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Rapid determination of vitamin B_2 and B_{12} in human urine by isocratic liquid chromatography

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ABSTRACT

A simple and rapid method for the identification and quantification of vitamin B_2 and B_{12} in human urine has been developed using reverse phase high performance liquid chromatography (HPLC) and the peaks identity were confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). HPLC separation was performed in single wavelength detector (λ_{365}) mode and separated isocratically using mobile phase methanol: 1 mM aqueous TFA (1:4) in C18 column. The calibration graphs plotted with different concentrations of vitamin B_2 and B_{12} was linear with a correlation coefficients (r^2) = 0.9975 and 0.9985, respectively. The recoveries of vitamin B_2 and B_{12} were above 87% and 90%, respectively. The results of this present study suggest that the proposed method may be simple and convenient way of identifying and quantifying vitamin B_2 and B_{12} from human urine.

1. Introduction

Vitamin B_{12} (cobalamin or cyanocobalamin) a tetrapyrrole complex which contains a cobalt atom in the molecule, assists the function of the nervous system and the formation of red blood cells. A deficiency of vitamin B_{12} may cause pernicious anemia and neuropathy in humans [1]. Vitamin B_2 (riboflavin) has a well-defined process in metabolism of fats, carbohydrates, and respiratory proteins. Riboflavin serves as a precursor for flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) that are involved in numerous reduction–oxidation reactions for the metabolism of other vitamins like vitamin B_6 and folate [2]. A deficiency of vitamin B_2 can result in skin lesions and light sensitivity [3].

Vitamin B_2 and B_{12} are lost from the body in urine and faeces. The concentration of vitamins in urine is greatly influenced by many factors such as dietary intake, nutritional supplement use, health and physical condition, etc. [4]. Vitamin B_2 and B_{12} deficiency may be more common than other vitamins in children, aged and women during pregnancy in developing countries [5–7].

Various analytical methods including radioisotope dilution assay [8], spectrophotometry [9], chemiluminescence [10], electrochemical [11], atomic absorption spectrometry [12], plasma desorption mass spectrometry [13], multinuclear solid-state NMR analysis [14], capillary electrophoresis [15], and high performance liquid chromatography [16,17] have been developed for the detection of Vitamin B_{12}.

Several analytical methods have been developed for the determination of vitamin B2 concentrations including fluorometric [18], liquid chromatographic [19], and capillary electrophoretic methods [20]. Most of the HPLC methods have been designed for the detection of high concentrations of vitamin B_2 and B_{12} in food, pharmaceutical preparations, and urine [21]. Moreover, the current literature on the separation of vitamins was carried out on C18 column with gradient elution with acetonitrile and aqueous phase and detection was performed in a broad range of wavelength (UV region) [22].

Some analytical techniques have been coupled to mass spectrometers like GC–MS and LC–MS, including ICP–MS (inductively coupled argon plasma), SCF–MS (supercritical fluid), NMR–MS (nuclear magnetic resonance) and IR–MS (infrared–MS). The main advantage of mass spectrometry technique is that it not only provides information on the molecular mass of a compound of interest, but also generates structurally significant information also. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is an efficient tool for biomolecules analysis [23]. MALDI-TOF MS has several advantages over other methodologies, including speed of analysis, high sensitivity, and wide applicability with a good tolerance toward contaminants, and ability to analyze complex mixtures [24]. In recent times, the applications of MALDI-TOF MS have been extensively used in

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biomolecules analysis because protonated analyte molecules are less fragmented than ESI–MS or LC– MS/MS [24]. Sangwon and Joon [25] have been used MALDI-TOF MS for rapid identification of folic acid and its derivatives in vitamin tablets and foods. MALDI MS is a suitable approach for rapid screening of vitamins in various foods and energy drinks [26].

We describe a simple, precise, low-cost, isocratic liquid chromatography method for simultaneous separation of vitamin B_2 and B_{12} from human urine and rapid identification was made using MALDI-TOF MS.

2. Materials and methods

2.1. Chemicals

HPLC-grade methanol and triflouroacetic acid (TFA) were purchased from SRL (India). Oxalic acid, vitamin B_2 and B_{12} were obtained from Sigma (St. Louis, MO, USA). Deionized water (18 MΩ) via an ultrapure-water purifier system from Millipore (Milford Bedford, MA, USA) was used throughout the study. Alpha-cyano-4-hydroxy cinnamic acid (CHCA) was procured from Applied Biosystem, USA.

2.2. Solutions preparation

The standard stock solution of vitamin B_2 and B_{12} (100 μg mL^{-1}) was prepared separately in 100 mL of deionized water. Standard solutions of each set were prepared in the concentration range of 100 ng mL^{-1} to 20 μg mL^{-1}. The solutions were stored at 4 °C in dark.

2.3. Sample collection and preparation

The urine samples used in the analysis were the first-voided morning specimens collected from volunteers. The samples were collected in clean, acid-washed, amber glass bottles. The specimens were thoroughly mixed with oxalic acid crystals as a preservative and centrifuged for 5 min at 5000*g* at 4 °C to remove any precipitates. The supernatant was transferred to a 20-mL vial either for immediate analysis or stored at 4 °C in the dark.

2.4. HPLC analysis

Absorbance at 300–600 nm of the stock solutions were scanned with (Beckman Coulter, DU 640B) spectrophometer equipped with 1 cm quartz cuvette. Samples were run through a HPLC system (Agilent 1100 series) coupled with UV–vis detector (G1315B). Sample injections of 20 μL were made from an Agilent 1100 Series auto-sampler; the chromatographic separations were performed on ZORBAX-EclipseXDB-C18 column (4.6 × 150 mm, particle size 5 μm) which was run with several mobile phases at a flow rate of 1 mL min^{-1} for 10 min and monitored at 365 nm.

2.5. MALDI-TOF MS analysis

Selected peaks of the HPLC chromatogram were collected using a fraction collector (GILSON, France) coupled with HPLC, each fraction was concentrated (100 μL) by Speed-Vac and then 10 μL of deionized water was added to each sample vial. Prior to MALDI analysis, 2 μL of each sample (HPLC purified fraction from urine and commercial standard vitamin solution) have been taken separately and mixed well with 8 μL of α-cyano-4-hydroxy cinnamic acid (10 mg mL^{-1}) as matrix. One microliter of each sample was spotted onto stainless steel MALDI sample plate and dried under laminar airflow. MALDI analysis was performed on an Applied Biosystem Voyager-DE PRO MALDI-TOF mass spectrometer equipped with a nitrogen laser (337 nm), operated in an accelerating voltage of 20 kV with a grid voltage of 93% and extraction delay time was 100 ns. Each spectrum was collected in the positive ion linear mode as average of 100 laser shots of predetermined or random positions across a spot. The data was externally calibrated using Calibration mixture-1 (Applied Biosystem, USA). Reproducibility of each spectrum was checked 20 times from duplicate prepared samples.

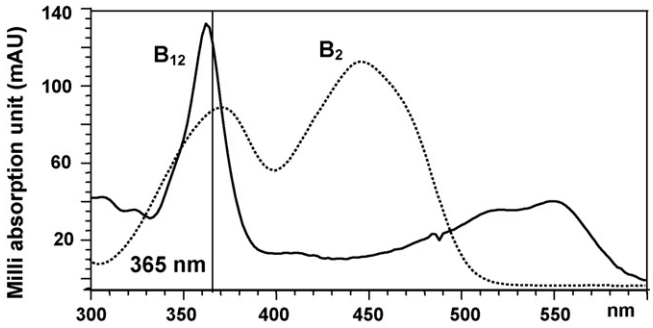

Fig. 1. UV–vis spectrum of vitamin B_2 and B_{12}. Spectra were overlaid after scanning from 300 to 600 nm.

3. Results and discussion

3.1. Optimization of separation conditions

The objective of the present work was to propose a precise and selective HPLC method with simultaneous UV detection for the quantification of vitamin B_2 and B_{12} in human urine. The UV–vis spectra of each standard vitamin (equal concentration) were recorded and overlaid (Fig. 1). UV–vis spectral analysis showed that the peak intensity was maximum at λ_{361} for vitamin B_{12} and two major peaks intensity at λ_{369} and λ_{448} was observed for vitamin B_2. We have selected the wave number at λ_{365} for the detection of vitamin B_2 and B_{12}, wherever both vitamins showed a simultaneous absorbance.

Isocratic elution using acetonitrile/water, acetonitrile/water containing 1 mM triethylamine (TEA)/0.1% formic acid, methanol/water, methanol/water containing 1 mM TEA/0.1% formic acid and methanol/acetonitrile as eluent were compared. Isocratic separation was found to be best when methanol/1 mM aqueous TFA are used as mobile phase. For optimization, different gradient elution conditions were also investigated and better resolution was achieved at a ratio (1:4) of methanol/1 mM aqueous TFA. Eclipse XDB-C18 column is made by first chemically bonding a dense monolayer of dimethyl-n-octadecylsilane stationary phase to a specially prepared ultra high purity (>99.995%) SiO2, that is highly useful for the separation of polar compounds by reversed phase liquid chromatography. The effect of C18 column on the separation of vitamins was explored earlier [21]. Both the vitamins are eluted within 10 min (Fig. 2) and no significant interfering peaks were observed at the retention times of the vitamins.

3.2. Method validation

Specificity of a method described as the ability to discriminate the analyte from potential interfering substances. Urine without preprocessing and internal standard was injected in HPLC system. The representative chromatogram in Fig. 2 shows that no obvious interference was observed on the separation of vitamins. The result expresses excellent specificity of the method for the determination of vitamin B_2 and B_{12}.

Under the optimum analysis conditions (the polar mobile phase comprised of methanol: 1 mM aqueous triflouroacetic acid (1:4) at