CHROMATOGRAPHIC SEPARATION AND DETERMINATION OF THIAMINE AND THIAMINE PHOSPHORIC ESTERS ON ION-EXCHANGE RESINS

L. DE GIUSEPPE AND G. RINDI
Institute of Human Physiology, University of Pavia (Italy)

INTRODUCTION

Thiamine and its mono-, di- and triphosphoric esters have been separated by paper chromatography employing different mixtures of solvents[1-8], by paper electrophoresis and by means of ion-exchange resins[6]. For such a separation on ion-exchange resins SILIPRANDI AND SILIPRANDI[6] used a weak cationic resin (Amberlite IRC 50), which, in the hydrogen form, allows TTP* to pass down, retards TDP and fixes TMP and T; consequently this procedure does not give a satisfactory separation of TTP and TDP.

We therefore developed a chromatographic method with the aim of obtaining a rapid, easy and complete separation of T and its mono-, di- and triphosphoric esters, which could be applied to biological materials.

EXPERIMENTAL

Principle

A mixture of T and its phosphoric esters was percolated through two superposed columns A and B (Fig. 1) containing a strong anion exchanger in the acetate and in the borate form respectively. TDP and TTP were retained in column A, while column B held TMP firmly and T very loosely. The elution of the substances was carried out with suitable eluents treating each column separately.

MATERIALS

Resin. Dowex-1,X-8, 200–400 mesh (Cl^-) (B.D.H.). The resin was submitted to the series of washings suggested by COHN[9], including the final one with concentrated HCl.

Preparation of the acetate form. A column of resin, purified as described above, was treated with 13% sodium acetate solution until the Cl^- reaction disappeared. The excess of the salt used was eliminated by thorough washing.

Preparation of the borate form. The resin (Cl^-) was treated, under the same conditions as above, with a 4.5% H_3BO_3 solution neutralized (pH 7–7.5) with concentrated NaOH, followed by careful washing with water.

* The following abbreviations have been used: T = Thiamine; TMP = Thiamine monophosphate; TDP = Thiamine diphosphate; TTP = Thiamine triphosphate.

References p. 551.

Thiamine. Thiamine hydrochloride (chloride)*.

TMP. Thiamine monophosphate (chloride)*

TDP. Thiamine diphosphate (chloride)*.

TTP. Thiamine triphosphates separated by column chromatography from a mixture of esters obtained by phosphorylation of T, according to VISCONTINI *et al.*[10]. The details of the method will be published elsewhere. The purity of each substance was checked by paper chromatography, according to SILIPRANDI AND SILIPRANDI[6].

Columns. The Pyrex glass tubes used (inner diam. 8 mm; height 80–90 mm) were fitted with a sintered glass disc, and fused to a ground glass stopcock. Suitable separatory funnels could be attached to the tubes by means of ground glass joints, which also joined the chromatographic tubes together (Fig. 1).

Size of the resin bed: 8 × 15 mm. *Flow rate*: 1 ml/75–90 sec.

METHOD

The columns A and B, containing the resin in acetate and borate form respectively, were superposed as shown in Fig. 1 so that the liquid flowing from A could pass into B.

0.1–2 mg of the mixture under examination, dissolved in a small volume of H_2O, was applied to column A and 24 ml of H_3BO_3 0.03 *M* were percolated through the columns without interruption. The boric acid readily displaced T, while it allowed TMP to remain fixed on column B and TDP and TTP on column A.

The columns were then separated in order to carry out further elution.

24 ml of 0.1 *M* NaCl in 0.1 *M* HCl were percolated through column B for the elution of TMP.

24 ml of 0.02 *M* sodium acetate in 0.04 *M* acetic acid to elute TDP, and 24 ml of 0.1 *M* HCl to elute TTP, were percolated in this order through column A.

Each fraction was collected in a 25-ml volumetric flask. 0.5 ml of 5 *M* HCl was added to the fractions containing T and TDP so that a final concentration of 0.1 *M* HCl was obtained in each flask.

All the volumes were brought to 25 ml with H_2O.

The optical density was measured at 270 mμ (isosbestic point)[11] in a Beckman spectrophotometer, Model DU, in silica cuvettes of 1 cm, against suitable blanks.

In order to check whether the proposed procedure of chromatographic separation allows a quantitative determination of the separated substances, some recovery assays were carried out.

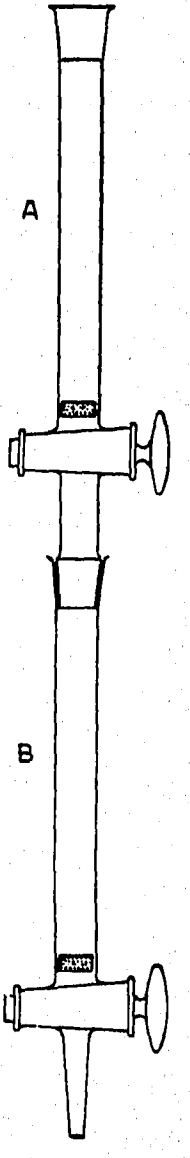

Fig. 1. Arrangement of the columns for the chromatography.

*The authors wish to thank Prodotti Roche, S.p.a., Milano, for the generous gift of these substances.

References p. 551.

TABLE I

RECOVERY OF T, TMP, TDP AND TTP APPLIED SINGLY ON THE COLUMNS AND CARRIED THROUGH THE WHOLE ANALYTICAL PROCEDURE

Added substance	*O.D$_{270}$ of the added solution*	*Eluent*	*O.D$_{270}$ of the eluted solution*	*Recovery %*
T	0.294	0.03 *M* H_3BO_3	0.280	95.2
		0.1 *M* NaCl in 0.1 *M* HCl	0.002*	—
		0.02 *M* Ac·ONa in 0.04 Ac·OH	0.002	—
		0.1 *M* HCl	0.000*	—
TMP	0.210	0.03 *M* H_3BO_3	0.002	—
		0.1 *M* NaCl in 0.1 *M* HCl	0.194*	92.3
		0.02 *M* Ac·ONa in 0.04 *M* Ac·OH	0.002	—
		0.1 *M* HCl	0.002*	—
TDP	0.238	0.03 *M* H_3BO_3	0.002	—
		0.1 *M* NaCl in 0.1 *M* HCl	0.003*	—
		0.02 *M* Ac·ONa in 0.04 *M* Ac·OH	0.230	96.6
		0.1 *M* HCl	0.001*	—
TTP	0.164	0.03 *M* H_3BO_3	0.000	—
		0.1 *M* NaCl in 0.1 *M* HCl	0.003*	—
		0.02 *M* Ac·ONa in 0.04 *M* Ac·OH	0.002	—
		0.1 *M* HCl	0.160*	97.5

* Corrected for the blank.

TABLE II

RECOVERY OF T, TMP, TDP AND TTP ADDED AS A MIXTURE

Mixture	*O.D$_{270}$**	*Eluent*	*O.D$_{270}$ of the eluted fractions*	*Recovery %*
T	0.147	0.03 *M* H_3BO_3	0.144	98
+				
TMP	0.109	0.1 *M* NaCl in 0.1 *M* HCl	0.099**	90.8
+				
TDP	0.115	0.02 *M* Ac·ONa in 0.04 *M* Ac·OH	0.110	95.6
+				
TTP	0.082	0.1 *M* HCl	0.080**	97.5
	0.453		0.433	95.8

* Optical density at 270 mμ of every constituent of the mixture before the chromatographic separation.

** Corrected for the blank.

In a first series of experiments the compounds were applied separately to column A and treated as described above. The results are shown in Table I.

In another series of assays, a suitable mixture of the four compounds was applied to column A and analysed as described under METHOD. The results are shown in Table II.

References p. 551.

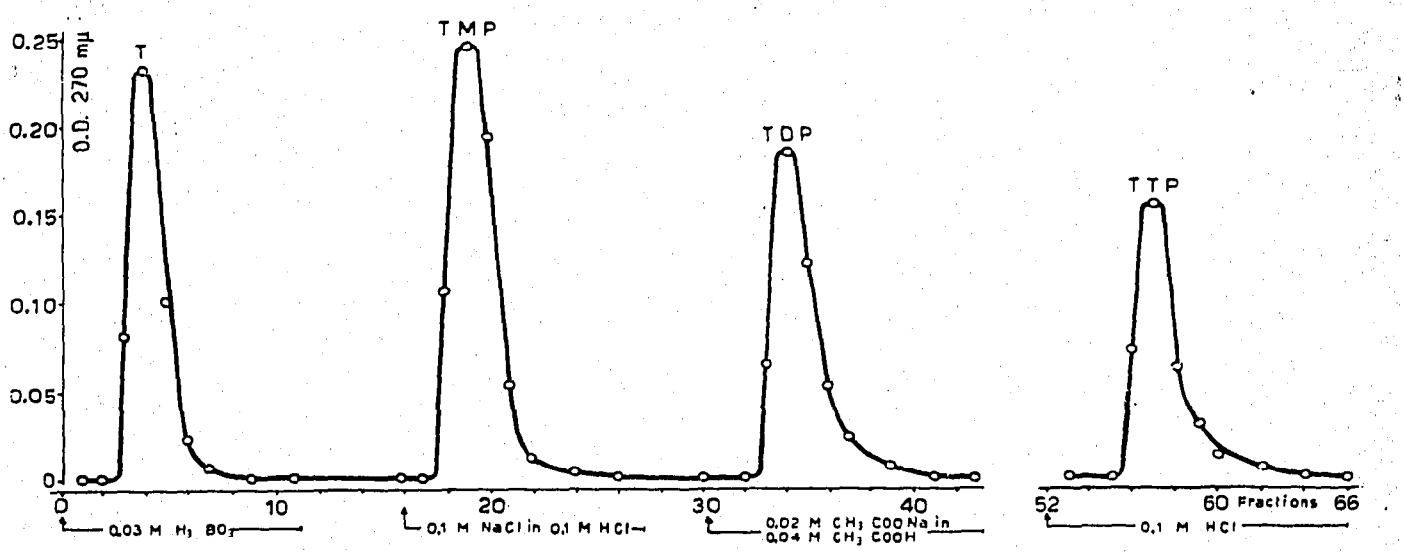

Fig. 2

Fig. 2. Separation of T, TMP, TDP and TTP. The solution applied contained: T 285 μg, TMP 270 μg, TDP 255 μg, TTP 275 μg, in a volume of 2 ml. Volume of the fractions: 1 ml. Readings made on the fractions tenfold diluted with 0.1 *M* HCl.

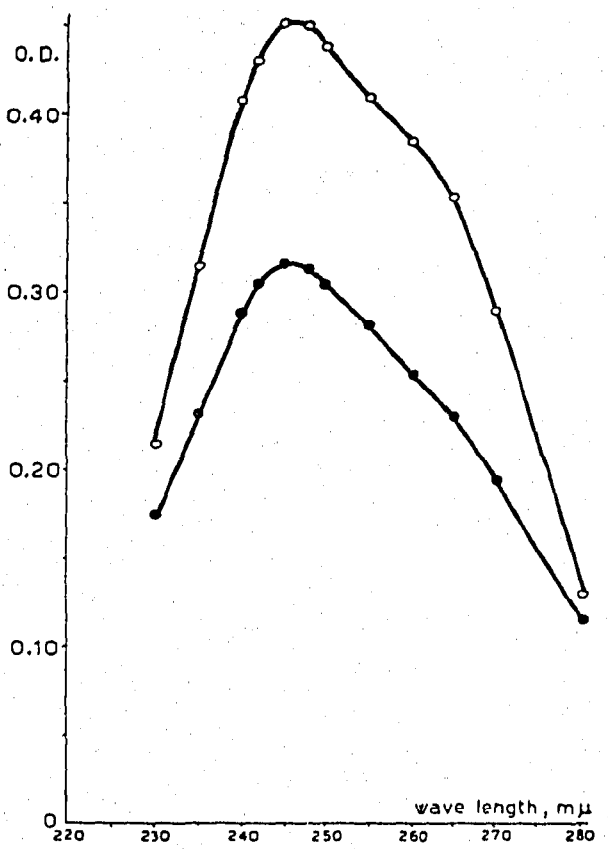

Fig. 3. Absorption spectra of the eluted T (•) and TDP (○) between 230–280 mμ.

References p. 551.

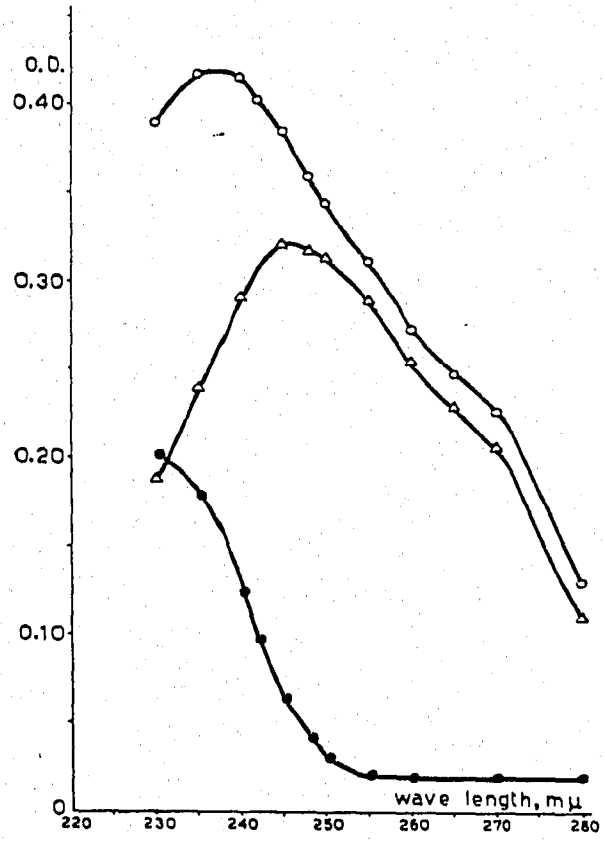

Fig. 4

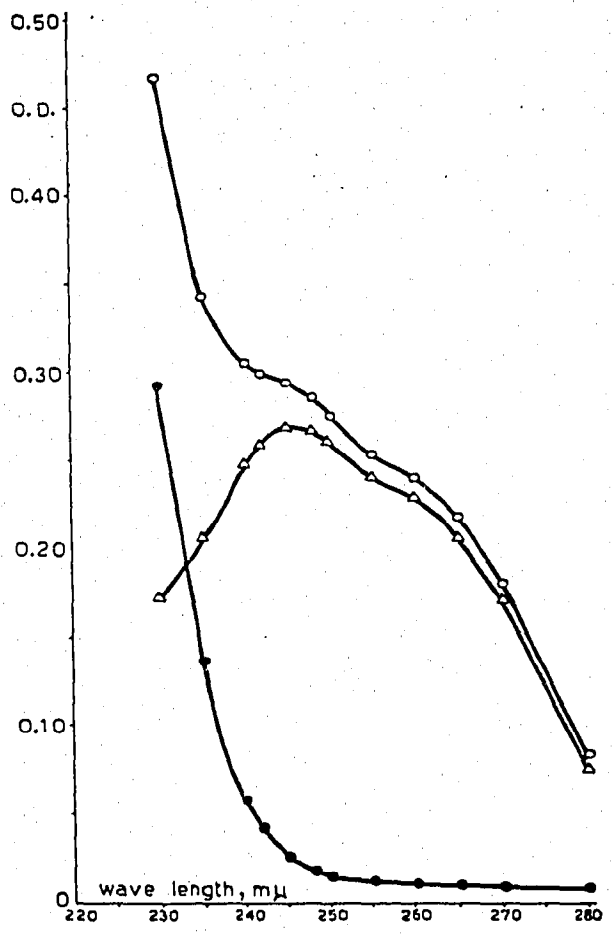

Fig. 5

Fig. 4. Absorption spectrum of TMP (△) between 230–280 mμ. The curve was obtained by deducting the O.D. values of the eluent, percolated through the column (•), from the corresponding O.D. values of the eluted TMP (○).

Fig. 5. Absorption spectrum of TTP (△) between 230–280 mμ. The curve was obtained by deducting the O.D. values of the eluent, percolated through the column (•), from the corresponding O.D. values of the eluted TTP (○).

RESULTS

The diagram in Fig. 2 shows that the separation of T and of its phosphoric esters, under the conditions described above, is sharp and efficient. The results in Table I show that the recoveries of the substances added separately are good, while the results in Table II show that the recoveries of the single components after chromatography of the mixture are of the same order as those obtained with the separate substances, indicating that the analytical procedure is practically quantitative.

Paper chromatography according to SILIPRANDI AND SILIPRANDI[6] of the separate eluted fractions, collected in the cold, lyophylized and taken up in 0.5 ml of HCl 0.05 *N*, showed the presence of a single consituent in each fraction.

Before lyophylization of the TMP fraction, centrifugation was necessary to remove a large amount of sparingly soluble H_3BO_3.

References p. 551.

The U.V. absorption spectrum of the eluates of each fraction was measured in the range between 230–270 mμ, using each eluent as a blank. As shown in Fig. 3, for the fractions T and TDP, the typical absorption spectrum of T was obtained with a maximum close to 245 mμ (pH = 1). However, for the TMP and TTP fractions this did not occur, because the respective eluents displaced not only the T esters, but also the anion bound to the resin (BO_3^{---} and CH_3COO^- respectively), which had a marked influence on the absorption in U.V. light measured against the eluent, as shown in Figs. 4 and 5. In order to obtain the typical T spectrum also for the TMP and TTP fractions, it is sufficient to use a blank obtained by percolating the eluents through a column without adsorbed T esters (Figs. 4 and 5, corrected). However, from the same figures it can be seen that the spectrophotometric measurement at 270 mμ is very little influenced.

DISCUSSION

The advantage of using a strongly anionic instead of a weakly cationic resin lies in the fact that with the former it is possible to fix and then to separate sharply the phosphoric esters of T, which have acid functions. When employing two superposed columns, the selectivity of the method can be increased by preparing the resin in a suitable form. This allows a preliminary separation of the substances, which on further elution with a suitable system of eluents can be made even more efficient.

For the determination of T and its esters in the separate eluates, the absorption in U.V. light at 270 mμ was measured. Similar results were obtained by measuring the thiochrome fluorescence after alkaline oxidation.

Obviously the transformation into thiochrome enhances both the sensitivity and the specificity of the method. This may constitute an important improvement for its application to biological materials, a point which we are now studying.

The method proposed for the separation of T and its mono-, di- and triphosphoric esters can be easily carried out and the time required for a complete analysis is about 3 hours.

While this manuscript was in preparation, SUZUOKI, YONEDA AND HORI[12], published a report in which the resin Dowex-1, X-10 (200–400 mesh) in the formate form, was used for the separation of the di-pentaphosphoric esters of T following the gradient elution technique.

SUMMARY

1. A method for the chromatographic separation of thiamine and its mono-, di- and triphosphoric esters on Dowex columns 1, X-8 (200–400 mesh) was studied, pure solutions being used.

2. The use of two columns containing the resin in borate and acetate form respectively proved to be particularly suitable for the preliminary separation of T and TMP from TDP and TTP.

3. The successive use of 0.03 *M* H_3BO_3 and 0.1 *M* NaCl in 0.1 *M* HCl allowed the elution of T and TMP, respectively, from the first column, while the elution of TDP and TTP was achieved by using 0.02 *M* Na acetate in 0.04 *M* acetic acid and 0.1 *M* HCl respectively.

4. The recoveries of the added substances amounted to 91–97%.

References p. 551.

REFERENCES

1 M. A. SPADONI AND G. TECCE, *Quaderni Nutriz.*, 11 (1950) 26.
2 M. VISCONTINI, G. BONETTI, C. EBNÖTHER AND P. KARRER, *Helv. Chim. Acta*, 34 (1951) 1384.
3 A. ROSSI FANELLI, N. SILIPRANDI AND P. FASELLA, *Science*, 116 (1952) 711.
4 K. H. KIESSLING AND G. LINDHAL, *Arkiv Kemi*, 6 (1953) 271.
5 W. BARTLEY, *Biochem. J.*, 56 (1954) 379.
6 D. SILIPRANDI AND N. SILIPRANDI, *Biochim. Biophys. Acta*, 14 (1954) 52.
7 E. GERLACH, E. WEBER AND H. J. DORING, *Arch. exptl. Pathol. Pharmakol., Naunyn-Schmiedeberg's*, 226 (1955) 9.
8 K. H. KIESSLING, *Acta Chem. Scand.*, 10 (1956) 1356.
9 W. COHN, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. III, Academic Press Inc., New York, 1955, p. 732.
10 M. VISCONTINI, G. BONETTI AND P. KARRER, *Helv. Chim. Acta*, 32 (1949) 1478.
11 J. J. DOHERTY, N. CANE AND F. WOKER, *J. Pharm. and Pharmacol.*, 7 (1955) 1053.
12 J. SUZUOKI, M. YONEDA AND M. HORI, *J. Biochem. (Japan)*, 44 (1957) 783.

Received April 21st, 1958