Journal of Chromatography, 88 (1974) 430–433
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 7082

Note

Electrophoretic separation of vitamin B_{12} derivatives

G. TORTOLANI and P. G. FERRI
Chromatography Laboratory, Quality Control Department, Alfa Farmaceutici S.p.A., Bologna (Italy)
(First received July 6th, 1973; revised manuscript received September 12th, 1973)

Mixtures of vitamin B_{12} derivatives have been analysed by means of column chromatography[1–4] and thin-layer chromatography on silica gel[5], alumina[6] and cellulose[7].

In the course of our investigations into the nature of vitamin B_{12} derivatives in the organs of different animal species[8] and into their photolytic stability, an electrophoretic technique for the separation of cyanocobalamin, hydroxocobalamin, methylcobalamin and cobamamide was developed. This electrophoretic technique also enables these cobalamins to be quantitatively assayed.

EXPERIMENTAL

Apparatus and materials

The tank for thin-layer electrophoresis was obtained from Desaga, Mannheim, G.F.R., and a Beckman DU-2 spectrophotometer was used. Cellogel® cellulose acetate electrophoretic strips and a microsyringe were obtained from Chemetron, Milan, Italy.

Cobalamins

Hydroxocobalamin ($B_{12}OH$) and cyanocobalamin ($B_{12}CN$) of chromatographically and spectrophotometrically pure commercial grade were obtained from Roussel-Uclaf, Paris, France, and methylcobalamin ($B_{12}CH_3$) and cobamamide (DBC) were prepared by partial synthesis in the laboratories of Alfa Farmaceutici S.p.A., Bologna, Italy.

Electrophoresis buffers

The following buffers were used: 0.5 *M* acetic acid (pH 2.9); 0.1 *M* sodium citrate–0.1 *M* hydrochloric acid (pH 4.0); 0.1 *M* sodium citrate–0.1 *M* sodium hydroxide (pH 5.0); 0.1 *M* sodium citrate–0.1 *M* sodium hydroxide (pH 6.0); pH 7.0 buffer according to Sörensen (39 ml of *M*/15 KH_2PO_4 and 61 ml of *M*/15 $Na_2HPO_4 \cdot 2H_2O$); and 0.2 *M* sodium borate–0.1 *M* hydrochloric acid (pH 8.0).

Application of the solutions

The electrophoretic strips were left in the buffer solution for about 30 min before the application of the cobalamins. The strips must be kept wet so as to prevent

changes occurring on the surface. For the purpose of qualitative assay, 10-μl drops of a solution containing approximately 5 μg of each cobalamin were applied to the strip; for the quantitative assay, *ca.* 80 μl of an aqueous solution containing *ca.* 40 μg of each cobalamin was applied to the strip in a line *ca.* 4 cm long by means of a microsyringe. The solutions were applied near the positive pole.

In order to prevent photolysis of the methylcobalamin and cobamamide, all operations were carried out in the dark or under a red lamp.

Electrophoresis

A potential difference of 200 V and a current of 5–10 mA were applied for 60–80 min; with the buffer of pH 2.9, a potential difference of 500 V and a current of 15 mA were applied for 180 min.

Detection

After the electrophoresis, the cobalamins were detected visually. Cyanocobalamin is violet, hydroxocobalamin bright red and methylcobalamin and cobamamide brick red.

Quantitative assay

After electrophoresis in buffer of pH 2.9, the bands relative to each compound were cut off and placed in 20-ml test-tubes with ground-glass stoppers and eluted from the Cellogel with water; cobamamide, however, was eluted with phosphate buffer according to Sörensen (pH 7.0). As in all previous operations, elution was performed in the dark, and was continued until all traces of the compound had been leached out of the electrophoretic strip.

The maximum time required for the procedure was 30 min; 4 ml of eluent were used, resulting in a solution containing *ca.* 10 μg/ml of each cobalamin. A drop of 0.1 *N* NaOH was added to the solution of methylcobalamin in order to increase the pH of the solution to between 5 and 8, in which range the spectrophotometric characteristics of $B_{12}CH_3$ remain constant.

The eluates were read spectrophotometrically at the maximum wavelength for each cobalamin, and readings compared against a blank obtained by cutting a piece of untreated Cellogel to the same size as the spots and eluting it normally.

RESULTS

The R_F values obtained for cobamamide by carrying out electrophoresis at different pH values are listed in Table I.

Fig. 1 shows the separation of the cobalamins by means of electrophoresis at pH 2.9. The separation is good and enables the quantitative assay of each cobalamin to be performed spectrophotometrically.

Quantitative assay

In order to eliminate errors due to the measurement of small volumes and to the effects of "creep back" and "capillation"[9], at least 40 μl of solution were applied to the strip. Table II lists the recoveries of the four cobalamins together with their $E^{1\%}_{1cm}$ values.

TABLE I

$R_{F(DBC)}$ VALUES OF VITAMIN B_{12} DERIVATIVES

pH	*$B_{12}CN$*	*$B_{12}CH_3$*	*$B_{12}OH$*	*DBC*
2.9	0.22	0.36	0.92	1.0
4.0	1.1	0.83	1.20*	1.0
5.0	1.1	0.90	1.25*	1.0
6.0	1.02	0.77	1.25*	1.0
7.0	1.02	0.78	1.05*	1.0
8.0	0.75	0.60	1.75*	1.0

* At pH values above 2.9, $B_{12}OH$ gives rise to the formation of a number of differently coloured spots (yellow, violet, pink and red). Experimental conditions are reported in the text.

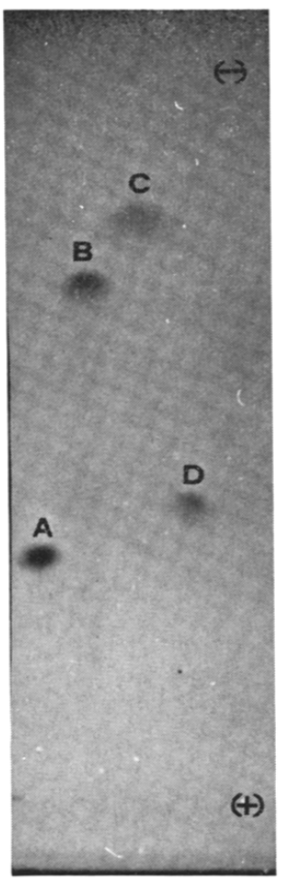

Fig. 1. Cellogel electrophoresis of $B_{12}CN$ (A), $B_{12}OH$ (B), DBC (C) and $B_{12}CH_3$ (D). Migration occurs towards the cathode. The electrophoretic buffer used was 0.5 *M* acetic acid (pH 2.9).

TABLE II

RECOVERIES AND SPECTROPHOTOMETRIC CHARACTERISTICS OF ELECTROPHORETICALLY SEPARATED COBALAMINS

Cobalamin	*Recovery (% ± S.D.*)*	$\lambda_{max.}$ *(nm)*	$E_{1\,cm}^{1\,\%}$
$B_{12}CN$	100 ± 0.5	361	207
$B_{12}CH_3$	96 ± 1.5	340	104
$B_{12}OH$	104 ± 1.3	351	195
DBC	105 ± 2.3	338	80

* Standard deviation calculated on 8 readings.

The following equation was used to calculate the recoveries

$$\text{Cobalamin } (\mu g) = \frac{E_l \cdot V_l}{E_{\lambda_l\,1cm}^{1\%} \cdot 10^{-3}}$$

where

E_l = extinction read for each cobalamin at its $\lambda_{max.}$;
V_l = 4 ml;
10^{-3} = conversion factor of $E_{\lambda_l\,1cm}^{1\%}$ from 1 g/100 ml to 10 μg/ml.

CONCLUSION

The procedure described gives a clear separation of the major cobalamins which have therapeutic applications in certain drugs. Owing to its simplicity, the technique has been used in research into the incorporation of the active forms in question into medicaments that offer a high degree of effectiveness and stability[10].

REFERENCES

1 J. I. Toohey and H. A. Barker, *J. Biol. Chem.*, 236 (1961) 560.
2 R. Scandurra, E. Barboni and V. Politi, *Acta Vitamin. Enzymol.*, 23 (1969) 92.
3 G. Tortolani, P. Bianchini and V. Mantovani, *Farmaco, Ed. Prat.*, 25 (1970) 772.
4 G. Tortolani, P. Bianchini and V. Mantovani, *J. Chromatogr.*, 53 (1970) 577.
5 L. Cima and R. Mantovan, *Farmaco, Ed. Prat.*, 17 (1962) 473.
6 Y. Popova, C. Popov and M. Ilieva, *J. Chromatogr.*, 24 (1966) 263.
7 R. A. Firth, H. A. O. Hill, J. M. Pratt and R. G. Thorp, *Anal. Biochem.*, 23 (1968) 429.
8 G. Tortolani and V. Mantovani, *Soc. Ital. Biol. Sper. Congress, Rome, 2nd–5th October, 1972.*
9 J. W. Fairbairn and S. J. Relph, *J. Chromatogr.*, 33 (1968) 494.
10 M. E. Colosi, G. Mascellani and A. Zannotti, *Farmaco, Ed. Prat.*, 28 (1973) 26.