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Simultaneous determination of hydroxocobalamin and its cyanide complex cyanocobalamin in human plasma by high-performance liquid chromatography Application to pharmacokinetic studies after high-dose hydroxocobalamin as an antidote for severe cyanide poisoning

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Abstract

Hydroxocobalamin (OHCbl) is a powerful antidote for cyanide poisoning, via the formation of non-toxic cyanocobalamin (CNCbl). Plasmatic cobalamins were measured at 361 nm, after enrichment and purification on a short C_{18} precolumn (1% acetic acid; 1 ml min^{-1}; 2 min), by back-flush elution on a C_{18} ODS-2 column [0.1 *M* sodium dihydrogenphosphate–methanol (63:27, v/v) (pH 4.0); 0.80 ml min^{-1}]. The precision was 3.21 and 3.54% for 10 μM OHCbl and CNCbl, respectively. The method was used to study the pharmacokinetics of OHCbl and the formed CNCbl in severely poisoned patients.

1. Introduction

The acute effects of cyanide poisoning are due largely to the inhibition of the mitochondrial respiratory chain by action on cytochrome oxidase and to the specific action on myoglobin and various enzymatic processes [1]. Many drug antidotes for cyanide poisoning involve direct complexation (cobalt derivatives) [2] or methaemoglobin generation (sodium nitrite) [3] and an increase in physiological cyanide elimination (sulfur donors such as sodium thiosulfate) [4,5]. As cobalt-containing compounds have a high affinity for cyanide, they have been widely used for antidotal purposes. However, as inorganic compounds, such as dicobalt edetate, are toxic, organic derivatives have been proposed by several workers, e.g., hydroxocobalamin (OHCbl) [6–9].

We have demonstrated that, in acute cyanide poisoning in humans, the use of a 5-g infusion of OHCbl led to a dramatic improvement in clinical status [10]. Further, in an experimental in vitro model of a rat cardiac papillary muscle, we have previously shown a nearly complete recovery of all the mechanical properties that had been greatly altered by cyanide poisoning (1 m*M* for

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10 min) after a 5-min period of OHCbl exposure in an equimolar bath concentration [11].

The rationale for administering OHCbl as an antidote for cyanide poisoning is based on the high affinity of CN ion for cobalt compounds that form non-toxic complexes such as cyanocobalamin (CNCbl), which are excreted in the urine. However, as cobalamins have been mainly used at low doses in humans (i.e., 5–10 mg daily) for their antianaemic properties, the available pharmacokinetic studies are limited. Moreover, data are restricted to the distribution and elimination of a particular cobalamin, administered alone. However, the pharmacokinetics of high doses of OHCbl (70–140 mg kg^{-1}) have been studied, but very recently and only in dogs [12]. Hence no pharmacokinetics data are available on OHCbl and its metabolite CNCbl formed by in vivo complexation of cyanide after administration to humans at high doses (5 g) for antidotal purposes.

Several methods have been reported for the determination of cobalamins in biological materials, such as microbiological assay [13], radioimmunoassay [14], resonance Raman spectrometry [15], inductively coupled plasma emission spectrometry (ICP) [12], derivative spectroscopy [16], TLC [17,18] and conventional liquid chromatography such as SP-Sephadex ion-exchange chromatography [18]. However, several of these methods either cannot distinguish between different species of cobalamins, e.g., ICP determines only the total plasmatic cobalt, or require the use of radioactive compounds or are complex or time consuming. Moreover, the chromatographic method described by Butte et al. [19] was limited to the determination of CNCbl for estimating the glomerular filtration rate. However, a chromatographic method has recently been used by Forsyth et al. [20] to study the pharmacokinetics of OHCbl in heavy-smoking normal volunteers.

In this paper, we describe a semi-automated HPLC method for this simultaneous determination using a precolumn purification and enrichment step with back-flush elution. This method was used to study the pharmacokinetics of OHCbl and the formed CNCbl in patients suffering from severe cyanide poisoning.

2. Experimental

2.1. Reagents

Potassium [^{14}C]cyanide (60 mCi $mmol^{-1}$) and tritiated water (5 mCi ml^{-1}) were obtained from Amersham (Amersham, UK). Sodium cyanide was purchased from Sigma (St. Louis, MO, USA). Stock standard radioactive cyanide solutions (500 μCi; 60 m*M*) were freshly prepared in 0.01 *M* NaOH and stored at 4°C, and their exact titres were determined by volumetric titration using silver nitrate immediately before use and adjusted if necessary. Working standard cyanide solutions were prepared by dilution immediately before use. OHCbl was obtained from Anphar Rolland Laboratories (Evry, France) and CNCbl from Lavoisier Laboratories (Paris, France) in 2.5 mg ml^{-1} sterile solutions (1.85 m*M*). The other chemicals used were purchased from Sigma or Merck (Darmstadt, Germany).

Working standard cobalamin solutions were prepared daily in 50 m*M* phosphate buffer (pH 7.0), protected from light using aluminium foil and stored at 4°C.

2.2. Equipment

A chromatographic system including two pumps (PU-880; Jasco, Tokyo, Japan) and two rotating injection valves (six-port sampling valve, Model 7025; six-ports switching valve, Model 7010; Rheodyne, Cotati, CA, USA) was used. A 200-μl injection loop was fixed to the sampling valve. The enrichment precolumn was fixed to the switching valve (C_{18}, ODS-2, 7 μm; 30 × 3.2 mm I.D.) (Shandon, SFCC, Paris, France). The analytical column was also linked to the switching valve, as shown in Fig. 1 (C_{18} ODS-2, 5 μm; 300 × 4.6 mm I.D.; Sup-Rs) (Prolabo, Paris, France). Detection was performed using a programming- and scanning-wavelength UV detector (Jasco UV-875). Quantification of cobalamins was performed by peak integration (Chromatopac C-R6A; Shimadzu, Kyoto, Japan).

^{3}H and ^{14}C radioactivity counts were determined in the dual-channel mode in aqueous scintillation fluid (Optiphase Safe; LKB Wallac,

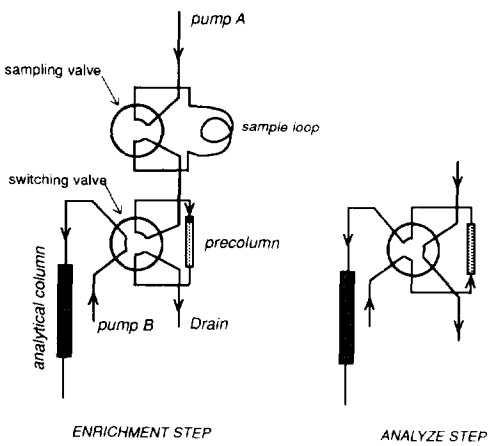

Fig. 1. Experimental design for the semi-automated HPLC determination of hydroxocobalamin (OHCbl) and cyanocobalamin (CNCbl) in plasma. During the enrichment step, the sample was loaded on to a precolumn and eluted with 1% acetic acid (pump A; 1 ml min^{-1}; 2 min). The switching valve then permitted the back-flush injection of the retained cobalamins by the mobile phase (pump B; 0.8 ml min^{-1}) on to the analytical column and their subsequent separation.

Turku, Finland) using a Model 1209 Rack Beta liquid scintillation counter (LKB) with automatic colour quenching and chemiluminescence correction. All radioactivity counts were corrected using a mean background of 40 dpm.

2.3. Procedure

Plasma samples were diluted 2–10-fold in 1% acetic acid according to the expected concentrations and centrifuged (10 000 *g*, 5 min). Diluted samples were then loaded using the sampling valve on to the precolumn fixed to the switching valve (Fig. 1). Sample purification and enrichment were performed using pump A with a 2-min elution step with 1% acetic acid in water (solvent A; 1 ml min^{-1}). Concentrated cobalamins were then injected on to the analytical column by back-flush elution by means of pump B using a mixture of sodium dihydrogenphosphate (0.1 *M*; pH 4.0) and methanol [solvent B (63:27, v/v); 0.80 ml min^{-1}]. Detection was performed at 351 nm (λ_{max} of OHCbl) or at 361 nm (λ_{max} of CNCbl) if required. Quantification of cobalamins was performed by peak integration.

Calibration graphs were obtained daily using plasma pool spiked with authentic OHCbl and CNCbl. The true concentration of the standard solutions was determined by spectrophotometry using the published specific molar absorptivity, a_M, of each cobalamine and adjusted if required.

The recovery of cobalamins was tested using aqueous solutions and plasma samples spiked with identical amounts of cobalamins at concentrations ranging from 10 to 100 μM ($n = 20$, in duplicate).

The precision (intra-day and inter-day) was determined using repeated determinations ($n = 10$) of plasma pool samples spiked with OHCbl and CHCbl (final concentrations 10 and 250 μM) with no dilution for 10 μM and a tenfold dilution for 250 μM.

The purity of the chromatographic peaks obtained from spiked plasma pool samples or from patients' samples was determined by recording the on-line spectra (200–600 nm) and comparison with the spectra obtained from aqueous standards.

The ability of OHCbl to form only CNCbl from cyanide was tested using [^{14}C]KCN. Plasma pool samples (1.8 ml) were introduced into 5-ml screw-capped tubes, buffered with 142 μl of 0.5 *M* phosphate buffer (pH 7.40) containing 2 μCi of tritiated water as a marker for non-retained peaks and spiked with 108 μl of a 1.85 m*M* solution of OHCbl (2 μmol). Radioactive cyanide (50 μl) was then added using various concentrated solutions, obtained from the stock standard solution (60 m*M*; 500 μCi) to give final concentrations ranging from 10 to 100 μM. The tubes were immediately sealed, vortex mixed and incubated for 5 min at room temperature. A 200-μl aliquot was then analysed by HPLC with collection of 1-ml fractions and concomitant monitoring of absorbance as above. The collected fractions were counted for ^{3}H and ^{14}C radioactivity after the addition of 5 ml of scintillation fluid.

The binding of OHCbl and CNCbl by plasma proteins was also tested. After determination of the protein content (Coomassie Blue method, Bio-Rad Kit; Bio-Rad Labs., Richmond, CA, USA), a human plasma pool was diluted with water, leading to protein concentrations ranging

from 10 to 50 g l^{-1}. The samples were then spiked with OHCbl and CNCbl to give a final concentration of 50 μM. The bound and free fractions were determined at equilibrium by centrifugal ultrafiltration (molecular mass cut-off 10 000, Ultrafree-MC; Millipore, Bedford, MA, USA). Cobalamins remaining in the ultrafiltrates (free fraction) were determined by HPLC. The bound fraction was obtained using the equation [bound fraction] = [theoretical concentration added] − [free fraction]. The binding parameters (maximum cobalamin bound, B_{max}, and dissociation constant, K_d) were determined using fitting software for the Michaelis–Menten model based on the Levenberg–Marquardt algorithm (Kaleidagraph; Synergy Software, Reading, PA, USA).

2.4. Clinical procedure

This HPLC method was used in pharmacokinetics studies in patients receiving high doses of OHCbl after severe cyanide intoxication (fire smoke inhalation or oral ingestion) [10]. At admission in our clinical toxicology unit, an initial blood cyanide determination was performed and a 30-min infusion of 5 g of OHCbl was immediately started. Blood samples (5 ml, in heparinized tubes) were obtained during a 240-h period. Samples were immediately centrifuged and the plasma was stored at −30°C until analysis.

3. Results

A typical chromatogram is shown in Fig. 2. The two cobalamins were well resolved since the retention times were typically 7.8 ± 0.2 min (k' = 1.18) for OHCbl and 16.5 ± 0.4 min (k' = 3.76) for CNCbl. However, the k' for CNCbl was strongly dependent on slight variations in the methanol percentage of the mobile phase (Fig. 3). No interference from plasma components was observed, as indicated by no peak after blank injection. Moreover, on-line spectra were identical with those obtained with standards throughout the determinations performed on spiked

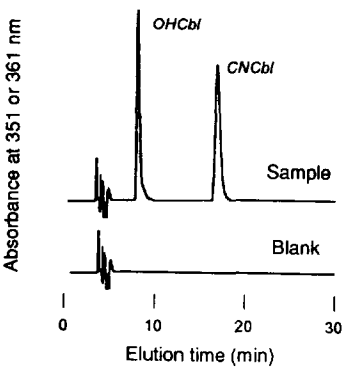

Fig. 2. Typical HPLC separation of cyanocobalamin (CNCBl) and hydroxocobalamin (OHCbl) from a human plasma sample from a patient severely poisoned by cyanide and treated with a 5-g infusion of OHCbl (sample taken 1 h after the end of infusion). The measured levels were 57 μM for OHCbl and 87 μM for CNCbl. A blank sample (patient plasma just before infusion of OHCbl) is shown to demonstrate the absence of interfering peaks.

plasma pool or patient's samples. Detection was performed at 351 nm, corresponding to the maximum absorption of OHCbl, but a wavelength of 361 nm (λ_{max} of CNCbl) gave similar results. However, a compromise value of 355 nm may be used without noticeable effect on the chromatographic characteristics.

The calibration graphs were linear over the range studied (5–100 μM, undiluted; r = 0.997 ± 0.03, n = 10). The recovery was 97 ± 3% for CNCbl and 72 ± 4% for OHCbl. The precision

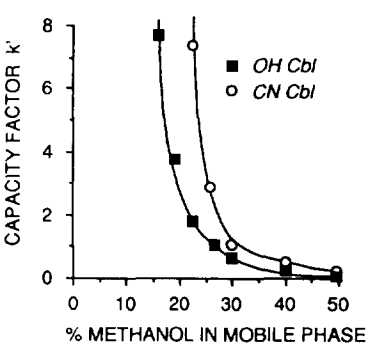

Fig. 3. Influence of the percentage of methanol in the mobile phase on the capacity factor, k', of (■) hydroxocobalamin (OHCbl) and (○) cyanocobalamin (CNCbl). This influence appears more pronounced for CNCbl than for OHCbl.

was excellent: 3.21 and 3.54% for OHCbl and CNCbl, respectively, at 10 μM and 1.17 and 1.54% at 250 μM.

The limit of detection (twice the baseline noise) was about 0.1 μM for OHCbl and 0.15 μM for CNCbl (undiluted samples).

The ability to OHCbl to form only CNCbl from cyanide was tested using the method described above. When cyanide was added at concentrations above the stoichiometric ratio to plasma samples containing OHCbl, a peak eluting at the same retention time as authentic CNCbl was always obtained and no other peak was observed. In addition, a proportional decrease in the OHCbl peak was noticed. Further, more than 95% of the total collected ^{14}C radioactivity was found under the CNCbl peak (Fig. 4). The remaining radioactivity was found in the non-retained fractions, indicated by ^{3}H elution and corresponding to non-complexed cyanide.

The influence of protein concentration on the binding of cobalamins in plasma was determined at equilibrium using an ultrafiltration method. As shown in Fig. 5, this binding obeyed to a non-linear and saturable process and differed between the two cobalamins. Indeed, the maximum binding, B_{max}, and K_d, calculated using a non-linear fitting algorithm, were 47.6 μM and 3.52 g l^{-1}, respectively, for OHCbl and 79 μM and 172 g l^{-1} for CNCbl ($r > 0.999$).

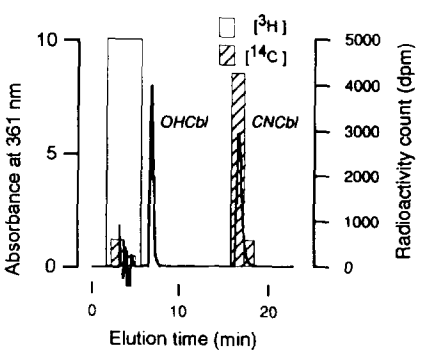

Fig. 4. Typical HPLC separation of cyanocobalamin (CNCBl) and hydroxocobalamin (OHCbl) after in vitro complexation of [^{14}C]cyanide in plasma under physiological conditions. Radioactive cyanide (50 μl; 0.5 μCi) was added to a 2-ml plasma sample buffered at pH 7.40 and containing 100 μM OHCbl (final cyanide concentration 50 μM). The incubation medium was then analysed by HPLC as described in the text. The open box denotes the chromatogram area where up to 99% ^{3}H radioactivity was found. The hatched boxes indicate the different fractions in which ^{14}C radioactivity was present. Absorbance was monitored at 361 nm (full-scale: 10 mAU). This chromatogram demonstrates the univocal complexation of cyanide by OHCbl.

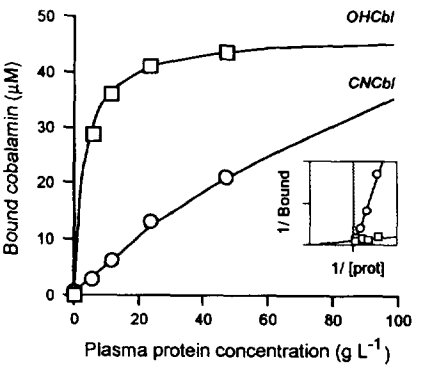

Fig. 5. Influence of protein concentration on the binding of (□) hydroxocobalamin (OHCbl) and (○) cyanocobalamin (CNCbl) by plasma proteins. OHCbl and CNCbl (50 μM) were added to various dilutions of plasma pool corresponding to 10–50 g l^{-1} of protein. The bound and free fractions were obtained by centrifugal ultrafiltration. The free fraction was determined for each concentration by HPLC and the bound fraction was calculated therefrom. Experimental points were fitted by computer according to a Michalis–Menten model ($r > 0.999$). Inset: double reciprocal plot of the data.

Fig. 6 shows a typical pharmacokinetic profile after administration of 5 g of OHCbl to a patient severely poisoned by cyanide (initial cyanide level 128 μM). During the 2 h after the start of OHCbl infusion, immediate formation of CNCbl indicating rapid complexation of cyanide was observed, followed by a biphasic elimination of both cobalamins. Using data obtained from several patients [10], the estimated pharmacokinetic parameters for OHCbl were 19.0 ± 5.2 h for the elimination half-life and 0.15 l kg^{-1} for the distribution volume V_D. For CNCbl the elimination half-life was shorter, 9.3 ± 3.2 h. Table 1 gives the maximum plasma levels of cyanide, OHCbl and CNCbl in poisoned patients after the administration of a 5-g infusion of OHCbl as a function of the source of cyanide (three patients after oral ingestion of cyanide salts and four patients after inhalation of fire smoke).

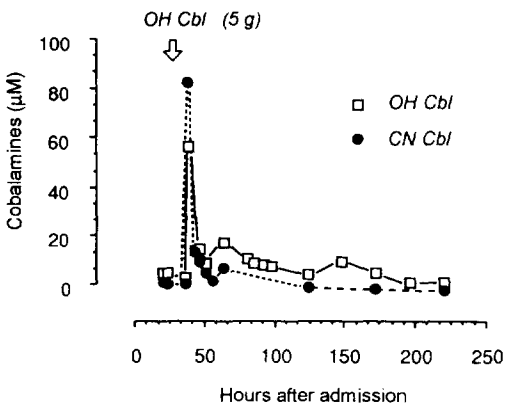

Fig. 6. Typical pharmacokinetic profile from a patient severely poisoned by cyanide and treated with a 5-g infusion of hydroxocobalamin (OHCbl). The initial cyanide level was 128 μM. Formation of cyanocobalamin (CNCbl) was immediately observed, indicating the rapid complexation of cyanide by OHCbl, followed by the elimination of the excess OHCbl and the formed CNCbl.

4. Discussion

The rationale to develop an HPLC method permitting the simultaneous determination of OHCbl and CNCbl was to study the pharmacokinetics of OHCbl and CNCbl after high-dose administration for cyanide antidotal purposes. CNCbl is a metabolite formed by complexation of cyanide by hydroxocobalamin. Most published methods cannot distinguish between the two cobalamins or were designed for the determination of a particular cobalamin [12–17]. However, accurate pharmacokinetic studies required a rapid but reliable method and the determination of the total amount of the both cobalamin species, i.e., including their bound and free forms.

Our method is based on purification and enrichment steps of the plasma samples using 1% acetic acid before analysis by reversed-phase HPLC. As shown by our binding experiments, plasma proteins are able to bind large amounts of cobalamins. This binding is non-linear and very different between OHCbl and CNCbl. Hence the necessary deproteinization step cannot be performed by ultrafiltration. Preliminary experiments showed that solvent precipitation of proteins gave poor recoveries and erratic values. Moreover, acid precipitation was avoided, considering the limited pH range of stability of the corrin ring. However, it has been demonstrated that the stability of the cobalamin moiety is excellent at pH 4.3 [21]. We therefore chose a deproteinization and enrichment step involving absorption of cobalamins on a short C_{18} column, followed by a 2-min elution of polar proteins by 1% acetic acid. This acidic solution and elution time were found to be a good compromise, leading to the elution of about 95% of injected proteins and giving a sufficient retention of OHCbl.

As indicated by the recovery experiments, this step permitted an almost complete recovery of CNCbl and an acceptable recovery of OHCbl, both with excellent reproducibility. The partial recovery of OHCbl may be explained by its stronger binding to plasma proteins than CNCbl

Table 1
Maximum plasma levels of cyanide (CN), hydroxocobalamin (OHCbl) and cyanocobalamin (CNCbl) in poisoned patients after administration of a 5-g infusion of OHCbl as a function of the source of cyanide (three patients after oral ingestion of cyanide salts and four patients after inhalation of fire smoke)

Compound	Plasma level (μM)[a]						
	BrCN	NaCN	HgCN	Fire smokes			
CN	16	260	217	22	123	129	208
OHCbl	325	15	83	514	74	131	12
CNCbl	39	275	226	10	215	221	259

[a] Each column represents data from an individual patient. The CNCbl formation was proportional and the OHCbl concentration was inversely proportional to the initial cyanide level.

and its polar nature, suggesting weak retention by the C_{18} purification column. It as been observed that during this step, OHCbl was partially eluted with proteins but this loss was sufficiently reproducible to permit an accurate determination if a calibration graph generated from spiked plasma pool was used.

As indicated by Fig. 3, the retention time was strongly dependent on slight variations in the percentage of methanol in the mobile phase, hence this percentage must be carefully controlled. Moreover, a buffer of pH 4.0 was used to limit possible degradation of cobalamins during the analytical process. It has been demonstrated that several aquocyano "incomplete" corrinoids, corresponding to stereoisomers, may be formed under various conditions, including pH variations, and may be separated by HPLC [22]. However, these degradation products were not observed in all our determinations. Indeed, on-line spectral analysis of eluting peaks showed no modification compared with the authentic standard, despite the γ band located at ca. 350–354 nm described for these products. Moreover, we tested the single-product formation for the reaction between cyanide and OHCbl in plasma under physiological conditions using [^{14}C]cyanide. As shown in Fig. 4, except for CNCbl, no radioactivity associated with an absorbing peak was found, suggesting that the reaction was unequivocal under our experimental conditions.

This method may be used without modification for the determination of cobalamins in urine samples. It is currently being used to study the pharmacokinetics of OHCbl and its cyanide complex CNCbl in humans severely poisoned by cyanide. As shown by our findings, the maximum concentrations of OHCbl and CNCbl were strongly dependent of the initial cyanide level, demonstrating that the concentration of CNCbl was directly proportional to this level with an inverse relationship for the respective OHCbl level.

In conclusion, we have described a simple, accurate and specific semi-automated HPLC method for the simultaneous determination of OHCbl and CNCbl in human plasma. This method has been fruitfully used for pharmacokinetic studies in patients severely poisoned by cyanide and receiving high doses of OHCbl for antidotal purposes.

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