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VISUAL EXPERIMENT

Estimation of vitamin B_{12} in plasma by High Performance Liquid Chromatography

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ABSTRACT

High performance liquid chromatography was used to develop and validate the detection of vitamin B_{12} in blood plasma sample. The mobile phase consists of a mixture of 0.025% trifluroacetic acid in deionized water and 30% methanol. The mixture used was adjusted to pH 2.9 and the flow rate was adjusted to 0.5 mL/min. The separation was achieved using C18 column maintained at 30ºC temperature and detection of vitamin B_{12} was conducted at maximum wavelength 230 nm.

INTRODUCTION

Vitamin B_{12} (cobalamin and its derivatives) is an essential water soluble micronutrient that plays an important role in regulation of brain functioning (Grotzkyj et al., 2012). The deficiency of vitamin B_{12} may lead to various complications such as neurological abnormalities like peripheral neuropathy, autonomic dysfunction, psychosis, memory impairment, Cognitive decline etc (Roynolds et al., 2006). The normal range of vitamin B_{12} in plasma of healthy subject is 200-900 pg/mL.

Vitamin B_{12} in plasma, serum and urine can be measured by radioimmunoassay, enzyme-linked immunosorbent assay, microbiological assay (using *Lactobacillus leichmannii;* Girdwood, 1954), HPLC (Stefova et al., 1997), capillary electrophoresis, mass spectroscopy, Raman spectroscopy (Mayer et al., 1973), Fourier transform Raman spectroscopy (Hancewicz and Petty, 1995), chemiluminescence (Song and Hou, 2003) and fluorescence quenching.

A deficiency vitamin B_{12} occurs when the level is less than 200 pg/mL (Shaik and Gan, 2013). Because of its very low concentration in plasma, it is very challenging to develop a method with good sensitivities and specificities. Currently, the available methods to estimate vitamin B_{12} in plasma are mainly based on microbiological assay which is time consuming, hazardous to the health of the operator and may lack specificity (Giorgi et al., 2012). Different analytical methods have been developed over recent years due to increasing demand of vitamins. However, most of these methods were applied in food particle, drinks, and vitamin supplements and so on.

There are very few analytical methods developed to detect vitamin B_{12} in plasma due to extensive time consumption in sample preparation, robustness and reproducibility.

Therefore, the aim of this study was to develop and validate a novel High Performance Liquid Chromatography (HPLC) methodology for rapid detection and quantitation of water soluble vitamin B_{12} in biological fluids (plasma) which can lead to both time and cost saving.

MATERIALS AND EQUIPMENTS

1. HPLC unit
2. Analytical balance
3. Sonicator (Sonorex Super 10 P)

4. Centrifuge (Labofuge 200)
5. Nitrogen/Argon gas cylinder
6. Filter paper (0.2 µm) for filtration
7. Microtip pipette (Thermo Scientific)
8. Vortex mixer (VM 2000 DIGI System)
9. pH paper (Fisher brand FB 33041)
10. Vitamin B_{12} (Sigma-Aldrich)
11. Dichloromethane (Merck. KGa, Germany)
12. Trifluoroacetic acid (Scharlab S.L, Spain)
13. Methanol (HPLC grade)
14. Deionized water

PREPARATION OF REAGENTS

Mobile phase: Solution A is prepared by adding trifluoroacetic acid (100 µL) into 400 mL of deionized water. The pH is adjusted to 2.9. Solution B consists of 150 mL methanol. Then 350 mL of solution A and 350 mL of solution B are mixed to make a final volume of 500 mL. Further, 500 mL of this solution is sonicated for 2 min before using as mobile phase. A fresh solution is prepared daily.

Standard solution of vitamin B_{12}: Weigh 1 mg of vitamin B_{12} into a test tube and dissolve it in 1 mL of water with TFA (1 mg/mL). Then make serial dilution into 100 µg/mL, 10 µg/mL, 1 µg/mL and 100 ng/mL.

VIDEO CLIP

Vitamin B_{12} assay: 4 min 9 sec

PROTOCOL

Preparation of the Sample

1. Take 400 µL of fresh plasma in a test tube
2. Add 800 µL of dichloromethane to precipitate protein
3. Vortex the sample for 30 sec
4. Centrifuge the sample at 3,500 rpm for 10 min to separate the precipitate from the supernatant
5. Collect the supernatant into another test tube
6. Add 400 µL of methanol to the supernatant
7. Wait for 10 min
8. Vortex the sample for 30 sec
9. Centrifuge the sample at 3,500 rpm for 10 min again to observe the two clear layer of solution
10. Separate the upper layer into another test tube
11. Evaporate the methanol using nitrogen/argon gas
12. Add 200 µL of solution A into the test tube to dissolve vitamin B_{12}
13. Vortex the sample for 30 sec

14. Adjust the pH of the sample to 2.9
15. Pour the sample/standard solution into a HPLC vial

Set-up of HPLC

1. Make sure that bottle contains >400 mL of mobile phase
2. Turn on instruments in the following order: computer, HPLC software, degasser, pump, column compartment, autosampler and detector
3. Set the flow rate to 0.5 mL/min
4. Set the injection volume to 50 µL
5. Set the column temperature to 30°C
6. Set the wavelength of UV detector to 230 nm

Injection of Sample and Data Collection

1. Keep the HPLC vial into the autosampler tray
2. Set the run time using the computer
3. Click the "Start" button
4. Inject 50 µL of the sample/standard into the C_{18} column [150 (length) x 4.6 mm (internal diameter), particle size 5 µm] using an autosampler
5. Data collection can be stopped at any time by either clicking on the Stop button
6. When data collection is complete, either through a manual stop run or when the preset run time is complete, a report will be generated
7. Identify the unknown peak from the standard known peak using the retention time
8. Printer the data in a A4 size paper
9. Turn off the instruments in this order: HPLC software, detector, autosampler, column compartment, pump, degasser and computer

CALCULATION

1. Identify the retention time of the standard peak
2. Confirm the unknown peak from the sample using the retention time of the standard peak
3. Inject series of vitamin B_{12} standard solutions (minimum 4) using the same procedure
4. Record peak areas
5. These four levels should be within the calibration range
6. Calculate the concentration of vitamin B_{12} using the following formula:

   (Peak area of sample/Peak area of standard) x Concentration of mixed standard solution, pg/mL x (Dilution volume of sample, mL/Sample weight, g)

DISCUSSION

In this study, vitamin B_{12} was measured by HPLC. In comparison to other sophisticated methods, HPLC method requires sample preparation whereas electroluminescence or radioimmunoassay does not (Karmi et al., 2011). In addition, HPLC method (68,000 pg/mL) is less sensitive than the radioimmunoassay (200 pg/mL) or electroluminescence (30 pg/mL). Cost and availability of the instrument are considered for estimation.

In HPLC, optimization of the method is required. It includes: a) suitable mobile phase, b) wavelength, c) with/without buffer and its type and concentration, d) pH of the mobile phase, e) column temperature, f) injection volume, and g) flow rate.

Vitamin B_{12} is a water soluble vitamin. Therefore, mobile phase should be a polar one. Methanol or acetonitrile is preferred.

The wavelength is also important. The reported wavelengths used for the estimation of vitamin B_{12} were 210 (Shaik and Gan, 2013), 230 (Giorgi et al., 2012), 260, 254, 360 (Shaik and Gan, 2013), 545 and 550 nm. We used 230 nm. In this study, methanol was used. The UV absorbance cutoff of acetonitrile or methanol is 200 or 205. Therefore, wavelength of 210 should better be avoided.

The pH is important factor for the determination of vitamin B_{12}. The peak is marked at low pH. A study was conducted on using mobile phases of different pH such as 2.3 (Shaik and Gan, 2013), 2.6 (Heudi et al., 2005), 2.9 (Giorgi et al., 2012), 4.0 and 6.0 and found that pH 2.3 was the best for vitamin B_{12} peak which is good in peak shape and height (Shaik and Gan, 2013). Because sometimes, the peak may be broaden.

At a flow rate of 0.1 mL/min, vitamin B_{12} was not eluted (Shaik and Gan, 2013).

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PRECAUTION

Prepare fresh buffer solution to avoid any bacterial contamination