoxal-\(P\), pyridoxamine, and pyridoxamine-\(P\) were chromatographed on a 400-
Fig. 1. Separation of vitamin B₆-forms on a column, height 400 mm, diameter 9 mm, of Dowex AG 50 W-X 8, (200–400 mesh). The column was eluted with ammonium formate buffers of increasing pH and concentration as described in the text. In the chromatogram the compounds appear in the following order: pyridoxal 5'-phosphate, pyridoxine 5'-phosphate, pyridoxamine 5'-phosphate, pyridoxal, pyridoxine and pyridoxamine. One mg of each compound had been applied to the column. The concentration in the effluent was determined by measuring the absorbance at 295 nm.

Fig. 2. Separation of pyridoxal 5'-phosphate, pyridoxine 5'-phosphate, 4-pyridoxic acid, and pyridoxamine 5'-phosphate (1 mg of each) on a column, height 400 mm, diameter 9 mm, of Dowex AG 50 W-X 8 (200–400 mesh). The column was eluted with ammonium formate buffers of increasing pH and concentration. UV absorbance at 295 nm (●—●), fluorescence at 425 nm, activating light 325 nm (4-pyridoxic acid) (□—□).

mm column of Dowex AG 50 W-X 8 is shown in Fig. 1. Pyridoxal-P and pyridoxine-P were eluted as separate peaks with the starting buffer. The gradient forming arrangement resulted in a S-shaped pH and concentration gradient between 200–450 ml of effluent. At the onset of the gradient a slight decrease in pH was regularly observed. Pyridoxamine-P appeared at about 275 ml of effluent followed by well separated peaks.
of pyridoxal, pyridoxine and pyridoxamine. With this procedure 4-pyridoxic acid was eluted immediately before, but well separated from pyridoxamine-P as seen in Fig. 2.

**TABLE I**

<table>
<thead>
<tr>
<th></th>
<th>Applied onto the column</th>
<th>Recovered in the eluate</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridoxine-P</td>
<td>22 700</td>
<td>24 800</td>
<td>109</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>73 000</td>
<td>70 600</td>
<td>97</td>
</tr>
</tbody>
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Fig. 3. Chromatography of a perchloric acid extract of a liver homogenate from a mouse injected with [3 H₈]pyridoxine. The chromatographic procedures as in Fig. 1. The isotope content in the fractions was determined by liquid scintillation counting. Pyridoxal 5'-phosphate (A), pyridoxine 5'-phosphate (B), pyridoxamine 5'-phosphate (C), pyridoxal (D), and pyridoxine (E).

Fig. 4. Chromatography of 50 µl of rat cerebrospinal fluid on a column, height 200 mm, diameter 4 mm, of Dowex AG 50 W-X 8 (200-400 mesh). The column was eluted with ammonium formate buffers of increasing pH and concentration. The fraction volume was about 0.4 ml. The isotope content in the fractions was determined by liquid scintillation counting. Pyridoxal 5'-phosphate (A), pyridoxine 5'-phosphate (B), pyridoxamine 5'-phosphate (C), pyridoxal (D), and pyridoxine (E).

Recovery

The recovery was estimated from an experiment in which $[^3]$H]pyridoxine-P and $[^3]$H]pyridoxine were chromatographed, Table I.

Application to biological material

Fig. 3 illustrates the result obtained with a perchloric acid extract of the liver from a mouse which had been injected with $[^3]$H]pyridoxine 5 min before decapitation. Well separated peaks were obtained of pyridoxal-P (A) pyridoxine-P (B) pyridoxamine-P (C) pyridoxal (D) and pyridoxine (E). The micro column has been used for instance in the separation of vitamin B$_6$ in rat cerebrospinal fluid after injection of $[^3]$H]pyridoxine. Fig. 4 illustrates the result from an experiment of this type in which 50 µl of cerebrospinal fluid was directly applied onto the column.

Discussion

Different techniques have been used for separation of the different forms of vitamin B$_6$, i.e. ion exchange-chromatography$^{1-6}$, paper chromatography and paper electrophoresis$^{7-11}$, thin-layer chromatography and thin-layer electrophoresis$^{12-14}$, high voltage electrophoresis$^{15}$ and gas chromatography$^{16}$. Many procedures give incomplete separations of all the compounds which are of particular interest in biological work, i.e. pyridoxine, pyridoxal, pyridoxamine, their respective 5'-phosphates and pyridoxic acid.

Several authors have published ion exchange chromatographic techniques$^{1-6}$. Complete separations have usually not been achieved and quantification has in some instances been based on specificity in the analytical procedure, e.g. fluorometry, rather than on complete chromatographic separation$^{3,10}$. These methods are therefore not well suited for metabolic studies with labeled compounds. For studies of this type it is important also that the chromatographic system should not be too sensitive to the presence of inorganic ions or other compounds present in biological extracts as appears to be the case with the method described by Bain and Williams$^2$. The present procedure results in well separated peaks of the different forms of vitamin B$_6$ and pyridoxic acid, none of which is eluted in the front of the chromatogram. It has been found to be well reproducible and to give a recovery around 100%. The fact that it is easily adapted to a very small scale makes it particularly useful in biological studies on small animals.

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References


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