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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF COBALAMINS

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SUMMARY

Physiological cobalamins were separated by means of high-performance liquid chromatography (HPLC). Optimal conditions for elution of methylcobalamin, adenosylcobalamin, hydroxycobalamin and cyanocobalamin were determined. Excellent separation and resolution of these physiological cobalamins by HPLC were achieved. In addition, several cobalamin analogues were also studied and shown to be separable from the physiological forms. HPLC provides a rapid, sensitive, reproducible means of characterizing physiological cobalamins.

INTRODUCTION

During the past two decades remarkable knowledge has accumulated regarding the biochemistry and biology of vitamin B_{12}. As evidence of the physiologic forms of B_{12} (methylcobalamin[Me-Cbl], adenosylcobalamin[Ado-Cbl], hydroxycobalamin [OH-Cbl], cyanocobalamin[CN-Cbl]) developed, our ability to examine these individual cobalamins was hindered by the lack of simple separation techniques. Linnell and coworkers[1–5] have provided evidence that the pattern of quantitative and qualitative changes in the cobalamin fractions has significant biological importance. Unfortunately, their contributions to the characterization of these fractions have required very tedious and complex chromatographic and bioautographic methods[1],

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not easily applicable to extensive studies and not generally adaptable to other laboratories.

The potential speed, sensitivity and resolution of high-performance liquid chromatographic (HPLC) technology appeared to provide an ideal approach for the investigation of the cobalamin fractions under varying pathophysiologic states. Data suggesting such feasibility for cyanocobalamin, which may not be a true physiologic moiety, were recently reported[6,7]. The present report describes the application of HPLC to the separation for characterization and quantification of the cobalamin fractions known to exist in man.

EXPERIMENTAL

Cobalamin preparations

Crystalline cobalamin standards (hydroxycobalamin and cyanocobalamin) were obtained from Sigma (St. Louis, Mo., U.S.A.); coenzyme-B_{12} and methyl-B_{12} were from Calbiochem (La Jolla, Calif., U.S.A.). Solutions were standardized by radioisotopic assay[8,9] and by spectrophotometric assay using the known extinction coefficients[10].

Sulfito-cobalamin was prepared from hydroxycobalamin by preparing a 10 mg/ml solution in 0.1 *M* sodium bisulfite[11]. The wavelength spectrum of the compound was characterized by a double maximum at 312 and 364 nm. This double peak reverted to a single maximum at 351 nm after photolysis; this was identical with authentic OH-Cbl.

Mixed monobasic acids of CN-Cbl and anilide derivatives of the acids[12] were provided by Dr. W. F. J. Cuthbertson of Glaxo Laboratories, Stoke Poges, Great Britain. The alkanolamine analogue of CN-Cbl, 2-amino-2-methylpropanol-B_{12} ("S-102") was provided by Dr. W. Friedrich, Universität Hamburg, G.F.R.[13].

Protection of cobalamins from photolysis

All extracts and solutions containing the photosensitive organocobalamins (*e.g.* Ado-Cbl, Me-Cbl, and CN-Cbl) were handled in the dark under the illumination of a photographic safelight (Foto 2001 Kindermann globe safelight with Varigam filter; 15 watt bulb, Ehrenreich Photo-Optical Industries, Garden City, N.Y., U.S.A.). Under these conditions no photolysis of endogenous ^{57}Co-labeled Ado-Cbl or Me-Cbl was observed for at least 2.5 h at room temperature at a distance of 25 cm from the safelight.

HPLC separation of cobalamins

Applicability of HPLC to the separation of the physiologic cobalamins was evaluated with two separate instruments under differing conditions. For clarity, these are denoted by temporal relationships.

In the initial studies, the cobalamins were separated by reverse-phase chromatography on a pre-packed stainless-steel column (30 cm × 3.9 mm I.D.) of μBondapak C_{18}, 10 μm particle size (Waters Assoc., Milford, Mass., U.S.A.) in a Model ALC-202/401 liquid chromatograph (Waters Assoc.) equipped with a Model 660 solvent programmer, and a Model 440 ultraviolet absorbance detector operating at a 254 nm wavelength. A reservoir of 0.05 *M* sodium acetate buffer, pH 4.0, was

connected to pump A and a reservoir of methanol ("distilled in glass", Burdick & Jackson Labs., Muskegan, Mich., U.S.A.) containing 0.05 *M* sodium acetate buffer, pH 4.0, to pump B; the buffers were prepared by mixing 2.9 ml of glacial acetic acid and 2.6 ml of 3.5 *M* sodium hydroxide with 1 l of glass-distilled water or methanol, and filtered through millipore. A solvent-inert filter (Millipore, Bedford, Mass., U.S.A.) was used for the methanol. The initial solvent concentration for the chromatography was 27% methanol. With sample injection an upward concave gradient (program No. 7 on the Model 660 programmer) was begun and completed in 10 min at final concentration of 95% methanol. The solvent flow-rate was 1.8 ml/min. Initial conditions were achieved by a linear program reversal of 5 min, followed by an equilibration period of 5 min at initial conditions. The column effluent was monitored spectrophotometrically at 254 nm and collected in (0.4 min) fractions for gamma counting.

In a second approach the cobalamins were separated by means of a DuPont Model 850 iiquid chromatograph equipped with a temperature controlled column oven (DuPont, Wilmington, Del., U.S.A.). In these studies solvent intake A was 0.05 *M* NaH_2PO_4 and solvent intake B was methanol. Removal of gas from the solvents was accomplished with continuous helium sparging. The columns used were the μBondapak C_{18}, 10 μm particle size (Waters Assoc.) and the Zorbax ODS, 4.6 mm × 25 cm, 6 μm particle size (DuPont). Flow-rate was 1.8 ml/min and column oven temperature was 40°. The initial solvent concentration was 23% methanol. Following the sample injection the initial conditions were maintained for 2 min. Then, a concave gradient (exponent +2) was begun with methanol concentrations of 23 to 70% spanning 10 min. After holding at 70% methanol for 1 min, "initial conditions" were obtained by a linear reversal of methanol concentrations from 70 to 23% over a 3-min period, followed by 5 min of equilibration at these "initial" conditions.

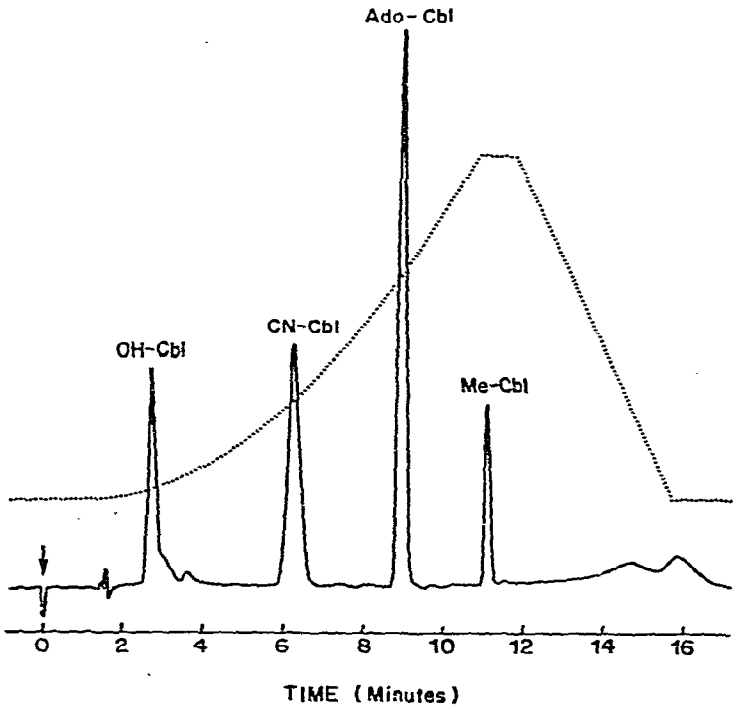
Cobalamin assay

The cobalamins were quantitated on pooled fractions by radioisotopic competitive inhibition assay, as previously described[8,14].

RESULTS

Separation of cobalamins

Complete separation of authentic cobalamin standards (OH-Cbl, CN-Cbl, Ado-Cbl, and Me-Cbl) was accomplished in approximately 11 min with the patterns shown in Fig. 1. Trials employing acetate in the solvent system produced significant "trailing" of the peaks. Substitution of phosphate for acetate resulted in a lesser retention on the columns, thereby permitting use of a lower concentration of methanol in the eluant. These changes eliminated the "trailing" phenomenon in the studies performed on the Waters μBondapak C_{18} columns (Fig. 1A). In studies on the DuPont Zorbax ODS columns (Fig. 1B) the trailing was reduced by the phosphate substitution, but not completely eliminated, particularly in the hydroxycobalamin peak. The minor peaks seen between OH-Cbl and CN-Cbl are corrinoid contaminants of OH-Cbl of unknown nature. Treatment with cyanide increased the retention times of these contaminants as well as OH-Cbl. The small, irregularly-shaped peaks at the end of the separation are solvent artifacts.



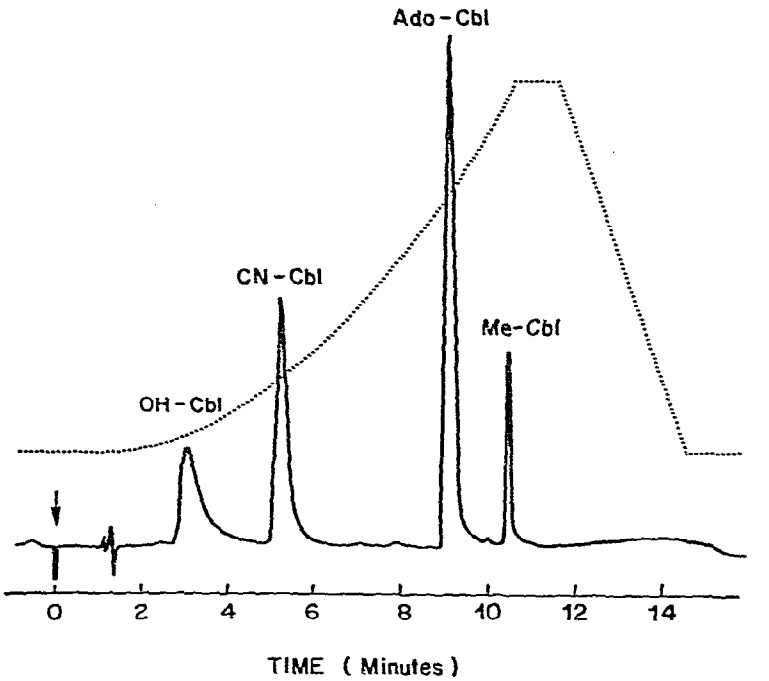


Fig. 1. HPLC separation of authentic cobalamin standards. A mixture of the cobalamins (hydroxycobalamin [OH-Cbl] 7.5 μg; cyanocobalamin [CN-Cbl] 9.8 μg; deoxyadenosylcobalamin [Ado-Cbl] 6.8 μg; and methyl cobalamin [Me-Cbl] 3.4 μg) in a 15 μl volume was injected. The effluent was monitored by ultraviolet absorption at 254 nm wavelength and 0.5 a.u.f.s. deflection. The dashed line represents the methanol concentration of the eluant. (A) The separation on the Waters μBondapak C_{18} column. (B) The separation on the DuPont Zorbax ODS column.

In studies not shown other solvent system trials demonstrated an interesting pattern when the cobalamins eluted with distilled water rather than the acid buffer. Under these conditions a reversal of the sequence of separation was seen with Me-Cbl being eluted first and OH-Cbl last. Unfortunately, a greater trailing artifact and incomplete elution precluded the application of these conditions for quantitative studies.

A variety of cobalamin analogues were also examined and their separation is shown in Fig. 2. Sulfitocobalamin (HSO_3-Cbl) was clearly identified by both systems and separated from OH-Cbl. On the Waters μBondapak C_{18} columns the HSO_3-Cbl had a longer retention time than OH-Cbl, whereas on the DuPont Zorbax ODS columns its retention was shorter than OH-Cbl. It is of interest that in earlier studies at ambient temperature using the acetate–methanol system the HSO_3-Cbl was not separable from the OH-Cbl.

The monobasic acids of CN-Cbl were resolved into two major and two minor peaks eluting beyond CN-Cbl (Fig. 2). The resolution of these peaks was clearer on the DuPont Zorbax ODS columns. When these analogues are admixed with CN-Cbl good resolution of the analogue M-1 from the CN-Cbl was achieved only when the acetate buffer system was employed. The S-102 and anilide analogues of CN-Cbl were well resolved from CN-Cbl in each of the systems studied (Fig. 2). The minor unlabeled peaks seen on Fig. 2 are contaminants of the analogue preparations.

Reproducibility of retention times and resolution of peaks were excellent regardless of the sample composition. A range of injection volumes of 2–100 μl displayed identical retention and resolution of cobalamins. Sample volumes in excess of 100 μl slightly decreased the sharpness of the peak. Reproducibility of the separation of a cobalamin extract (day to day and/or week to week) showed less than 2% variation.

The recovery of cobalamins injected onto the HPLC system was virtually 100% ($99 \pm 1\%$); *ca.* 0.2–0.3% was eluted on the subsequent run.

Quantitation of eluted cobalamins

Standard curves were prepared for each cobalamin by plotting known quantities of the cobalamin injected against the peak area obtained from the spectrophotometric detector. For each cobalamin a clear linear quantitative relationship was obtained between 100 ng and 10 μg of the injected material. At any given concentration the standard deviation of the points on the standard curve was less than 2%.

Profile of photolysis of cobalamins

Experiments on the photolytic cleavage of the physiologic cobalamins are shown in Fig. 3. Photolysis of both Ado-Cbl and Me-Cbl is clearly demonstrable by HPLC techniques with the expected corresponding increase in OH-Cbl. No significant photolysis of the CN-Cbl was seen under these conditions of light exposure.

DISCUSSION

The present studies demonstrate the applicability of HPLC technology to the separation and fractionation of individual cobalamins. HPLC provides excellent resolution of the cobalamins, adequate sensitivity for application for tissue cobalamin

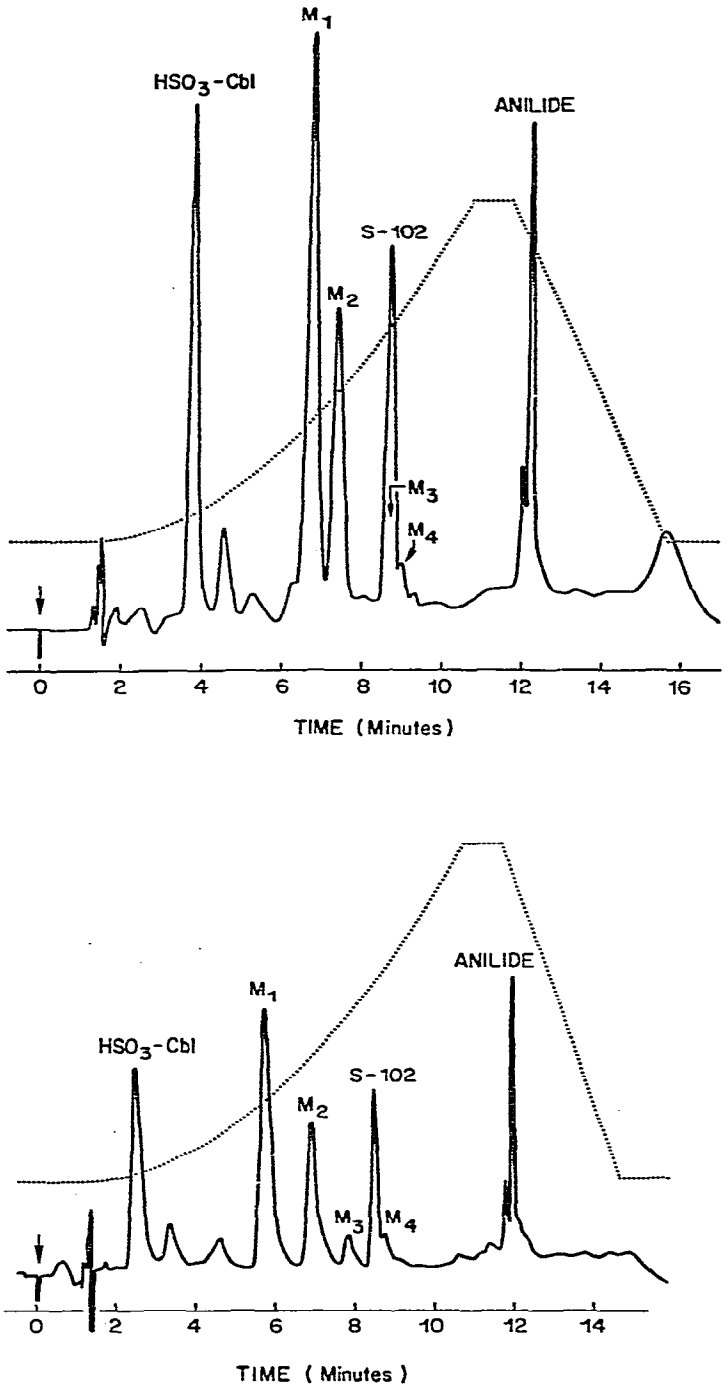


Fig. 2. HPLC separation of cobalamin analogues. Under the conditions noted in Fig. 1, 50 μl volumes (sulfito-cobalamin [HSO_3-Cbl]; mixed isomers of monobasic acids of cyanocobalamin [M_1, M_2, M_3, M_4]; alkanolamine analogue of cyanocobalamin [S-102]; and anilide derivative of the monobasic acids [anilide]) were injected. The full scale deflection was 0.2 a.u. (A) The separation on the Waters μBondapak C_{18} column. (B) The separation on the DuPont Zorbax ODS column.

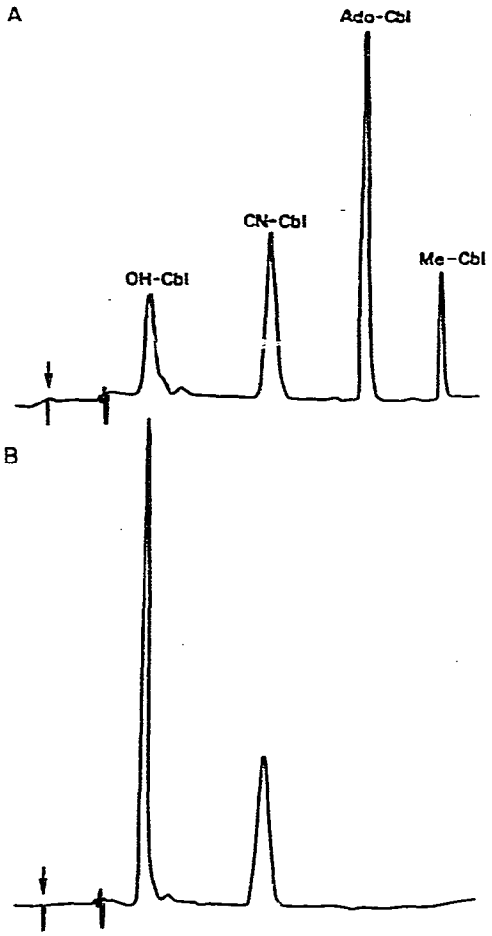


Fig. 3. HPLC of cobalamins (A) before and (B) following light exposure to high intensity light for 15 min at 4°. Photolysis of Ado-Cbl and Me-Cbl was complete, as shown in (B).

analysis enhanced by coupling with a B_{12} competitive inhibition assay[8,9,14], the technical advantage of ease and rapidity of analyses, and documented reproducibility. Studies are now in progress to characterize the fractions in tissues in a variety of clinical states.

Pathophysiological or clinical correlative relationships to individual cobalamin fractions have had only preliminary exploration[2–5], because of the serious technological limitations in separating and quantifying these cobalamins (*i.e.* methylcobalamin, adenosylcobalamin and cyanocobalamin). Although the data are limited, evidence suggests that B_{12} deprivation results in an asymmetric decline in the individual cobalamins[4,5], and that other metabolic changes (*e.g.* alcohol) have different effects on methylcobalamin and adenosylcobalamin[15] function. Thus, critical need exists for the characterization of the cobalamin fractions under a variety of clinical states. HPLC offers technological promise for a direct approach to these important pathophysiological issues.

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