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Note

SP-Sephadex column chromatography of cobalamins contained in liver extracts

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Various chromatographic techniques are described in the literature on paper[1], silica gel thin layers[2], alumina and cellulose[3–5], Dowex 50W columns[6–10] and ion-exchange cellulose [10,11] for the separation and analysis of certain forms of vitamin B_{12}. We recently described a chromatographic technique on a single column of SP-Sephadex for the separation of a mixture of cyanocobalamin, cobamamide, methylcobalamin and hydroxycobalamin[12].

This technique was employed to determine the cobalamine and nucleoside contents of a liver extract obtained from various animal species. The experimental findings are reported in this paper.

EXPERIMENTAL

Apparatus and materials

A Fractomat Y-3 fraction collector, a Beckman Model 746 peristaltic pump (1–10 ml/min) and a Stratomat automatic stratifier were used.

SP-Sephadex C-25 (Na^+) and QAE-Sephadex (Cl^-) were obtained from Pharmacia (Uppsala, Sweden), microcrystalline cellulose and silica gel GF_{254} from Merck (Darmstadt, G.F.R.).

Methylcobalamin and cobamamide were obtained by means of partial synthesis in the research laboratories of Alfa Farmaceutici S.p.A.; hydroxycobalamin and cyanocobalamin, spectrophotometrically and chromatographically pure, were obtained from Roussell-Uclaf (Paris, France).

Preparation of liver extracts

Albino guinea-pig livers weighing 20 g, New Zealand rabbit livers weighing 40 g and portions of ox liver weighing 40 g were homogenized with 70% ethanol and heated for 30 min at 35°. The homogenate was cooled to room temperature and centrifuged at 6000 rpm. The supernatant was filtered on a G4 porous septum, vacuum concentrated at 35° and the residue reconstituted with 20 ml of distilled water. The aqueous solution was extracted with water-saturated phenol and the phenol phase, to which were added one volume of acetone and three volumes of ether, was re-extracted with distilled water. The resultant extract was vacuum concentrated in a rotary evaporator to a volume of about 15 ml and 0.1 ml of *m*-cresol was added.

All the operations were carried out in red light so as to avoid photolytic decomposition of the cobalamins.

Column chromatography on SP-Sephadex

Preparation of the column. The column, 0.9 cm I.D., containing SP-Sephadex to a depth of 20 cm, was prepared as previously described[12].

Elution. A known quantity of each extract, corresponding to the liver of a given animal, was placed on the column, where it was adsorbed. Elution was first carried out with water, eighteen fractions of 2 ml being collected at a constant rate of 0.4 ml/min, and then with sodium acetate buffer (0.05 *M*, pH 5.0). Each fraction was developed microbiologically on an *Escherichia coli* mutant 113-3 slide, and the cyanocobalamin activity was used as a term of reference (U.S.P.) to express the activities of hydroxycobalamin, cobamamide and methylcobalamin.

The fractions corresponding to the first peak (20 ml) were pooled and vacuum concentrated in a rotary evaporator (Fraction A).

Column chromatography on QAE-Sephadex

Preparation of the column. An electromagnetic shaker was used to suspend 5 g of QAE-Sephadex in a solution of 1.0 *M* sodium chloride. The supernatant was decanted and replaced with fresh buffer. The above operation was repeated twice more and the ion excess removed by washing with distilled water. The gel was then poured to a depth of 20 cm (after settling) into a glass column, 1.5 cm I.D.

Elution. Fraction A was placed on the column and eluted with distilled water and 0.005 *M*, 0.05 *M*, 0.1 *M* and 1 *M* aqueous solutions of sodium chloride. Each 5-ml fraction was determined microbiologically using *E. coli* 113-3 mutant.

The fractions eluted from the QAE-Sephadex column were pooled and vacuum concentrated in a rotary evaporator to a volume of 1–2 ml (Fraction B).

As standard cyanocobalamin chromatographed on QAE-Sephadex is eluted with distilled water according to the peak for fraction B, we decided to carry out further chromatography on a thin layer of cellulose in order to trace its presence in the liver extracts.

Thin-layer cellulose chromatography

The chromatographic determination of cyanocobalamin in fraction B was performed using the method described by Johnson *et al.*[1] and employing a 250-μm thin layer of cellulose in place of paper.

Deposition and development. Four strips, 2.5 cm wide, were marked on the 20×20 cm chromatographic plate. Approximately 150 μl of Fraction B and 0.5 μg each of cyanocobalamin and hydroxycobalamin, used as standards for comparison, were applied separately to each of three strips. The fourth strip was left blank.

Elution was carried out with *n*-butanol–isopropanol–water (1:0.7:1). When development was complete (after about 6 h), rectangular pieces measuring 1×2.5 cm were marked off on each strip, scraped and the scrapings placed in centrifuge tubes containing 4 ml of water.

After 15 min the suspension was centrifuged and the supernatant microbiologically determined by means of the *E. coli* test.

Thin-layer chromatography on cellulose and silica gel layers

Identification of the nucleic acid derivatives present in the liver extracts was carried out by means of mixed thin-layer chromatography.

Preparation of chromatographic plates. A 0.40-mm layer of a suspension of cellulose and silica gel GF_{254} (2: 1) and distilled water in the ratio 1 : 3 was deposited on the 20 × 20 cm plates.

Development. A 100-μl amount of aqueous solution containing 250 μg/ml of each standard compound was deposited, using a Chemetron microsyringe, on the chromatographic plates in a 2.5-cm-long line. Chromatographic development was carried out in the first direction with chloroform–diethyl ether–ethanol–acetone–distilled water (2:6:5:3:1.5) and in the second direction with distilled water only. Development time at room temperature was about 2 h for the first direction and 1 h 15 min for the second. When elution was complete, the plates were observed in UV light at 254 nm. The constituents appeared as violet blotches on the fluorescent yellow-green background of the chromatographic plate.

RESULTS

Fig. 1 shows the separation scheme for the cobalamins extracted from rabbit, guinea-pig and ox liver. The yellow fractions corresponding to the first peak (Fraction A) might contain neutral or acid cobalamins, decomposition products of cobamamide and traces of cyanocobalamin. The second peak, occurring between 20 and 40 ml, corresponds to methylcobalamin, identified by re-chromatographing the individual fractions on a thin layer of cellulose against standard methylcobalamin.

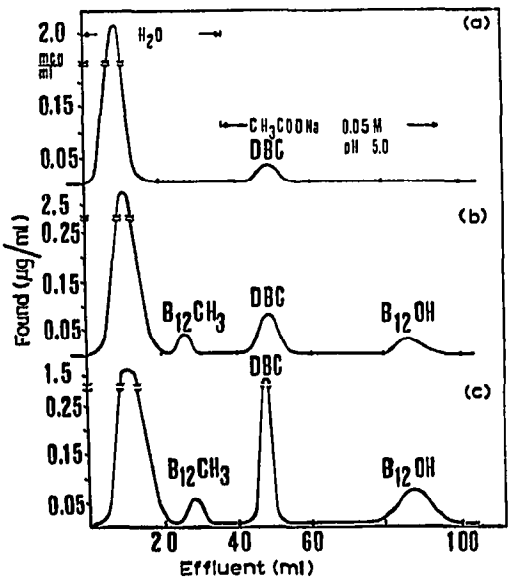

Fig. 1. Chromatographic separation on SP-Sephadex (Na^+) of cobalamins extracted from (a) rabbit, (b) guinea-pig and (c) ox liver. Eluents, water and 0.05 *M* CH_3COONa, pH 5.0; Column, 20 × 0.9 cm I.D.; flow-rate, 0.4 ml/min. Each 2-ml fraction was developed microbiologically by means of the *E. coli* test. DBC = Cobamamide; $B_{12}CH_3$ = methylcobalamin; $B_{12}OH$ = hydroxycobalamin.

Cobamamide was eluted from the fractions between 45 and 55 ml and hydroxycobalamin from those between 80 and 100 ml.

In Fraction A, the following nucleic acid derivatives were identified by means of two-dimensional chromatography on a mixed thin layer of cellulose and silica gel GF_{254} (ref. 13): inosine, xanthine, hypoxanthine, uracil and uridine. Adenosine, adenine, thymine and riboflavine were separated and identified in the fractions ranging from 40 to 100 ml. These substances were identified both by means of a chart of standard nucleic acid derivatives chromatographed under the same experimental conditions and by means of the spectrophotometric curves of the compounds themselves, after chromatographic development, in the wavelength range 200–300 nm in 0.1 *N* hydrochloric acid.

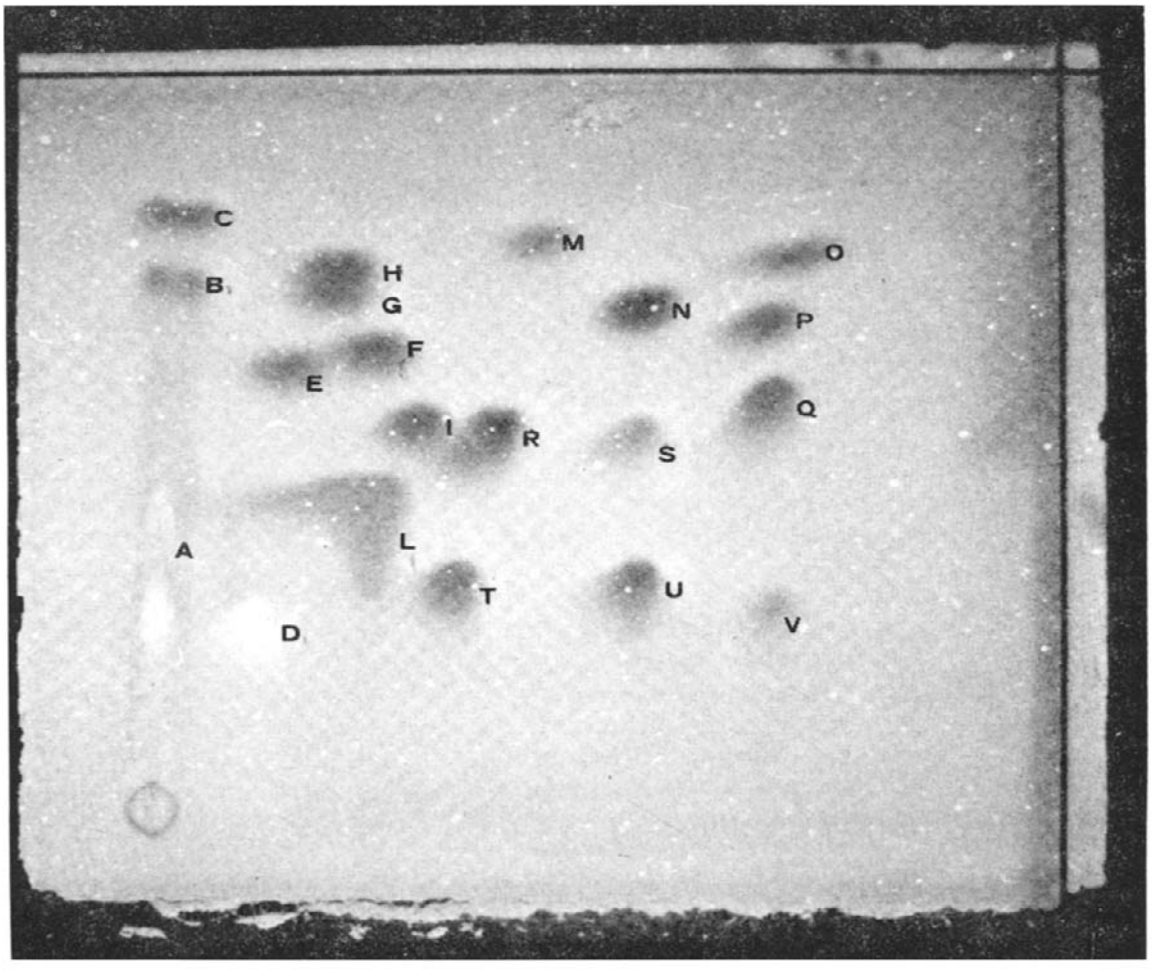

Fig. 2. Chromatogram of the separation of a mixture of nucleic acid derivatives. A = Riboflavine-5'-phosphate; B = AMP; C = orotic acid; D = riboflavine; E = guanosine; F = cytosine; G = inosine; H = cytidine; I = hypoxanthine + adenosine; L = xanthine; M = uridine; N = uracil; O = thymidine; P = thymine; Q = nicotinic acid; R = 1-methylhypoxanthine; S = 7-methylxanthine; T = adenine; U = N-methylaminopurine; V = N-dimethylaminopurine.

Fig. 2 shows a chromatogram of the separation of a mixture of nucleic acid derivatives used as a reference standard.

Fig. 3 shows the re-chromatography of Fraction A on the QAE-Sephadex column. There are three microbiologically active peaks. The fractions corresponding to the first peak (Fraction B) do not contain cyanocobalamin, for when re-chromatographed on cellulose they do not exhibit a microbiological activity when tested against *E. coli*, which is in agreement with the findings for the cyanocobalamin used for reference.

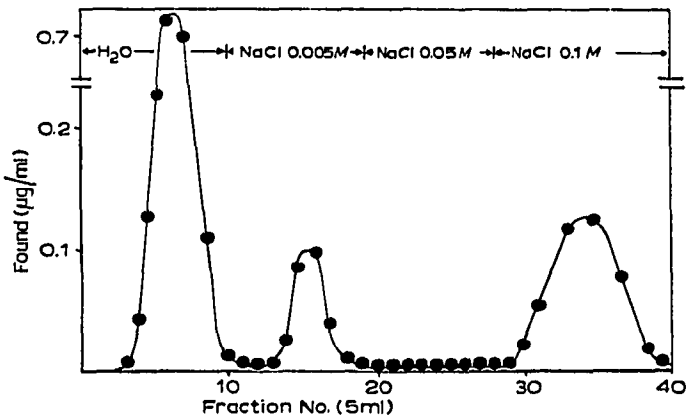

Fig. 3. Chromatographic separation on QAE-Sephadex A-25 of Fraction A isolated on SP-Sephadex. Eluents, as indicated in the figure; column, 20 × 1.5 cm I.D.; flow-rate, 0.4 ml/min. Each 5-ml fraction was determined microbiologically by means of the *E. coli* test.

An unidentified compound is found the R_F value of which (0.2) corresponds neither to that of cyanocobalamin (R_F 0.5) nor to that of hydroxycobalamin (R_F 0.015). Using the technique described by Heathcote and Haworth[14], the following amino acids were identified in Fraction B: leucine, isoleucine, phenylalanine, valine, thyroxine and tryptophan.

Methionine, which, as is known, is responsible for the growth of the bacterium in question, is not present.

DISCUSSION

From the above analyses it is seen that the cobalamin content of the liver extracts investigated is formed largely by cobamamide and, to a lesser extent, by methylcobalamin. The presence of hydroxycobalamin may be due to the decomposition of cobamamide or of methylcobalamin during extraction and purification. However, no trace of cyanocobalamin was found in the extracts. Methionine, which, as is known, is responsible for the growth of *E. coli* mutant 113-3, is also absent. Further tests are now being carried out to determine the unidentified and microbiologically active fractions. The chromatographic technique proposed is used in conjunction with thin-layer chromatography for the determination of nucleic acid derivatives and vitamins[15] in our control laboratories for the assay of commercial liver extracts.

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