Journal of Chromatography, 608 (1992) 311–315
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 332

Identification of vitamin B_{12} and analogues by high-performance capillary electrophoresis and comparison with high-performance liquid chromatography

D. Lambert, C. Adjalla, F. Felden, S. Benhayoun, J. P. Nicolas and J. L. Guéant

INSERM U308, Mechanisms of Regulation of Alimentary Behaviour, Group of Biochemistry, Faculty of Medicine, Avenue Forêt de Haye, B.P. 184, 54505 Vandoeuvre Cedex (France)

ABSTRACT

High-performance capillary electrophoresis (HPCE) was compared for the identification and determination of corrinoids (hydroxy-, cyano-, 5'-desoxyadenosyl- and methyl-cobalamin and cyano-cobinamide) with high-performance liquid chromatography (HPLC). The within-run reproducibility of the retention times in HPCE and HPLC were similar (2.4 and 2.2%, respectively). The detection limit in HPCE was 20 μg/ml. HPLC can be used, in combination with radioisotope dilution assay, when very low concentrations (100 pg/ml) have to be determined in biological material. HPCE is more efficient than HPLC for the identification of corrinoids after conversion into the CN-cobalamin and CN-cobinamide forms.

INTRODUCTION

Human biological fluids contain different forms of vitamin B_{12}: hydroxo-cobalamin (OH-cbl), cyano-cobalamin (CN-cbl) and the two coenzymatic forms 5'-deoxyadenosyl-cobalamin (Ado-cbl) and methyl-cobalamin (CH_3-cbl). Cobalamins can be distinguished from potentially harmful analogues of vitamin B_{12}, devoid of enzymatic activity [1]. These analogs can be divided in two groups: cobamides that lack the nucleotide moiety (5,6-dimethylbenzimidazole) and cobamides that contain a modified nucleotide. Only analogues with a nucleotide close to that of cbl bind intrinsic factor (IF). Haptocorrin (Hc) binds both cobinamides and cobamides [2].

Human [2–4] and animal [5] tissus contain corrinoids analogues that do not have any activity, and that sometimes inhibit growth and development and cause severe demyelinization of nerve fibres [6].

The β aquo ligand (Ado or CH_3) can be replaced by an aquo group after exposure to light. The β ligand is replaced by a CN group after incubation with KCN. The consecutive treatment of a mixture of cobalamins and cobinamides by exposure to light and addition of KCN produces therefore the two forms CN-cbl and CN-cobinamide.

The determination of cobalamins has been used in order to diagnose cobalamin deficiencies in humans [7,8]. In recent years, both thin-layer chromatography and high-performance liquid chromatography (HPLC) have been developed for the determination of the different forms of cobalamin present in plasma and other biological samples [9,10]. Methods combining chromatography and isotope dilution assay can be used to separate and to identify the different forms of corrinoids [11–14].

In this study, two methods for the separation of corrinoids were compared: high-performance capillary electrophoresis (HPCE) and HPLC. The application of HPCE was tested on multi-vitamin prep-

Correspondence to: Dr. D. Lambert, INSERM U308, Mechanisms of Regulation of Alimentary Behaviour, Group of Biochemistry, Faculty of Medicine, Avenue Forêt de Haye, B.P. 184, 54505 Vandoeuvre Cedex, France.

0021-9673/92/$05.00 © 1992 Elsevier Science Publishers B.V. All rights reserved

arations for parenteral nutrition, the corrinoids stability of which can be altered by physical conditions and interactions with other components [15,16].

EXPERIMENTAL

Chemicals

Crystalline OH-cbl, CN-cbl, Ado-cbl, CH_3-cbl and CN-cobinamide were obtained from Sigma (St. Louis, MO, USA) and used as standards for both HPLC and HPCE. Standards were dissolved in distilled water (concentrations were chosen to obtain a good detector response), and filtered through a 0.45-μm filter (Millipore).

NaH_2PO_4 and orthophosphoric acid respectively were obtained from BDH (AnalaR grade) and Prolabo, respectively. For both HPCE and HPLC, buffers were filtered (0.45 μm) and degassed under vacuum. A multi-vitamin mineral preparation (Soluvit) was obtained from Kabi-Pharmacia.

C_{18} cartridges (5-μm silica, 250 mm × 5 mm I.D.) and Sep-pak C_{18} cartridges were purchased from Waters.

High-performance capillary electrophoresis

Capillary electrophoresis was performed using a Waters Quanta 4000 apparatus. The system was equipped with a fused-silica capillary tube (100 cm × 75 μm I.D.) with an effective length of 93 cm. The applied voltage was 30 kV. Sample injection was maintained for 15 s and the column temperature was maintained at 26°C. The anode and cathode buffer was 20 m*M* NaH_2PO_4, adjusted to pH 2.5 with 20 m*M* orthophosphoric acid solution. Absorbance was measured at 214 nm.

Reversed-phase high-performance liquid chromatography

HPLC was performed at room temperature using a two-pump gradient system (Waters). The detection wavelength was 254 nm (Lambda-max 480 spectrophotometer, Waters).

HPLC separation of corrinoids was carried out in 40 min, on a C_{18} column, using a 10–50% linear gradient of acetonitrile in 0.085 *M* phosphoric acid as mobile phase. The pH was adjusted to 3.0 with triethanolamine. The flow-rate was 0.5 ml/min.

Exposure to light and KCN treatment

Standard corrinoids were first exposed to light (45-min exposure to a 60-W tungsten lamp at a distance of 50 cm) and then subjected to KCN treatment (incubation with one volume of $0.2 \cdot 10^{-2}$ *M* KCN for 2 h) in order to convert corrinoids into the CN forms.

RESULTS

A mixture of the five standards, OH-, CN-, Ado- and CH_3-cbl and CN-cobinamide, was resolved in five well separated peaks in HPCE (Fig. 1A). The retention times of the corrinoids are given in Table I. Identification was achieved by injecting either the corrinoid mixture or a sample of each corrinoid form. A better separation of CN-cobinamide, OH-cbl and Ado-cbl was obtained with a lower voltage (15 kV), the retention times of CH_3-cbl and CN-cbl then being longer than 16 and 25 min, respectively (not shown).

The peaks of OH-cbl, Ado-cbl and CH_3-cbl disappeared after exposure to light and treatment with KCN. Only two peaks, whose retention times corresponded to CN-cobinamide and CN-cbl, were present (Fig. 1B). The detection limit of CN-cbl was 20 μg/ml.

The multi-vitamin mineral preparation (Soluvit) was passed through a Sep-pak C_{18} cartridge to obtain water-soluble components [9]. The eluent was 20% 2-butanol. The identification by HPCE allowed four main peaks to be obtained. One of these had a retention time corresponding to that of CN-cobalamin (not shown). The three other peaks had retention times below those of corrinoids (2.2, 3.0 and 3.9 min). There was no similar peaks between Soluvit and corrinoid standards.

The five different forms of cobalamin were also identified by HPCL (Fig. 2A). The differences in retention times (Table I) were not as marked as with HPCE. Morever, at pH 3, CN-cobinamide injected alone appeared to be present as two peaks corresponding to CNα- and CNβ-cobinamide [12,17]. The retention time of CN-cbl was intermediate between those of CNα- and CNβ-cobinamide.

After exposure to light and treatment with KCN, only three peaks, whose retention times corresponded to CN-cobinamide and CNα- and CNβ-cbl, were present on the HPLC trace (Fig. 2B).

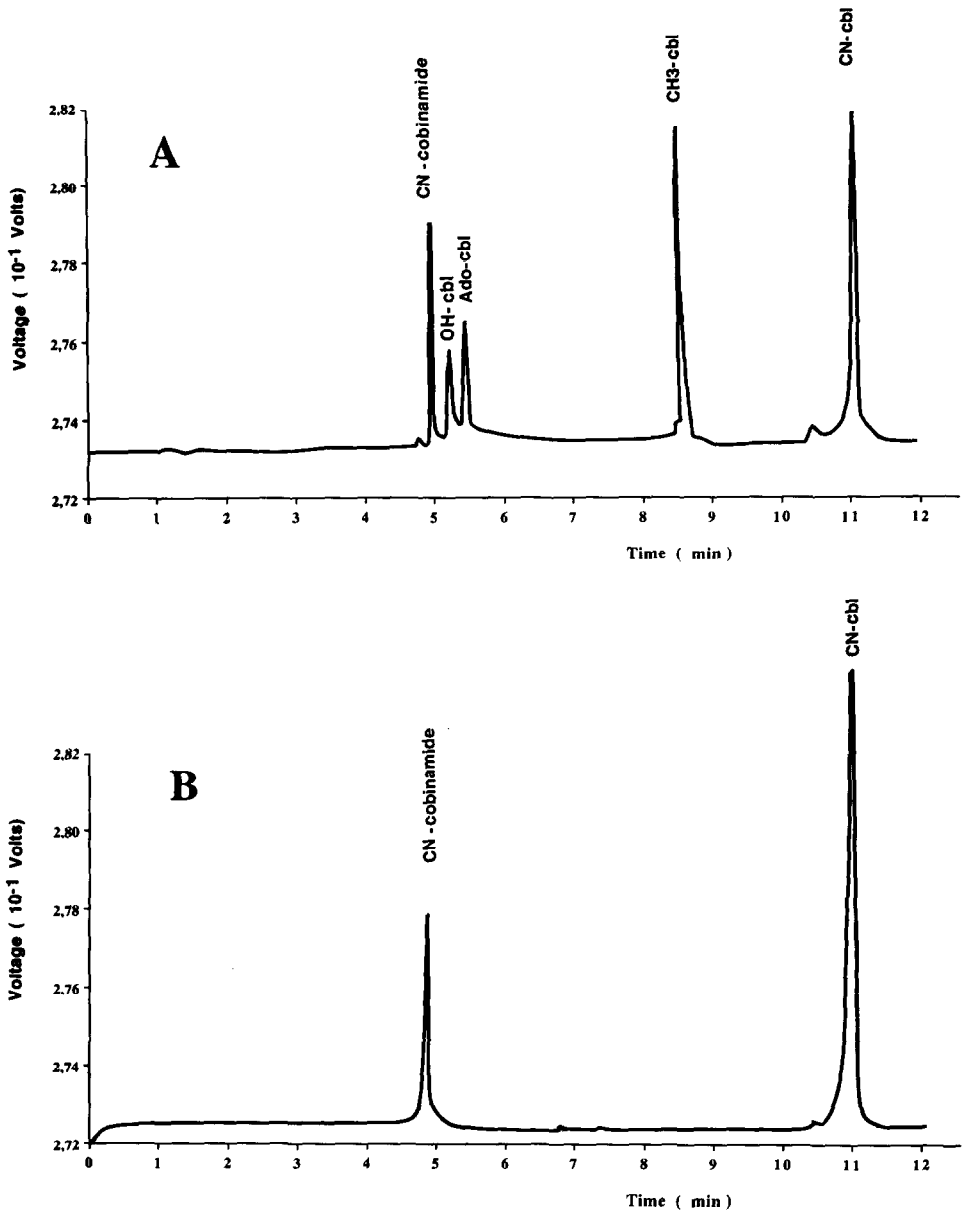

Fig. 1. HPCE of standard corrinoid samples. (A) Untreated samples; (B) samples after exposure to light (45 min, 60-W tungsten lamp; distance 50 cm) and then KCN treatment (incubation with one volume of $0.2 \cdot 10^{-2}$ *M* KCN for 2 h). Electrophoretic conditions as in Table I.

The within-run reproducibilities of the retention times in HPCE and HPLC were similar, with coefficients of variation of 2.4 and 2.2%, respectively.

DISCUSSION

In HPCE, the retention times of CN-cbl and CN-

TABLE I

RETENTION TIMES OF CORRINOIDS SEPARATED BY HPCE AND HPLC

HPCE: capillary size, 100 cm × 75 μm I.D.; applied voltage, 30 kV; sample injection, 15 s; temperature, 26°C; anode and cathode buffer, 20 m*M* NaH_2PO_4 (pH 2.5, adjusted with 20 m*M* orthophosphoric acid); detection wavelength, 214 nm. HPLC: C_{18} column with a 10–50% linear gradient of acetonitrile in 0.085 *M* phosphoric acid as mobile phase (pH 3.0, adjusted with triethanolamine); run time, 40 min at room temperature; flow-rate, 0.5 ml/min; detection wavelength, 254 nm.

Corrinoid	Retention time (min) ± S.D. (n = 6)	
	HPLC	HPCE
OH-cobalamin	14.08 ± 0.50	5.24 ± 0.10
CNα-cobinamide	15.82 ± 0.31	4.95 ± 0.12
CN-cobalamin	17.21 ± 0.31	10.85 ± 0.25
CNβ-cobinamide	18.48 ± 0.18	4.95 ± 0.12
Ado-cobalamin	18.86 ± 0.45	5.50 ± 0.10
CH_3-cobalamin	21.10 ± 0.47	8.45 ± 0.18

cobinamide differed by *ca.* 5.5 min. This result allowed errors of identification that could sometimes occur in HPLC, where the difference was only 1.2 min, to be avoided. For this reason, HPCE is more efficient than HPLC for the identification of corrinoids after conversion into the CN forms. HPCE allows a better discrimination between active and harmful corrinoids. However, only HPLC was able to distinguish CNα-cobinamide from CNβ-cobinamide. The elution of both CNα- and CNβ- cobinamide in the same peak in HPCE can be explained by the absence of a difference in the net charge of the two molecules.

HPLC allows fractions of the eluate to be collected which can be used for radioisotope analysis of corrinoids (radioisotope dilution assay, RIDA). For this reason we use HPLC, in combination with RIDA, when very low concentrations (100–1000 pg/ml) in biological material have to be determined. With this method for the quantification of corrinoids, the "true cbl" forms can be distinguished from cbl analogues, using intrinsic factor (IF) and haptocorrin (HC) as binders of the RIDA [14]. Such a combination with RIDA is not possible with HPCE; the amount of sample is of the order of nanolitres and the amount of corrinoid eluted in each peak is too small to be detected.

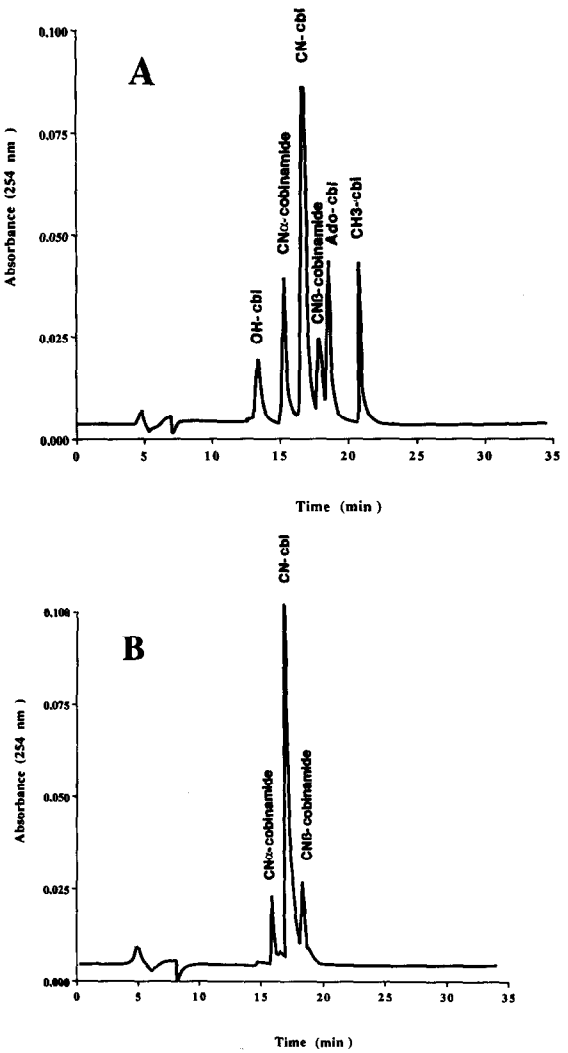

Fig. 2. HPLC of standard corrinoid samples. (A) Untreated samples; (B) samples after exposure to light (45 min, 60-W tungsten lamp; distance 50 cm) and then KCN treatment (incubation with one volume of $0.2 \cdot 10^{-2}$ *M* KCN for 2 h). Chromatographic conditions as in Table I.

When concentration is not a limiting factor, HPCE is easier to perform than HPLC. For example, HPCE can be used for the analysis of multivitamin preparations for parenteral nutrition and to check the stability of corrinoid compounds to light exposure and temperature and the interaction of corrinoids with other vitamins and trace elements.

In conclusion, both HPCE and HPLC are effi-

cient methods for separating corrinoids. The choice between the techniques depends on the type of sample to be analysed. HPCE can be performed when the corrinoid concentration is high enough, whereas a combination of HPLC with RIDA is recommended for low-concentration samples such as biological fluids.

REFERENCES

1 J. F. Kolhouse and R. H. Allen, *J. Clin. Invest.*, 60 (1977) 1381.
2 J. F. Kolhouse, H. Kondo, N. C. Allen, E. Podell and R. H. Allen, *N. Engl. J. Med.*, 299 (1978) 785.
3 J. L. Guéant, B. Monin, P. Boissel, P. Gaucher and J. P. Nicolas, *Digestion* 30 (1984) 15.
4 S. Kanazawa and V. Herbeth, *Am. J. Clin. Nutr.*, 37 (1983) 77.
5 H. Kondo, J. F. Kolhouse and R. H. Allen, *Proc. Natl. Acad. Sci. U.S.A.*, 77 (1980) 817.
6 R. C. Siddons, J. A. Spence and A. D. Dayan, *Adv. Neurol.*, 10 (1975) 239.
7 I. Chanarin, *The Megaloblastic Anemias*, Blackwell, Oxford, 1979.
8 E. Nexo and H. Olesen, in D. Dolphin (Editor), *B12: Biochemistry and Medicine*, Vol. 2, Wiley, New York, 1982, p. 87.
9 P. Gimsing and E. Nexo, in C. A. Hall (Editor), *The Cobalamins*, Churchille Livingstone, Edingburgh, 1983, p. 1.
10 P. Gimsing and W. S. Beck, *Scand. J. Clin. Lab. Invest.*, 194 (Suppl.) (1989) 37.
11 P. Gimsing, *Anal. Biochem.*, 67 (1983) 288.
12 P. Gimsing and W. S. Beck, *Methods Enzymol.*, 123 (1986) 3.
13 J. Van Kapel, L. M. J. Spijkers, J. Lindemans and J. Abels, *Clin. Chim. Acta*, 131 (1983) 211.
14 M. Djalali, J. L. Guéant, S. El Kholty, D. Lambert, M. Saunier and J. P. Nicolas, *J. Chromatogr.*, 529 (1990) 81.
15 V. Herbeth, G. Drivas, R. Foscaldi, C. Manusselis, N. Colman, S. Kanazawa, K. Das, M. Gelernt, B. Herzlich and J. Jennings, *N. Engl. J. Med.*, 307 (1982) 255.
16 H. Kondo, M. J. Binder, J. F. Kolhouse, W. R. Smythe, E. R. Podell and R. H. Allen, *J. Clin. Invest.*, 70 (1982) 889.
17 D. F. W. Jacobson, R. Green, E. V. Quadros and Y. D. Montejano, *Anal. Biochem.*, 120 (1982) 394.