Separation of isopropylnoradrenaline and the corresponding O-methyl derivative using a tandem chromatographic procedure on cellulosophosphate and carboxymethylcellulose. For the separation of both catecholamines from epinephrine and norepinephrine two columns 0.6 × 40 cm combined in series were used. These columns were filled up to 35 cm with cellulosophosphate Whatman CP 11 (Column No I) and carboxymethyl cellulose Whatman CM 32 (Column No II). Columns were eluted by a linear gradient of ammonium acetate buffer with a concentration change from 0.05 to 0.25 *M*. The pH of the buffer was adjusted previously to 6.1; flow rate on both columns was less than 0.8 ml/min. Fractions of 2 ml were collected. Columns were loaded with 0.5 ml of a sample obtained after either purification procedure. The elution of the column was stopped after 100 ml of the eluant had passed through the system. Under these conditions *O*-methylisopropylnoradrenaline is eluted in fractions No. 3–7, isopropylnoradrenaline in fractions No. 10–15 (see Fig. 1).

Oxidation of isopropylnoradrenaline and its O-methyl derivative to the corresponding lutines. Combined fractions 3–7 and 10–15 were evaporated to a final volume of 0.2 ml, diluted to 1 ml with borate buffer (0.66 *M*, pH 7) and the following reagents were added subsequently: 0.05 ml of 0.02 % $CuCl_2 \cdot 2H_2O$ and 0.05 ml of 0.25 % potassium ferricyanide. The reaction was stopped after 3 min by adding 0.05 ml of 10 % BAL in 25 % formaldehyde. After 10–20 sec 0.2 ml of 10 *N* NaOH was added, and after another 5 min 0.15 ml of glacial acetic acid was pipetted into the sample. The reaction mixture was vigorously mixed and subjected to fluorimetric evaluation.

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Separation and determination of cobalamins on an SP-Sephadex column

In a previous paper[1] we have described the separation of a mixture of methylcobalamin, cobamamide, cyanocobalamin and hydroxocobalamin by means of successive chromatograms carried out on CM-cellulose and Dowex 50 W-X2 columns. The present note describes a simple method of separating the above derivatives of vitamin

B_{12} using a single ion-exchange column of SP-Sephadex. The proposed method is also used for the quantitative determination of cobalamins.

Experimental

Apparatus and materials. The following apparatus was employed: a Beckman DU-2 spectrophotometer; a Fractomat Y-3 with photoelectric cell for the automatic collection of fractions; a Photocrom twin-ray spectrophotometer and a Sargent S.R. recorder for the analysis of effluents and a Beckman 746 peristaltic pump (1–10 ml/min). SP-Sephadex C-25 (Na^+) was obtained from Pharmacia, Uppsala, Sweden. Cobalamins: cyanocobalamin USP, methylcobalamin and cobamamide were obtained by partial synthesis in the Research Laboratories of Alfa Farmaceutici S.p.A. by DR. VITALE and DR. GUERRA. Commercial hydroxocobalamine was spectrophotometrically and chromatographically pure.

Preparation of the resin and the column. SP-Sephadex was dispersed in a beaker with a 0.05 *M* sodium acetate buffer (pH 5.0) by means of an electromagnetic shaker and then poured into the chromatographic column (diameter 0.9 cm) until it reached a height of 20 cm after settling. It was repeatedly washed with distilled water to remove excess ions, and the eluate was checked for spectrophotometric purity in the wavelength range 260–400 nm.

Elution. 2 ml of a solution containing 50 μg/ml of each cobalamin were placed at the top of the column containing the cationic Sephadex. First, elution was carried out with 40 ml of distilled water and then with 70 ml of 0.05 *M* CH_3COONa–CH_3COOH buffer (pH 5.0), 2-ml fractions being collected. The rate of elution was maintained at a constant value of 0.4 ml/min.

Cyano- and methylcobalamin were eluted separately with distilled water, while cobamamide and hydroxocobalamin remained fixed at the top of the resin. They can be separated by increasing the ionic force of the eluent. Each 2-ml fraction was read against a blank of the same eluent at the wavelength corresponding to the absorption peak typical of each cobalamin (Table I). All the operations were conducted in dim red light to avoid photolytic degradation of the cobalamins.

Results

Fig. 1 shows the separation of the cobalamins on SP-Sephadex which proves particularly suitable for the quantitative determination of labile substances.

Quantitative determination. Table I shows the percentages of the four cobalamins recovered, the wavelengths at which the spectrophotometric readings of the single

TABLE I

% RECOVERY OF COBALAMINS SEPARATED ON COLUMN OF SP-SEPHADEX C-25 (Na^+)

Cobalamin	% *Recovery* ± *S.D.*[a]	λ_{max}	$\varepsilon \times 10^{-3}$
Cyanocobalamin	103 ± 1.2	361	28.056 (ref. 2)
Methylcobalamin	96.6 ± 0.5	340	14.002 (ref. 3)
Hydroxocobalamin	99.2 ± 0.8	351	25.985 (ref. 4)
Cobamamide	97.2 ± 1.7	338	12.636 (ref. 5)

[a] Standard deviation based on 8 determinations.

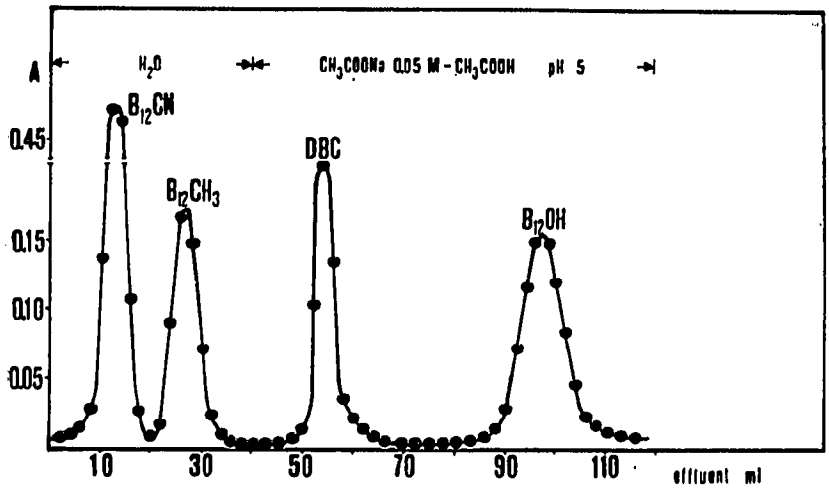

Fig. 1. Chromatographic separation of cyanocobalamin ($B_{12}CN$), methylcobalamin ($B_{12}CH_3$), hydroxocobalamin ($B_{12}OH$) and cobamamide (DBC) on a 0.9 × 20 cm column. $B_{12}CN$ and $B_{12}CH_3$ eluted with distilled water at flow rate of 24 ml/h. DBC and $B_{12}OH$ eluted with 0.05 *M* acetate buffer (pH 5.0). The volume of each fraction is 2 ml. Absorbance measurements at the absorption of each cobalamin.

fractions were carried out and the molar absorption values used to calculate the amounts recovered.

Discussion

The chromatogram in Fig. 1 shows good separation of the cobalamins which were eluted in symmetrical peaks with very few ml of eluent. Cyanocobalamin is neutral and is therefore the first to be eluted with distilled water separately from methylcobalamin, which, presumably on account of the inductive effect of the methylic group imparting a very slight basicity to the molecule, is delayed.

The molecule of adenine and the hydroxyl group bonded to the cobalt atom impart a certain basicity to cobamamide and hydroxocobalamin. With distilled water they remain at the top of the column and their elution is only possible by increasing the ionic force of the eluent.

Since cobamamide and hydroxocobalamin have different basicities it is not necessary to adopt a gradient of concentration and/or pH.

The data reported in Table I show that the technique used is satisfactory from a quantitative point of view as well, errors being of the order of ± 5 %.

The method described is easily performed and requires about 4 h.

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