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Short communication

Evaluation of coupling of cobalamin to antisense oligonucleotides by thin-layer and reversed-phase liquid chromatography

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Abstract

We have evaluated by chromatography two strategies of oligonucleotide binding to vitamin B12 (cobalamin). The first one was based on a covalent linkage of aminooligonucleotide to carboxycobalamin in presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC). Carboxycobalamin and EDC–cobalamin were eluted with a retention time of 16.5 and 21.6 min, respectively, in RP-HPLC, while aminooligonucleotide and oligonucleotide–cobalamin were coeluted at 19.4 and 19.8 min. In the second strategy, avidin was coupled to both biotinylated oligonucleotide and vitamin B12. Aminocobalamin and biotinylated cobalamin had respective retention times of 13 and 15.7 min in RP-HPLC and respective R_f values of 0.3 and 0.8 in thin-layer chromatography. Incubation of avidin with biotinylated cobalamin produced, in Superose 12 gel permeation, a peak with a retention time of 28 min, which corresponded to avidin–biotinylated cobalamin as it disappeared with an excess of either biotin or biotinylated oligonucleotide. In conclusion, we have prepared and purified by RP-HPLC and gel permeation chromatography an oligonucleotide–avidin–cobalamin complex which will be used as a vector complex of antisense oligonucleotides.

Keywords: Cobalamin; Vitamin B_{12}; Oligonucleotides

1. Introduction

Antisense therapy of diseases that are due to aberrant gene expression is an exciting possibility. Of central importance to the long-term future of therapeutic oligonucleotides has been the rationalization of the oligonucleotides in the context of modern pharmacology [1–3]. Progress has been gratifying in that most of the fundamental concerns about the oligonucleotides are now answered. With six oligonucleotides in clinical trials, numerous demonstrations of potent systemic activities, and clarification of potential toxicities, it is reasonable to conclude that substantial progress has been achieved.

Two of the significant challenges that remain to be overcome before these compounds can realize their full potential as therapeutic agents are: (1) their limited oral bioavailability [1], (2) their pattern of broad and unspecific distribution after parenteral administration [4]. Conjugation to appropriate vectors may help to solve both these problems [5–8].

Vitamin B12 (cobalamin) is absorbed in the distal ileum [9] by receptor-mediated endocytosis of a complex formed with a protein termed intrinsic factor. After passage through the enterocyte, the vitamin enters portal blood, is transported to the liver and in this way gets access to the systemic circula-

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tion [10,11]. The rate of absorption of a vitamin B12-derivatized oligonucleotide from the intestine should, thus, be greater than that of the corresponding underivatized compound. Furthermore, since vitamin B12 is indirectly involved in the production of thymidilate and DNA, it has increased uptake in rapidly dividing tissues. Up-regulation in the number of transcobalamin II receptors, a carrier protein responsible for cobalamin transport within the intra/extracellular space, has been demonstrated in several malignant cell lines during accelerated thymidine incorporation and DNA synthesis [12–14]. Vitamin B12 conjugation to oligonucleotides may, thus, help delivering oligomers to rapidly dividing cells, such as cancer cells, in vivo.

In this work, we have tested two strategies for antisense oligonucleotide conjugation to vitamin B12. In the first one, oligonucleotide was covalently linked to an aminocobalamin derivative according to the method described by Russel-Jones et al. [15] for conjugating vitamin B12 to peptides and proteins. The second strategy consisted of preparing a biotinylated cobalamin which was subsequently complexed with biotinylated oligonucleotide via binding of biotinylated residues of these two compounds to avidin tetramers [16,17]. These two methods were evaluated by analysing the conjugate products by thin-layer chromatography (TLC), reversed-phase chromatography (RP-HPLC) and gel permeation chromatography.

2. Experimental

2.1. Chemicals

Crystalline cyanocobalamin (Cbl) and 2-(N-morpholino)-ethanesulfonic acid (MES) were obtained from Sigma (St. Louis, MO, USA); 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and sulfosuccinimidyl 6-(biotinamido)-hexanoate (sulfo-NHS-LC-biotin) were purchased from Pierce (Rockford, IL, USA); ethylenediamine and trifluoroacetic acid (TFA) from Aldrich (Steinheim, Germany). [^{57}Co]Cobalamin (spec. act. 284550 μCi/μmol) was obtained from Amersham (Les Ulis, France). AG1 X2 column (500×25 mm I.D.), C_{18} LiChrospher column (250×4 mm I.D., 5 μm) and Superose 12 column (250×10 mm I.D.) were purchased from Bio-Rad (Watford, UK), Interchim (Montluçon, France) and Pharmacia (Uppsala, Sweden), respectively.

2.2. Preparation of oligonucleotide

20-mer phosphorothioate oligonucleotides bearing at their 3′ end either amine or biotinyl linker arms were prepared using an automated synthesizer (model 392, Applied Biosystem, Roissy, France) by solid-phase phosphoramidate technique according to the manufacturer's protocol. Functionalization at the 3′ end was performed during the synthesis by suitable derivatized controlled pore glass solid supports (Clontech, Montigny-le-Bretonneux, France). After cleavage from the column and deprotection in concentrated ammonium hydroxide, purification of the modified oligonucleotides was accomplished using oligonucleotide purification elution cartridges (Applied Biosystem).

2.3. Preparation and purification of monocarboxycobalamin (carboxy-Cbl)

Carboxy-Cbl was prepared according to the method of Houts [18]: 0.74 nmol Cbl was hydrolysed in 200 ml of 0.5 *M* HCl at 37°C for 3 h, the reaction mixture was neutralized with 0.5 *M* NaOH and desalted by extraction into phenol [19]. The Cbl derivatives were back-extracted into water by adding diethyl ether (3 volumes) and acetone (2 volumes). The aqueous layer was then concentrated to 50 ml in a rotary evaporator (bath temperature 50°C) and applied to a column of AG1 X2 (acetate form, 200–400 mesh, 500×25 mm I.D.). The column was washed with water to remove unreacted Cbl. The monocarboxylic acids were separated by isocratic elution with 0.04 *M* sodium acetate (pH 4.7) at a flow-rate of 0.5 ml/min. On the basis of their elution positions, the isomers were identified as *b*-acid, *e*-acid and *d*-acid of Cbl. The fractions corresponding to the *d*-acid were collected and desalted by phenol extraction and crystallized from aqueous acetone. The yield of the whole procedure of *d*-acid synthesis was estimated at 4% for the *d*-acid. Identification of the isomers was confirmed by TLC [20].

2.4. Attaching carboxycobalamin to amine linker arms oligonucleotides

A 2.1-nmol quantity of the Cbl monocarboxy-derivative (0.7 μl of a solution at 4 mg/ml in 0.1 *M* MES, pH 6.0) was mixed with 42 nmol of EDC in 1 μl of MES buffer. After 3 min, 42 nmol of antisense amino-oligonucleotide in 20 μl of water were added. The mixture was stirred overnight at room temperature in the dark. Oligonucleotide derivatives were analysed by RP-HPLC using a Gilson HPLC pump, model 305 (Gilson Medical Electronics, Bishheim, France), on a C_{18} LiChrospher column (250×4 mm I.D., 5 μm). A 20-min linear gradient of 5–29% acetonitrile in 0.1 *M* triethylammonium acetate (pH 7.0) was used at a flow-rate of 0.5 ml/min [21]. Oligonucleotides and oligonucleotide derivatives were detected at 260 nm using an UV–Vis absorbance dectector (Spectra-Physics UV 100, Spectra-Physics, Fremont, CA, USA), connected to a Chromato-integrator (Merck D-2000, Merck, Darmstadt, Germany). Vitamin B12 was assayed using the ACS vitamin B12 test following the instructions of the manufacturer (Abbott Labs, Abbott Park, IL, USA). The Cbl was quantified using a calibration curve ranging from 0 to 1498 p*M*.

2.5. Preparation of the biotinylated derivative of Cbl

The reaction was performed using a 20-fold molar excess of ethylenediamine (20 μmol) over the *d*-isomer (1 μmol) and a 20-fold molar excess of EDC (20 μmol) in MES buffer at pH 6.0. The amino-derivative was analyzed by RP-HPLC on a C_{18} LiChrospher column (250×4 mm I.D., 5 μm) using a linear gradient of 5–60% acetonitrile in 0.1% TFA at a flow-rate of 0.5 ml/min and detected at 360 nm using an UV–Vis detector. The fractions corresponding to the peaks were pooled and dried in a Speed Vac Plus (Savant Instrument, Farmingdale, NY, USA). The product was then dissolved in 0.05 *M* sodium phosphate buffer (pH 7.4) containing a 10-fold molar excess of sulfo-NHS-LC-biotin (10 μmol). After a 4-h incubation at room temperature, the biotinylated product was separated by RP-HPLC as described above, pooled and dried. The product was dissolved in 300 μl distilled water.

2.6. Preparation and separation of the biotinylated Cbl–avidin complex

Avidin was saturated with an excess of biotinylated Cbl in 20 m*M* Tris–HCl buffer (pH 7.4) containing 0.15 *M* NaCl. The Cbl–avidin complex was separated from the free Cbl by gel permeation chromatography. The mixture was applied to a Superose 12 column (250×10 mm I.D.) and eluted with the Tris buffer described above at a flow-rate of 0.5 ml/min. The product was detected at 360 nm.

2.7. Inhibition of [^{57}Co]cobalamin binding to IF by avidin–biotinylated Cbl complex

A 0.7-pmol amount of intrinsic factor (IF) from human gastric juice was incubated with 0.7 pmol of [^{57}Co]cobalamin (0.2 μCi) in 20 m*M* Tris–HCl buffer (pH 7.4) 0.15 *M* NaCl. This sample was applied to a Superose 12 column (300×10 mm I.D.), eluted with the Tris–HCl buffer at a flow-rate of 0.5 ml/min. A second sample containing 0.7 pmol of [^{57}Co]cobalamin (0.2 μCi) and 100 μl of the fraction of the avidin–biotinylated Cbl collected from gel permeation (Fig. 3a) was incubated with 0.7 pmol of IF and applied to the Superose 12 column.

2.8. Thin-layer chromatography

The different Cbl derivatives were analysed by TLC using Kieselgel 60 plates (Merck). The elution buffer contained 71.5% of 2-butanol (v/v), 0.5% acetonitrile (v/v) and 28% water (v/v). The R_f values of the different derivatives were expressed as the ratio of the distance migrated by carboxy-cobalamin used as standard to that of the studied compounds.

3. Results

The reaction products corresponding to the coupling of carboxycobalamin to the amino-oligonucleotide were identified by RP-HPLC. When the effluent was detected at 260 nm, the reaction produced one main peak at 19.6 min and three small peaks at 15.3, 16.5 and 21.6 min (Fig. 1a). Injection of the amino-oligonucleotide also revealed a 19.6 min peak and

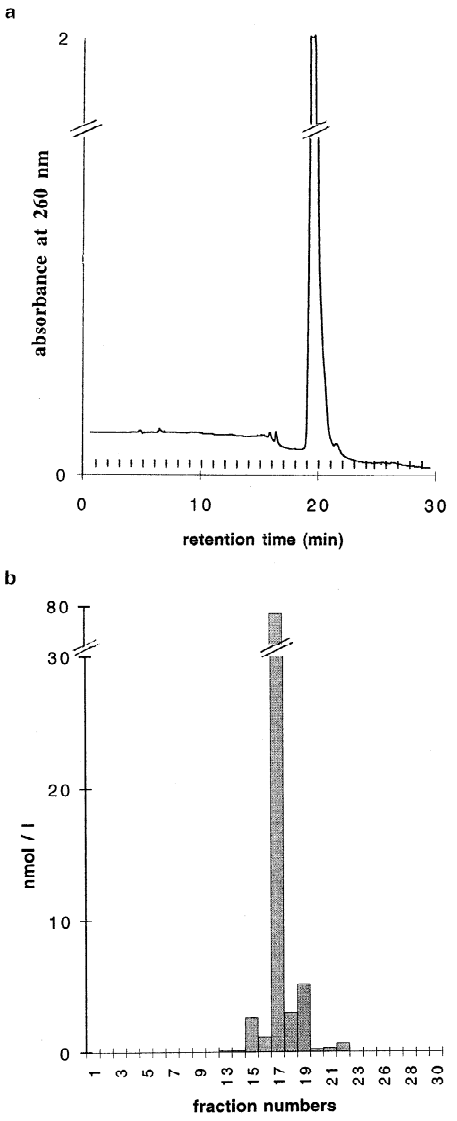

Fig. 1. (a) RP-HPLC elution profile of a sample of the reaction mixture carboxycobalamin–EDC–aminooligonucleotide. Solid-phase was C_{18} LiChrospher column (250×4 mm I.D., 5 μm). The mobile phase A was 0.1 *M* triethylammonium acetate (pH 7.0). Chromatography grade acetonitrile was used as mobile phase B. The sample was eluted using a linear gradient of 5–29% of mobile phase B for 20 min. The flow-rate was 0.5 ml/min. UV detection was performed at 260 nm. The retention time of oligonucleotide was 19.6 min. Carboxycobalamin and EDC–cobalamin were eluted at a retention time of 16.5 and 21.6 min, respectively. (b) Vitamin B12 assay of the fractions corresponding to the RP-HPLC profile shown in (a). Fraction 17 corresponded to free carboxycobalamin (78.5 n*M*) and fraction 22 to EDC–cobalamin (0.5 n*M*). This assay allowed to localize the conjugate in the 19th fraction with a concentration at 5.0 n*M*.

that of carboxycobalamin a 16.5 min peak. A sample corresponding to carboxycobalamin/EDC reaction mixture produced two peaks at 16.5 and 21.6 min which were assigned respectively to the unreacted carboxycobalamin and to an EDC–cobalamin compound. The fractions eluted in RP-HPLC were assayed for cobalamin (Fig. 1b). A high concentration (78.5 n*M*) of cobalamin was detected in the 17th fraction and much lower in the 15th and 19th fractions (2.5 and 5 n*M*, respectively). It was hypothesized from the comparison between these data and the RP-HPLC profiles that the 19th fraction contained the cobalamin–oligonucleotide conjugate which, thus, was not separated by the chromatographic method used. It was also concluded from these data that the yield of the coupling procedure was close to 5%.

The second strategy consisted of preparing a biotinylated cobalamin which was subsequently complexed with the biotinylated oligonucleotide via the binding of biotin residues of the two compounds to avidin tetramers. The carboxycobalamin was coupled to ethylenediamine in presence of EDC, as described by Russell-Jones et al. [15]. The aminocobalamin derivative was then coupled to sulfo-NHS-LC-biotin. The different products obtained at the two steps of the procedure were separated by RP-HPLC and identified by TLC (Fig. 2). The data are summarized in Table 1. The analysis of the mixture carboxycobalamin–diamine–EDC showed two peaks at 13 and 14.5 min in RP-HPLC and each peak showed one spot in TLC with R_f values of 0.3 and 0.2, respectively (Fig. 2a). Carboxycobalamin migration was used as a reference with an R_f value of 1.0. Carboxycobalamin may, therefore, be transformed into an aminocobalamin and an EDC–cobalamin, both being more polar than carboxycobalamin. In contrast, the two RP-HPLC 14.5 and 15.7 min peaks observed with an aminocobalamin/EDC/cobalamin/sulfo-NHS-LC-biotin mixture showed two spots per peak when analysed by TLC. EDC–cobalamin (lower spot) had an R_f of 0.2 and the biotinylated cobalamin (upper spot) an R_f of 0.8 (Fig. 2b). The second chromatographic step, gel permeation with chromatography Superose 12, was performed to separate the avidin complexes from the free biotinylated derivatives. Detection was at 360 nm (Fig. 3). Incubation of avidin with an excess of

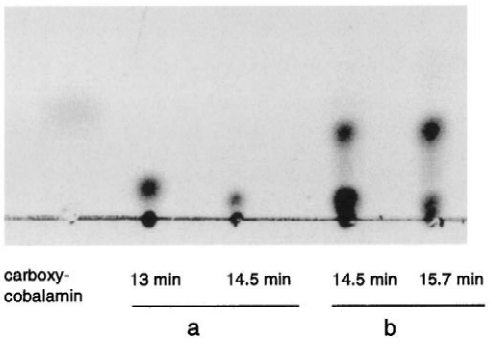

Fig. 2. Thin-layer chromatography of reaction products of carboxycobalamin biotinylation. (a) Analysis of fractions from the 13th and 14.5th min RP-HPLC peaks of carboxycobalamin/EDC/ethylenediamine reaction mixture. The 13.0 and 14.5 min peaks corresponded to aminocobalamin and EDC–cobalamin, with R_f values of 0.3 and 0.2, respectively. (b) Analysis of the fraction from 14.5 and 15.7 min RP-HPLC peaks of cobalamin derivatives–sulfo-NHS-LC-biotin reaction mixture. Two spots were obtained in each fraction, corresponding to EDC–cobalamin and biotinylated cobalamin with R_f values of 0.2 and 0.8, respectively.

biotinylated cobalamin gave two peaks at 28 and 39 min, which corresponded respectively to an avidin–biotinylated cobalamin complex and to free cobalamin derivatives (Fig. 3a). When the same experiment was repeated after preincubating avidin with biotin, the 28 min peak disappeared. This confirmed that this peak corresponded to an avidin–biotinylated cobalamin complex. As estimated by comparison of peak area, the percentage of the total biotinylated cobalamin bound to the tetrameric form of avidin may be estimated at about 8%. The peak corresponding to the complex was still observed when avidin and biotinylated cobalamin were incubated with a similar concentration of biotinylated oligonucleotide (Fig. 3b), but disappeared when the compound was in excess (Fig. 3c). Each fraction collected during the Superose 12 gel permeation step was analysed by UV–Vis spectrophotometry at 260, 280 and 360 nm, in order to follow the elution of oligonucleotide, avidin and cobalamin. The absorbance profile of avidin showed tetrameric, dimeric and monomeric forms with respective retention times of 30, 35 and 37 min. The absorbance profile obtained with a similar concentration of biotinylated oligonucleotide and biotinylated cobalamin in the presence of avidin showed that the 28 min peak contained biotinylated oligonucleotide, tetrameric avidin and biotinylated cobalamin (Fig. 3b). In contrast, with an excess of biotinylated oligonucleotide, the absorbance profile revealed a peak at 33 min containing biotinylated oligonucleotide, and dimeric avidin (Fig. 3c). This confirmed that biotinylated cobalamin competed with the oligonucleotide derivative in its binding to avidin. We, then, tested the ability of the avidin–biotinylated cobalamin complex to compete with [^{57}Co]cobalamin for binding to intrinsic factor (IF). IF–[^{57}Co]cobalamin eluted with a retention time of 38 min in Superose 12 gel permeation chromatography. This peak decreased significantly, corresponding to the release of free [^{57}Co]cobalamin (Fig. 4), after incubation of [^{57}Co]cobalamin with IF in presence of a sample of the avidin–biotinylated cobalamin complex collected

Table 1
Identification of biotinylated cobalamin (biotinylated Cbl) by RP-HPLC[a] and TLC

Reaction mixture	Expected compounds	Retention time of peak from RP-HPLC at 360 nm (min)	R_f^b of the peak in TLC
Carboxycobalamin	Carboxycobalamin	14.8	1
Carboxycobalamin/diamine/EDC	Aminocobalamin, EDC–cobalamin	13.0 14.5	0.3 0.2
Carboxycobalamin/diamine/EDC/sulfo-NHS-LC-biotin	EDC–cobalamin, biotinylated cobalamin	14.5 15.7	0.2 0.8

[a] RP-HPLC using as aqueous solution 0.1% TFA with a gradient of 5–60% acetonitrile in 25 min. Flow-rate of 0.5 ml/min.
[b] R_f was determined, using carboxycobalamin as marker reference (R_f=1).
Abbreviations used: EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride; sulfo-NHS-LC-biotin, sulfo-succinimidyl 6-(biotinamido)-hexanoate; RP-HPLC, reversed-phase high-performance liquid chromatography; TLC, thin-layer chromatography.

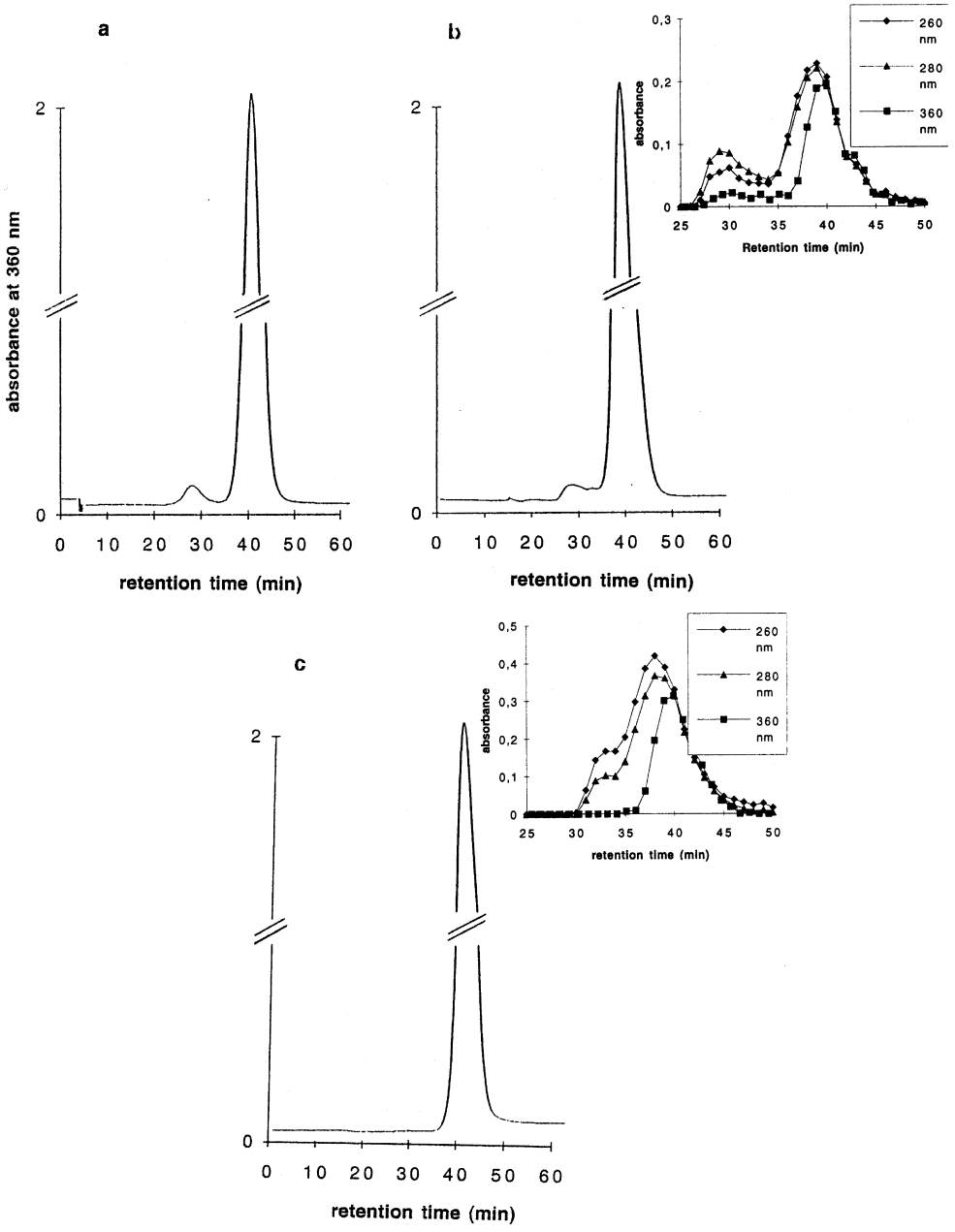

Fig. 3. Superose 12 gel permeation of avidin incubated with biotinylated cobalamin. The samples applied to the Superose 12 column (250×10 mm I.D.) were eluted with 20 m*M* Tris (pH 7.4) containing 150 m*M* NaCl at a flow-rate of 0.5 ml/min. UV absorbance detection was performed at 360 nm. (a) When avidin was incubated with biotinylated sample, the cobalamin–avidin complex and the free cobalamin derivatives were eluted with the retention times of 28 min and of 39 min, respectively. (b) When the same experiment was repeated in presence of a similar concentration of biotinylated oligonucleotide, identical peaks were obtained. Inlet, the absorbance profiles at 260, 280 and 380 nm measured in each collected fraction showed the simultaneous presence of cobalamin, oligonucleotide and tetrameric avidin, in the 28 min peak. (c) When the same experiment was performed with an excess of biotinylated oligonucleotide, the 28 min peak disappeared, showing a competitive inhibition of the biotinylated cobalamin–avidin binding by biotinylated oligonucleotide. The same result was obtained with an excess of biotin. Inlet, the absorbance profile measured at 260, 280 and 360 nm in each collected fraction showed the simultaneous presence of oligonucleotide and the dimeric avidin in the 33 min peak.

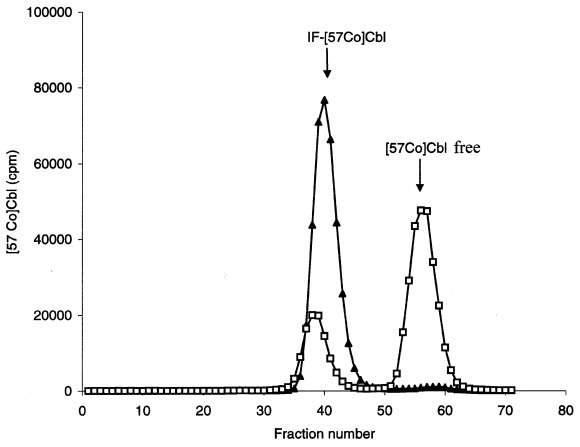

Fig. 4. Inhibition of binding of ^{57}Co-labelled cobalamin to intrinsic factor (IF) by the avidin–biotinylated cobalamin complex. A 0.7 pmol amount of intrinsic factor from human gastric juice was incubated with 0.7 pmol of ^{57}Co-labelled cobalamin (0.2 μCi). The ^{57}Co-labelled cobalamin–intrinsic factor was eluted with a retention time of 38 min in Superose 12 gel permeation (-▲-). This peak disappeared, corresponding to the release of free labelled cobalamin, when the experiment was repeated in presence of 100 μl of the fraction of the avidin–biotinylated cobalamin collected from gel permeation (Fig. 3a) (-□-). The chromatographic conditions are defined in Fig. 3.

from gel permeation chromatography as shown in Fig. 3a.

4. Discussion

In both of these strategies, the cobalamin–oligonucleotide compound can theoretically use the natural transport and endocytosis pathway of cobalamin. The subcellular distribution of the two compounds may be different as the first one is a covalent conjugate and the second one makes use of avidin as a noncovalent cross-linker of cobalamin and oligonucleotide.

Mild hydrolysis of vitamin B12 yields a mixture of mono- and dicarboxylic acids and one tricarboxylic acid [22,23]. These acids derive from the propionamide side chains *b*, *d* and *e* which are more susceptible to hydrolysis than the amide groups on the acetamide side chains *a*, *c* and *g*. Each monocarboxylic acid derivative of cobalamin has a distinct affinity for IF. The affinity of the *d*-isomer is threefold less than that of the unmodified vitamin B12, while isomers *e* and *b* have affinities which are 250 and 2500-fold reduced, respectively [19]. We, thus, chose to couple the oligonucleotide to the *d*-carboxyvitamin B12, even if this isomer represents only 25% of the mixture of the mono-carboxylic acids [24].

In the first strategy, it appeared that the RP-HPLC procedure used was unadapted to separate the free oligonucleotide from the conjugate. The 15th and 19th fraction collected contained cobalamin derivatives detectable by the ACS vitamin B12 assay. These fractions corresponded neither to carboxycobalamin nor to EDC–cobalamin. Both were, therefore, candidates for containing the cobalamin-oligonucleotide conjugate. In fact, it is likely that cobalamin detected in the 19th fraction corresponded to the cobalamin–oligonucleotide conjugate, as this compound had a retention time similar to that of the free oligonucleotide. Taking this hypothesis into consideration, the yield of the coupling procedure was estimated at 5%.

In the second strategy, biotinylated and EDC–cobalamin had too close retention times to be separated by RP-HPLC. Superose 12 gel permeation chromatography was used to separate the biotinylated cobalamin complexed with avidin from the free derivative. We paid attention to the binding of biotinylated conjugates to the tetrameric form of avidin, as only the tetramer may act as an efficient noncovalent cross-linker of both cobalamin and oligonucleotide derivatives. In fact, avidin was cleared in tetrameric, dimeric and monomeric forms in Superose 12 gel permeation, with respective retention times of 30, 35 and 37 min. The incubation of biotinylated cobalamin with an excess of avidin allowed determination of the fraction of the total cobalamin bound to tetrameric avidin in gel permeation (Fig. 3a). This fraction was evaluated at 8%. The presence of biotinylated cobalamin complex to avidin was attested by the ability of the complex to inhibit the binding of [^{57}Co]cobalamin to the intrinsic factor (Fig. 4). When avidin was incubated with biotinylated vitamin B12 and biotinylated oligonucleotide (Fig. 3a), the chromatogram showed only two peaks, one corresponding to free derivatives (39 min) and the other to the cobalamin–avidin–oligonucleotide complexes (28 min). This complex had the same retention time as the vitamin B12–avidin complex. Superose 12 gel permeation was therefore

an efficient tool for the separation of cobalamin–avidin–oligonucleotide complexes from free derivatives. Using this method it will be possible to prepare avidin–biotinylated vitamin B12 complexes, and use them as vectors for all kinds of biotinylated oligonucleotides. It remained to be determined if vitamin B12 in the vitamin B12–avidin–oligonucletide complexes can recognise its membranes receptors. Transcytosis experiments are now performed in our laboratory using the Caco-2 adenocarcinoma cell line as endocytosis cell model [25,26].

In conclusion, we have prepared and purified by RP-HPLC and gel permeation an oligonucleotide–avidin–cobalamin complex which may be used as a vector for antisense oligonucleotide therapy.

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