Liquid-Chromatographic Measurement of Cyanocobalamin in Plasma, a Potential Tool for Estimating Glomerular Filtration Rate

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We describe a method for measuring unlabeled cyanocobalamin in human plasma, based on its absorbance at 368 nm after reversed-phase sample-cleanup on a cartridge filled with octadecylsilylated silica followed by "high-pressure" liquid chromatography. The diminution of the cyanocobalamin concentration in plasma follows first-order kinetics after about 30 min and may be used to calculate the glomerular filtration rate.

The classical method for measuring the glomerular filtration rate (GFR) requires a constant intravenous infusion of inulin and accurate urine collection and plasma sampling. The disadvantages of the inulin clearance method are well known: the considerable burden on the patients, the possibility of bacterial infection during the bladder catheterization, the lengthy time required, and the tedious chemical analysis for inulin (1). With the aim of simplifying the evaluation of the GFR, workers have applied other substances, such as 51Cr-EDTA (2-5) or 57Co/58Co-cyanocobalamin (6, 7). These made it possible to calculate, after one injection ("single shot"), the clearance of the test substance from measurements of three or four subsequent concentrations in the blood, the rate of diminution being used to calculate the clearance ("slope technique"). Use of radioactive materials such as are required in the slope technique is undesirable.

We report here a procedure for quantifying cyanocobalamin in plasma by "high-pressure" liquid chromatography (HPLC). The results for the clearance of cyanocobalamin indicate this substance to be a tool for the evaluation of the GFR.

Materials and Methods

Apparatus

The HPLC apparatus was that of Knauer (Berlin, F.R.G.): a Model 52.00 pump, a Model RH 17 (100-μL loop) injection system, a 250 × 4.6 mm column filled with 5-μm Lichrosorb SI 60 (E. Merck, Darmstadt, F.R.G.), and a Model 85.00 variable-wavelength detector. Column temperature (30 °C) was controlled with a Model 60.00 oven (Knauer). Sep-Pak C18 cartridges (Waters Associates, Milford, MA 01757) were applied for the reversed-phase cleanup.

Reagents and Standards

Methanol, bromcresol green, sodium cyanide, trichloroacetic acid (TCA), and cyanocobalamin (p.i. grade Cytobion, 2000 and 5000 μg) were of analytical grade, except as noted, from E. Merck, Darmstadt, F.R.G. Cyanocobalamin as a standard for the analytical work was supplied by Sigma, München, F.R.G.

Solutions of sodium cyanide (1 g/L), TCA (3 mol/L), bromcresol green (20 mg/L) and 10, 20, 30, and 50 mg/L cya-

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Fig. 1. Absorption spectra of cyanocobalamin (—) and dicyanocobalamin (—)
Cyanocobalamin were all made in doubly distilled water.

Mobile phase: Methanol/sodium cyanide, 0.1 g/L (40/60 by vol); flow rate 0.8 mL/min.

Procedures

It is presupposed that patients must be in a balanced hydration state if clearance values are to be reliable.

Clearance schedule: Take 3 mL of EDTA-treated blood as a blank and intravenously inject 200 μg of cyanocobalamin, to saturate binding sites in blood and cells; 8–14 h later, inject 5000 μg of cyanocobalamin intravenously and 5, 10, 15, 30, 45, 60, and 90 min later withdraw blood samples into EDTA-containing tubes.

Cyanocobalamin measurement: Obtain plasma from the blood by centrifugation at 3000 × g for 10 min. Add 0.5 mL of TCA solution and 5 mL of water to 1 mL of plasma, while stirring. After centrifugation, add 10 μL (200 ng) of brom cresol green solution, which serves as the internal standard, to the supernatant and force this mixture through a C18 cartridge with a valve–piston type of pipette. Cyanocobalamin and the internal standard are adsorbed by the reversed-phase material. Wash the cartridge with 5 mL of water. Convert the cyanocobalamin into dicyanocobalamin by forcing 1 mL of the sodium cyanide solution through the cartridge. Then wash with another 3 mL of water. Elute the cyanocobalamin and the internal standard from the reversed-phase material with 1 mL of methanol. Evaporate this solution in a nitrogen stream, dissolve the residue in 80 μL of sodium cyanide solution, and inject the solution into the HPLC system.

Calculation of the cyanocobalamin concentration: Quantify the area of the peaks representing cyanocobalamin and the internal standard by the height times width at half height method and calculate the relative peak area with respect to the internal standard. For quantification of cyanocobalamin in plasma, use the relative peak area as compared to the standard calibration curve for cyanocobalamin.

Calculation of the GFR: Calculate the GFR by using the formula (8):

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Cl_{corr} = \frac{D}{y_0} \cdot k \cdot K_t
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where \(Cl_{corr}\) is clearance, corrected for a body surface area of 1.73 m² (mL/min); D is dose, i.e., quantity of cyanocobalamin (ng) injected; \(y_0\) is the concentration of cyanocobalamin in plasma at zero time (μg/L = ng/mL); \(k\) is the elimination constant (1/min); and \(K\) is the correction factor for 1.73 m².
Results and Discussion

Analytical Variables

Calibration: We established a curve correlating cyanocobalamin concentration in plasma with relative peak area by using pooled blank plasma supplemented with cyanocobalamin. With a constant concentration of brom cresol green as internal standard, the calibration curve proved to be linear between 20 and 500 µg of cyanocobalamin per liter of plasma.

Recovery rate: The relative analytical recovery was calculated by comparing the relative peak area ratio of a 300 µg/L cyanocobalamin solution in plasma with that of an aqueous cyanocobalamin solution, 300 µg/L. Both solutions contained 200 µg of brom cresol green per liter. The recovery rate was calculated to be 65.3% (SD 3.0%) for n = 12.

Precision: Within-day precision was evaluated by using plasma samples supplemented with cyanocobalamin. The CVs varied from 3.98% for a 500 µg/L to 6.94% for a 200 µg/L cyanocobalamin concentration in plasma.

Stability: The high stability of cyanocobalamin in plasma when stored cool or frozen has already been reported (10).

Enrichment procedure: Cyanocobalamin is separated from other plasma components by trapping it on octadecylsilated silica (the "reversed-phase" C18 cartridge). After the cartridge is washed, the cyanocobalamin is converted into dicyanocobalamin by treatment with cyanide. This is done because dicyanocobalamin is more stable and has a higher absorption coefficient at 368 nm than does cyanocobalamin (see Figure 1). The dicyanocobalamin is then eluted from the cartridge with methanol. It is stabilized by the cyanide that is present in the HPLC eluent. For the cleanup procedure it is important not to use a syringe with brass parts (even if they are chrome plated), because cyanocobalamin is extremely unstable, with exchange of the central metal ion, under acidic conditions (11).

HPLC separation: Figure 2 illustrates examples of HPLC chromatograms, showing cyanocobalamin in plasma at different intervals after its injection. As demonstrated by the zero-time chromatogram, nothing in plasma absorbs at 368 nm to disturb the measurements of cyanocobalamin and the internal standard.

Calculation of the GFR

Plotting the ln of cyanocobalamin concentration (y) vs time (t) (Figure 3) results in a linear relationship according to equation 3 from 30 to 90 min after the injection. When substances such as 51Cr-EDTA or cyanocobalamin are injected into the blood, they show a biphasic decrease (12). Their pharmacokinetics may be described by a two-compartment model with first-order kinetics of elimination. The first (short) phase stands for the distribution of cyanocobalamin into the extra-vascular fluid compartment. The second, long phase—which is attained about 20 to 30 min after the injection—reflects the renal elimination of cyanocobalamin.

Using measurements of cyanocobalamin concentrations in plasma collected 45, 60, and 90 min after the injection, one can easily obtain the virtual zero-time concentration y0 and the elimination constant k needed for the calculation in equation 1, by either regression analysis or graphic extrapolation. With these cyanocobalamin concentrations we calculated the clearances for six healthy persons to lie between 120.5 and 175 mL/min.

Unfortunately, we could test only a few patients, but the values we obtained agree well with those found for inulin clearance, 93–159 mL/min (9), the classic method for evaluating GFR. Furthermore, 57Co labels have been used to show that cyanocobalamin is suitable for use in the evaluation of the GFR (7).

Thus our results suggest that unlabeled cyanocobalamin may be a potential tool to measure the GFR under "slope technique" conditions when the compound is determined quantitatively in plasma by HPLC. This technique is harmless to the patient and easy for medical and laboratory personnel to use, in contrast to the "constant infusion technique" used for inulin. However, more work is needed to evaluate the correlation between the cyanocobalamin and the inulin clearance in more detail.

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“Rocket” Immunoelectrophoresis Assay of Vitamin D-Binding Protein (Gc Globulin) in Human Serum

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The anodic properties of the binding protein for vitamin D and its metabolites in human serum prompted us to develop a “rocket” immunoelectrophoresis assay for this alpha globulin. Only 0.2 mL of antiserum is required for assay of 72 samples in 5-µL wells on two plates. The assay is sensitive (0.1 µg) and precise (intra-assay CV 1.4%; inter-assay CV 7.9%), and permanently recorded assay plates can be produced in 24 h. These features are attractive for the convenient measurement of this protein, which is important in anti-rachitic sterol transport.

The binding protein for vitamin D and its metabolites (DBP) in plasma is now recognized to be identical to the plasma group-specific component (Gc) (1-4). A 58 000-dalton alpha globulin, binds anti-rachitic sterols with mole per mole stoichiometry (2-4), but circulates predominantly in the apo (5). Absence of this protein is believed to be a lethal mutation, and genetic variations, inducing co-dominant alleles were recognized (6). Quantitation of plasma DBP has received attention recently, and radial immunodiffusion (7-9) and radioimmunoassay (5) methods have been reported. The concentration of DBP in plasma is normally approximately 6 µmol/L, so immunoprecipitation techniques are suitable for small plasma volumes. Although we have achieved good results with a radial immunodiffusion assay (10), the anodic property of DBP suggested that we could develop a more precise, rapid, and reproducible assay by using the technique of “rocket” immunoelectrophoresis (11).

Materials and Methods

Materials

Pure DBP was prepared from Cohn Fraction IV of human plasma as previously described (2), and monospecific antisera to it were raised in rabbits as reported (5). Alternatively, anti-Gc antisera is available from Calbiochem-Behring Co., La Jolla, CA 92112, or from Dako Corp., Santa Barbara, CA 93103.

Agarose, type III (high electroendosmosis), and Tris (Trizma) base were from Sigma Chemical Co., St. Louis, MO 63178. Diethyl barbituric acid (barbital) was from Fisher Scientific Co., Fair Lawn, NJ 07410, and Coomassie Blue R250 was from Miles Laboratories, Inc., Elkhart, IN 46514.

For electrophoresis we used a Model 1415 electrophoresis cell with electrode/buffer chambers (Bio-Rad Laboratories, Richmond, CA 94804) and a dc power supply (Buchler Instruments, Inc., Fort Lee, NJ 07024). Glass plates (200 × 100 × 3 mm) were cut locally, and solvent saturation pads (Gelman Sciences, Inc., Ann Arbor, MI 48106) were used as electrode wicks. A plate-leveling apparatus was purchased from Hoeffer Scientific Instruments, San Francisco, CA 94107, and wells were cut into the agarose with a 2-mm (o.d.) gel punch (LKB Instruments, Inc., Rockville, MD 20852). We used Plexiglas template with 40 perforations, 5 mm apart, made for us by Graham Morris, MRC Dunn Nutritional Laboratory, Cambridge, CB4 1XJ England.

Barbital, 25 mmol/L in Tris, 75 mmol/L, pH 8.6, was the buffer used in preparing gels, in the electrophoresis, and for dilutions of reference DBP and serum. Gels were stained in a solution of Coomassie Blue R250, 250 mg/L, in glass-distilled water/isopropanol/acetic acid (65/25/10 by vol).

Methods

A 10 g/L solution of agarose was prepared in the Tris–barbital buffer by heating until clear, and then cooled to 55 °C. An appropriate volume of antiserum (0.1–0.3 mL) was stirred into 30 mL of the agarose solution, and the solution was poured onto an ethanol-washed, pre-warmed (55 °C) glass plate that had been leveled. After the gel formed, the template was moved over the plate and wells were cut with the vacuum-connected gel punch positioned 20 mm from the long edge of the plate. Allowing for 10-mm margins, 36 wells were made.

The plate was then placed into the tank, with the wells nearest the cathode. Pre-moistened wicks were gently pressed onto the 10-mm margins at the long edge of the plate, and the wick edges were saturated with buffer from a Pasteur pipette...