*Journal of Chromatography*, 529 (1990) 81–91
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 5301

# High-performance liquid chromatographic separation and dual competitive binding assay of corrinoids in biological material[a]

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(First received November 13th, 1989; revised manuscript received March 2nd, 1990)

## ABSTRACT

Corrinoids were extracted with hot ethanol from human plasma and faeces and separated by high-performance liquid chromatography. The corrinoids (cobalamin and cobalamin analogues) were quantified in the eluted fractions by a dual radioisotope assay using as binders intrinsic factor and haptocorrin to detect cobalamin and total corrinoids, respectively. Recoveries ranged from 37.7 ± 5.1% for hydroxycobalamin to 75.0 ± 9.1% for cyanocobalamin. In plasma, the main forms of cobalamin were the coenzymes methylcobalamin and 5′-deoxyadenosylcobalamin (32.1 ± 13.4 and 28.4 ± 12.3%, respectively, of total corrinoids). The cobalamin analogue fraction of plasma was eluted with a retention time close to that of cobinamide and of deoxyadenosylcobalamin. In the faeces, most of the corrinoids separated were detected better by the haptocorrin assay than by the intrinsic factor assay. One corrinoid peak was eluted with the same retention time as cobinamide. This peak was detected by haptocorrin assay but not by intrinsic factor assay. It could therefore correspond to cobinamide.

## INTRODUCTION

Five different cobalamins (Cbl) have been identified in human plasma: methylcobalamin (MeCbl), 5′-deoxyadenosylcobalamin (AdoCbl) (the two coenzymatic forms of Cbl), hydroxycobalamin (OH-Cbl), cyanocobalamin

[a]This work was presented in part at the First International Symposium on Biomedicine and Physiology of Vitamin $B_{12}$, London, September 1988.

0378-4347/90/$03.50 

(CN-Cbl) and traces of sulphitocobalamin [1–4]. The separation of the different forms of Cbl has been achieved by thin-layer chromatography [3,4], paper chromatography, ionophoresis [1,5], ion-exchange chromatography [6] and more recently by high-performance liquid chromatography (HPLC) [7–9]. Before the separation, Cbl must be extracted from biological samples [2].

The extraction led to denaturation of OH-Cbl [8]. This problem was solved by adding cadmium acetate or N-ethylmaleimide to the extraction medium [8,10]. The quantification of corrinoids must distinguish the cobalamin forms from the other corrinoids, which are named Cbl analogues. These Cbl analogues lack vitamin $B_{12}$ activity. They can be divided into two groups: cobinamides (Cbi), which lack the nucleotide moiety, and cobamides, which contain nucleotide other than the 5,6-dimethylbenzimidazole involved in the structure of cobalamin. At present, only one indirect method is available, which distinguishes the "true Cbl" from total corrinoids. This method is a dual radioisotope dilution assay, which uses intrinsic factor and haptocorrin as binders [11,12].

Previous reports suggested that fairly large amounts of unidentified Cbl analogues are present in human and animal tissues [11–15]. They originate from the intestinal bacterial synthesis [16–19]. Very little is known about the nature or even the presence of Cbl analogues in plasma. Their affinity for haptocorrin and intrinsic factor is unknown. Thus, the radioisotope dilution assays used for quantifying Cbl analogues in plasma remain dubious [20]. A definitive answer to their hypothetical presence in plasma has to be given since these analogues are potentially harmful to the organism [14,15].

This paper describes how corrinoids have been extracted from plasma and faeces, analysed by reversed-phase HPLC, and quantified by dual radioisotope dilution assay.

## EXPERIMENTAL

### *Chemicals*

Crystalline OH-Cbl, CN-Cbl, MeCbl and AdoCbl were obtained from Sigma (St. Louis, MO, U.S.A.), crystalline CN-Cbi from Calbiochem (La Jolla, CA, U.S.A.) and $^{57}$Co-labelled CN-Cbl (specific activity 220 Ci/$\mu$g) from The Radiochemical Centre (Amersham, U.K.). Cadmium acetate, glacial acetic acid, triethanolamine, *tert.*-butanol, methanol, acetonitrile and Amberlite XAD2 were obtained from Merck (Darmstadt, F.R.G.). The Sep-Pak $C_{18}$ cartridges were purchased from Waters Assoc. (Milford, MA, U.S.A.) and the LiChrospher RP18 glass column (5-$\mu$m silica, 250 mm × 5 mm I.D.) from Merck.

### *Biological fluids*

All samples were handled in the dark or in dim-red illumination. Plasma was separated from the whole blood within 1 h after puncture, to minimize the

liberation of corrinoids from red cells, which contain substantial amounts of corrinoids [21].

The faecal samples were treated immediately after 24-h collection: 1 g of faeces was mixed with 3 ml of 0.1 *M* sodium phosphate buffer (pH 7.4), containing 5000 U/l aprotinin (Laboratoire Choay, Paris, France), 0.02 m*M* phenylmethylsulphonyl fluoride, 3 m*M* sodium azide and 0.05% (v/v) Triton X100, under rotative agitation for 24 h at 4 °C. The faecal extract was removed after centrifugation at 19 000 *g* for 60 min. The faecal supernatants were stored at −80 °C and analysed within twenty days of collection.

*Extraction of corrinoids*

The volume of samples was 10 and 3 ml, respectively, for plasma and faeces extracts. Corrinoids were extracted with hot ethanol to denature and to precipitate the corrinoid-binding proteins. To prevent the non-specific binding of OH-Cbl to histidine residues of proteins, the samples were preincubated for 2 h at room temperature with an excess of cadmium acetate (0.2 *M*) [10]. The samples were added to four volumes of absolute ethanol preheated to 80 °C and mixed vigorously. The mixture obtained was incubated for 20 min at this temperature. The sample was centrifuged at 2000 *g* for 10 min after cooling it in an ice-bath. The supernatant was kept, and the precipitate was mixed with two volumes of cold 80% (v/v) ethanol. After centrifugation, the two supernatants were pooled and evaporated to dryness in a rotary evaporator at 40 °C.

*Desalting*

The extracts of plasma were desalted on a Sep-Pak $C_{18}$ cartridge as described previously [9]. The faecal extract was desalted by two successive treatments with Amberlite XAD2 and with Sep-Pack $C_{18}$. The Sep-Pak $C_{18}$ cartridges were successively rinsed with 2 ml of acetonitrile and 6 ml of bidistilled water. Dry extracts were dissolved in 2 ml of bidistilled water and applied to the cartridges. Each cartridge was then washed with 12 ml of bidistilled water and eluted with 6 ml of *tert.*-butanol (20%, v/v). The eluate was evaporated to dryness.

Amberlite XAD2 (Merck) was prepared as described by Gimsing and Beck [9] and packed in a 30-ml glass column. Dry extracts were dissolved in 2 ml of solvent A [1% (v/v) acetic acid in bidistilled water] and applied to the column. The column was washed successively with 12 ml of solvent A, 12 ml of solvent A containing 10% methanol (v/v) and 30 ml of solvent A containing 5% methanol (v/v). After use, the column was washed with 20 ml of methanol containing 0.1 *M* potassium hydroxide.

*Reversed-phase HPLC separation*

HPLC was carried out at room temperature using a two-pump gradient system (Waters Assoc.). The corrinoid peaks were detected at 254 nm (UV1, Pharmacia, Uppsala, Sweden) and at 365 nm (lambda-max 480, Waters As-

soc.). Corrinoids were separated on a $C_{18}$ column (Merck) using the method of Gimsing and Beck [9]. The mobile phases consisted of 0.085 *M* phosphoric acid titrated to pH 3.0 with triethanolamine (phase A) and acetonitrile (phase B). A 10–50% linear gradient of phase B was performed in 20 min at a flow-rate of 0.5 ml/min. The effluent was collected in 0.5-min fractions.

*Radioisotope dilution assay*

The concentration of corrinoids was determined in duplicate in the HPLC fractions using a radioisotope dilution assay [20] adapted from Kolhouse et al. [12]. The eluted fractions were evaporated to dryness and redissolved in 500 μl of distilled water; 100 μl of the sample were needed per test-tube. The test-tubes were protected from light during the assay. The samples were heated at 100°C for 15 min after the addition of 24 fmol of $^{57}$Co-labelled CN-Cbl in 1 ml of 0.082 *M* borate buffer (pH 9.2) containing 0.02 *M* sodium azide, 0.1 *M* urea, 0.3 m*M* potassium cyanide, 0.3% thioglycerol, 0.05% dithiothreitol (w/v) and 0.02% bovine serum albumin (BSA) (w/v). Hog haptocorrin and hog intrinsic factor (9.1 fmol per test-tube) were purified [23,24] and used as binders. A haemoglobin-coated charcoal suspension was used to adsorb the free corrinoids and to remove them by centrifugation at 2000 *g* for 15 min as described by Gottlieb et al. [25]. The results obtained with intrinsic factor (intrinsic factor assay) were considered to represent the "true Cbl" concentration and those obtained with haptocorrin (haptocorrin assay), the total corrinoid concentration. The standard curve of the two assays was established using six solutions with respective CN-Cbl concentrations of 55, 110, 220, 440, 886 and 1770 p*M*.

*Estimation of recovery of added cobalamins*

Samples of plasma and faeces extracts were incubated for 20 min at room temperature with 0.4 pmol of $^{57}$Co-labelled CN-Cbl, which was extracted and analysed as described above. The recovery was estimated by measuring the radioactivity before extraction and after HPLC. The recovery of each cobalamin form was determined in plasma by incubating 10 ml of pooled plasma poor in Cbl with 11 pmol of CN-Cbl, OH-Cbl, AdoCbl, MeCbl or $(CN)_2$-Cbi at room temperature for 20 min. The recovery was estimated by measuring the amount of corrinoids by radioisotope assay in the initial sample and in the HPLC fractions.

## RESULTS

A sample containing 3 μ*M* OH-Cbl, 3 μ*M* CN-Cbl, 2 μ*M* AdoCbl, 1.5 μ*M* MeCbl and 4 μ*M* $(CN)_2$-Cbi was analysed by HPLC. The corrinoids were eluted in the following order: OH-Cbl, CN-Cbi, CN-Cbl, CN-Cbi, AdoCbl and MeCbl (Fig. 1). The retention times obtained in nineteen different experi-

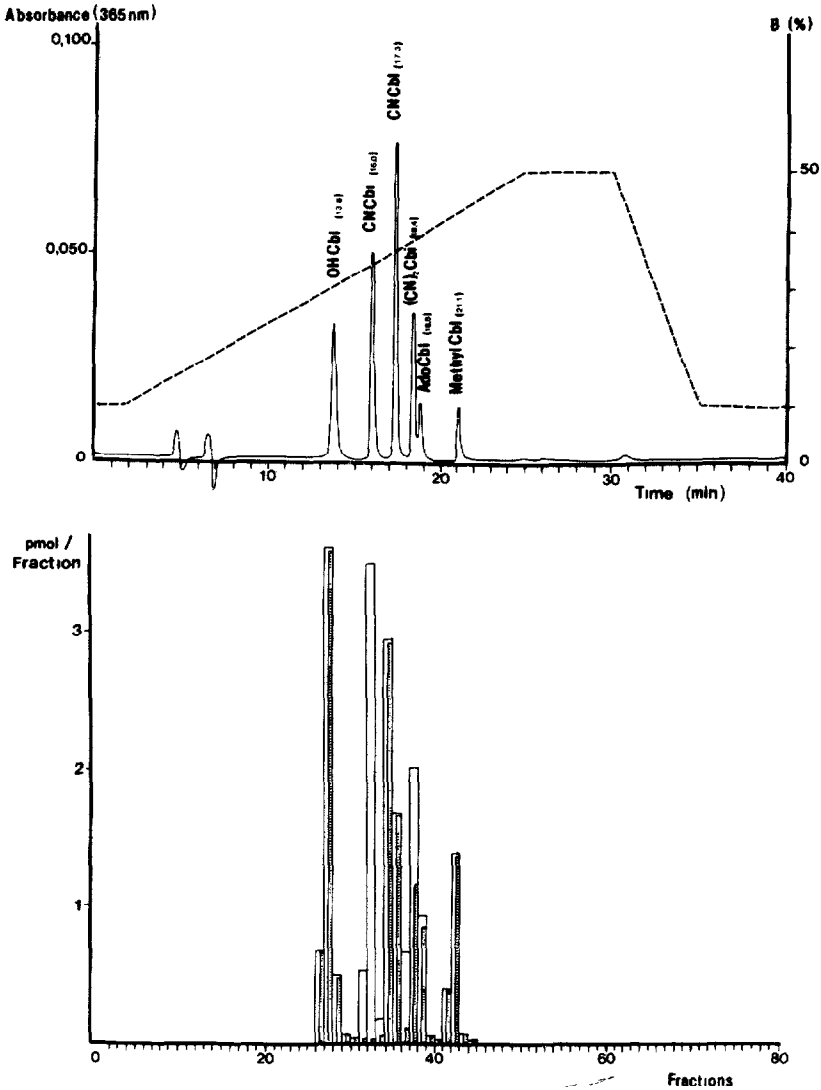


Fig. 1. Separation by reversed-phase HPLC (top) and detection by radioisotopic assay (bottom) of a mixture of reference cobalamins and cobinamide. HPLC was performed with a 250 mm×5 mm I.D. LiChrospher RP 18 column eluted with a gradient of acetonitrile (%, B, ---). The corrinoids in the eluted fraction were quantified by a dual radioisotope assay. Intrinsic factor was used as binder for the detection of cobalamins (▤), and haptocorrin was used for the quantitation of all the corrinoids (▭), e.g. cobalamins and cobalamin analogues.

TABLE I

EFFECT OF THE EXTRACTION PROCEDURE ON THE RECOVERIES AND RETENTION TIMES OF COBALAMINS AND COBINAMIDE

| Corrinoid | Retention time[a] (min) | | Recovery[b] (%) |
|---|---|---|---|
| | Before extraction | After extraction in hot ethanol | |
| OH-Cbl | 14.68 ± 0.63 | 14.47 ± 0.80 | 37.7 ± 5.1 |
| Cbi | 15.82 ± 0.51/18.48 ± 0.18 | 15.97 ± 0.58/18.26 ± 0.56 | 68.7 ± 5.2 |
| CN-Cbl | 17.21 ± 0.31 | 17.22 ± 0.25 | 75.0 ± 9.1 |
| AdoCbl | 18.66 ± 0.45 | 18.49 ± 0.28 | 45.3 ± 3.0 |
| MeCbl | 20.60 ± 0.47 | 20.39 ± 0.43 | 59.7 ± 7.2 |

[a]Data are means ± S.D. from nineteen determinations on nineteen different days.
[b]Data are means ± S.D. from six determinations of the concentration of corrinoids added to plasma and of corrinoids recovered in HPLC fractions. The corrinoid concentration was determined with the haptocorrin radioisotope assay.

ments were determined with standard corrinoids either treated or untreated by the extraction procedure (Table I). These retention times were very reproducible. Each reference corrinoid had an identical retention time before and after the extraction procedure. Cbi injected alone appears as two peaks, as described by others [7,9]. The treatment of standard corrinoids with hot ethanol did not modify the absorption spectrum (data not shown). The peaks corresponding to the different forms of Cbl had the same intensity when measured by the haptocorrin assay or by the intrinsic factor assay. In contrast, Cbi was detected by the haptocorrin assay but not by the intrinsic factor assay. The amount of cobinamide detected per fraction by the intrinsic factor assay represented less than 2% of the amount detected with haptocorrin. The elution position of Cbi was very close to that of AdoCbl, which resulted in their co-elution when detected by the radioisotope assay in the corresponding fraction (Fig. 1).

The recovery of the different standard solutions of corrinoids varied considerably (Table I). When $^{57}$Co-labelled CN-Cbl was added to plasma and faeces, the yield of the total procedure was estimated to be 74 and 60%, respectively.

Plasma samples from fifteen healthy subjects were analysed. The criteria for identification of each form of Cbl in plasma were that the HPLC retention time was identical with the corresponding standard substance and that the corrinoid was detected in the corresponding HPLC fraction by the intrinsic factor assay, as well as by the haptocorrin assay (Fig. 2). The distribution of corrinoids in plasma is presented in Table II: MeCbl and AdoCbl were the main forms of Cbl found, representing 32.1 ± 13.4 and 28.4 ± 12.3% of total corrinoids, respectively. In fact, the peak that eluted in the position of AdoCbl was detected better with the haptocorrin assay than with the intrinsic factor assay

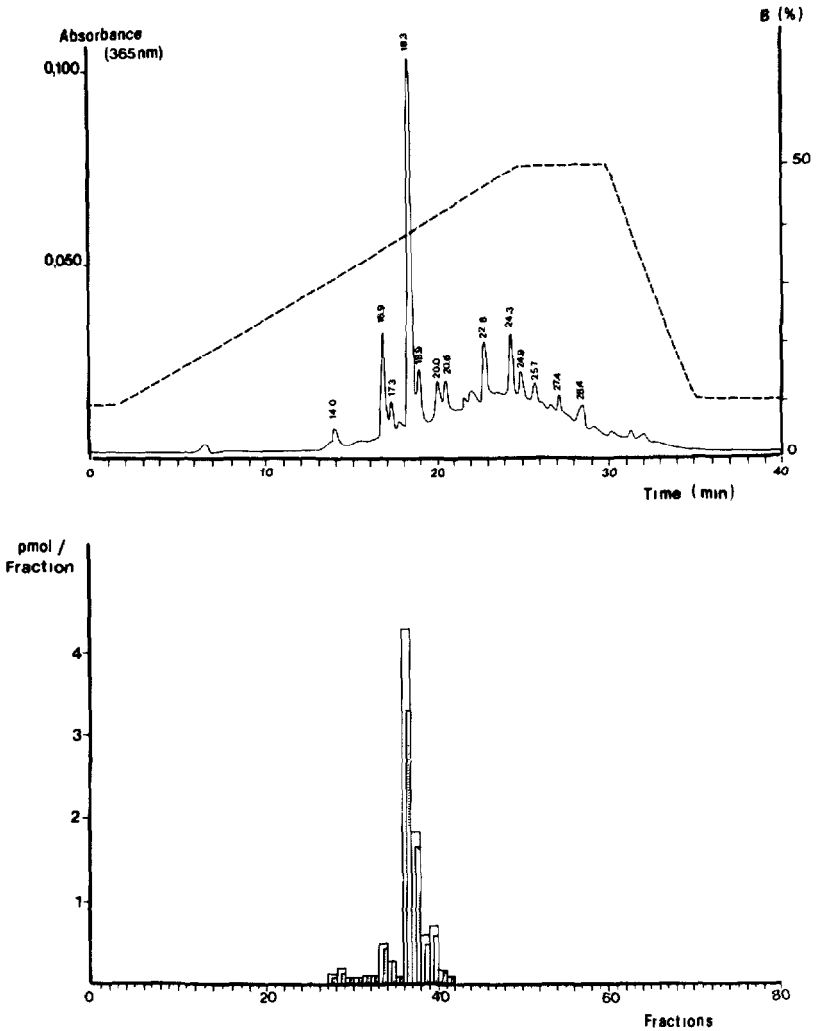


Fig. 2. (Top) HPLC separation of corrinoids from plasma using a gradient of acetonitrile (% B, ---). (Bottom) The corrinoids were detected by a dual radioisotope dilution assay using haptocorrin and intrinsic factor, respectively, as binders to quantify the total corrinoids (□) or only the cobalamins (▤).

(Fig. 2). This could correspond to the presence of cobinamide in the sample, as observed with a mixture of standard corrinoids (Fig. 1). In the faeces extracts, the corrinoid peaks were mainly detected by the haptocorrin assay, showing a high proportion of Cbl analogues (Fig. 3). The first eluted peak had the same retention time as Cbi; it was detected only by the haptocorrin assay and not by the intrinsic factor assay. The concentration of total corrinoids in

TABLE II

PERCENTAGE DISTRIBUTION OF CORRINOIDS IN PLASMA OF NORMAL SUBJECTS

| Corrinoid | Distribution (mean ± S.D., $n=15$) (%) |
|---|---|
| OH-Cbl | 18.26 ± 7.60 |
| CN-Cbl | 1.4 ± 1.0 |
| MeCbl | 33.1 ± 13.8 |
| AdoCbl | 38.6 ± 16.7 |
| Cbl analogue[a] | 8.7 ± 5.2 |

[a]The Cbl analogue corresponds to the difference in the concentrations observed with the haptocorrin assay and with the intrinsic factor assay in the fraction corresponding to the elution of AdoCbl and Cbi (see Fig. 1).

faeces was estimated to be 83.7 pmol/g, and the faecal output of corrinoids to be 17 $\mu$g per day.

## DISCUSSION

Incubation with hot ethanol did not modify the retention times of reference corrinoids (Fig. 1, Table I). This could indicate that corrinoids were not chemically altered during extraction with hot ethanol. It was observed earlier that the methods for the extraction of Cbl from plasma were responsible for the selective loss of OH-Cbl. One explanation was that OH-Cbl binds to histidine residues of proteins [8]. Improved extraction procedures of OH-Cbl have been described in presence of the thiol-blocking agent N-ethylmaleimide [8] or cadmium ions [14]. The latter procedure was used in our work.

The yield obtained with $\gamma$-counting of $^{57}$Co-labelled CN-Cbl was similar to that estimated by radioisotope assay of CN-Cbl (74–75%). The recovery of the different standard corrinoids varied from 37.7 ± 5.1% for OH-Cbl to 75.0 ± 9.1% for CN-Cbl ($n=6$). These data showed the necessity to take into account the recovery of each corrinoid for the calculation of the distribution of corrinoids.

The order of elution of reference corrinoids was similar to that previously reported [7,8]. Cbi was eluted in two peaks: the nucleotide base is absent in this Cbl analogue and the CN can be present as either the upper or the lower axial ligand [7,27].

The elution position of the second peak of Cbi was very close to that of AdoCbl. This resulted in superimposed detection of the two corrinoids by the radioisotope assay in the corresponding fractions: in these fractions, the levels of corrinoids were higher with the haptocorrin assay than with the intrinsic factor assay. The concentration of AdoCbl corresponded theoretically to that

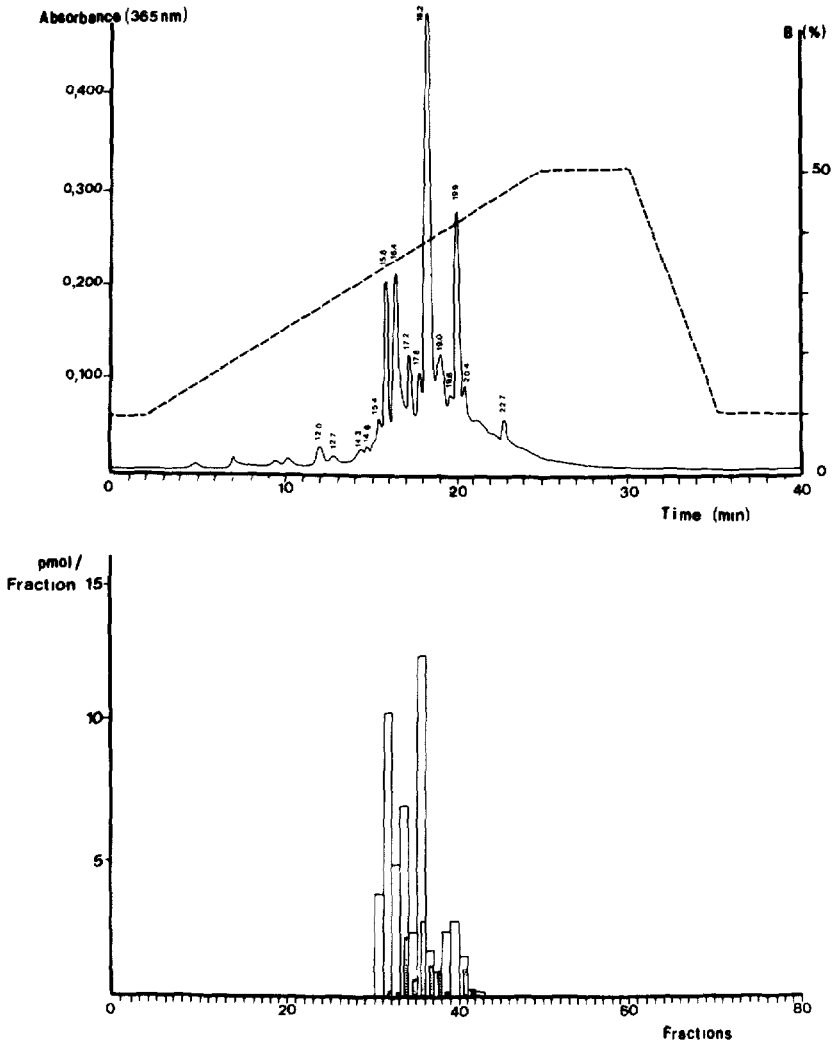


Fig. 3. (Top) HPLC separation of corrinoids from a faeces extract by gradient elution with acetonitrile (% B, ---). (Bottom) The corrinoids were detected mostly by the haptocorrin radioisotope assay (□). Some peaks were also detected in part by the intrinsic factor radioisotope assay (▤), but this was not the case for the first eluted peak. This peak had the same retention time as cobinamide.

measured with the intrinsic factor assay. The intrinsic factor assay gave the same concentrations as the haptocorrin assay for the other peaks of Cbl.

No direct identification of Cbi in plasma has previously been described using either thin-layer chromatography with bioautography [3,26] or HPLC with radioisotope assay [8]. This could be because Cbi is eluted in a position close

to that of AdoCbl in reversed-phase chromatography, and also because the bacteria that are used in bioautography (*Escherichia coli*) give the same growth response for Cbi as for AdoCbl [28].

In plasma, the fraction collected at the retention time of 18–18.5 min contained a higher amount of corrinoids when assayed by the haptocorrin assay than by the intrinsic factor assay (Fig. 2). This is in agreement with the results obtained with standard solutions of AdoCbl and Cbi (Fig. 1). This fraction could therefore contain a mixture of Cbi and of AdoCbl. The distribution of corrinoids in plasma was estimated by taking into account the recovery of the different reference substances (Table II). The two predominant forms of Cbl were the coenzymes AdoCbl and MeCbl (Table II). We observed a higher proportion of AdoCbl than that reported by others [8,26]. In fact, the distribution of the different forms of Cbl showed a wide variation (Table II), and this phenomenon has been also observed by Gimsing et al. [8] in the plasma of normal subjects. Van Kapel et al. [8] separated the corrinoids of plasma by HPLC and measured the fractions by a dual radioisotope assay with intrinsic factor and haptocorrin as binders. They observed a higher content of corrinoids with the haptocorrin assay than with the intrinsic factor assay in all the fractions of corrinoid collected. In our study, such a difference was observed only with the "AdoCbl/Cbi" fraction (Figs. 1 and 2), which suggests the presence of a predominant Cbl analogue in the plasma of normal subjects.

The total level of corrinoids in faeces was $83.7 \pm 22$ pmol/g, corresponding to a daily excretion of the order of 17 $\mu$g. These results must not be considered as a definitive estimation of the faecal excretion of corrinoids since they have been obtained with only three samples. The level was four-fold higher than that in faeces of children, using gel permeation and radioisotope assay [28]. Treatment with ethanol seemed to be an efficient method for the extraction of corrinoids from the faeces. The desalting on Amberlite XAD2 was done to eliminate biliary salts and porphyric derivatives, which give artefacts in the radioisotope assay. In our study $94.8 \pm 3.9\%$ of the corrinoids present in faeces corresponded to Cbl analogues (Fig. 3). Herbert et al. [19] estimated the excretion of total corrinoids to be 100 $\mu$g per day. This value is ca. six-fold higher than our estimation. However, these authors did not centrifuge the faecal extracts and therefore measured the soluble corrinoids as well as the corrinoids contained in bacteria. One form of Cbl analogues present in faeces extracts could correspond to CN-Cbi, since it had the same retention time as CN-Cbi and it was detected by the haptocorrin assay but not by the intrinsic factor assay.

In conclusion, we have combined an HPLC method [9] for separating corrinoids in plasma and faeces extracts and a dual radioisotope assay for quantitating them in collected fractions. Faeces extracts are an important source of Cbl analogues and one of these analogues could be cobinamide. In plasma, one

predominant Cbl analogue is detected, which is eluted with a retention time close to that of AdoCbl.

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