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The LC–MS method for the simultaneous analysis of selected fat-soluble vitamins and their metabolites in serum samples obtained from pediatric patients with cystic fibrosis☆

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ABSTRACT

Cystic fibrosis (CF) is one of the most common genetic diseases in children and affects mainly respiratory and digestive system functions. Despite the prolonged supplementation of vitamins, malnutrition manifested by poor growth and weight loss in children is a major complication in CF related to pancreatic insufficiency and difficulty in absorbing fat-soluble vitamins. In the present study, we have developed and validated a sensitive and accurate high-performance liquid chromatography coupled to mass spectrometry (LC–MS) method for the simultaneous quantification of three fat-soluble vitamins (A, E and K_1) and two vitamin D_3 active metabolites: 25-hydroxyvitamin D_3 and 1,25-dihydroxyvitamin D_3 in serum samples obtained from pediatric patients with CF. In optimized conditions, the LC–MS method was highly sensitive and presented excellent linearity with a regression coefficient higher than 0.999. The accuracy was in the range of 87.55–95.58 % for all analytes. The precision of the method, expressed as% RSD, ranged from 1.36 % to 3.74 % as the intra-day variability and from 2.35 % to 7.98 % as the inter-day precision for all the studied compounds. Sample preparation included a protein precipitation step with the use of methanol followed by liquid-liquid extraction with *n*-hexane. The statistical analysis (*t*-test and principal component analysis (PCA)) of the obtained results revealed significant changes in the plasma level of the analyzed compounds, with 25-hydroxyvitamin D_3, vitamin E and K_1 present at extremely low concentrations in patients with cystic fibrosis in comparison to healthy controls. The elaborated method reached the expectations for the fast and reliable assessment of fat-soluble vitamin status in children with cystic fibrosis in order to diagnose the disease and monitor the treatment process.

1. Introduction

Cystic fibrosis (CF) is an autosomal recessive genetic disease caused by mutations in the *CFTR* gene, mainly associated with the Caucasian population [1]. It is an aggressive and multi-organ disease, classically leading to abnormalities of the respiratory, gastrointestinal, pancreatic, and reproductive systems. Progress in clinical care made during the last 40 years has resulted in more effective treatment of lung disease and an improvement in nutrition, which in consequence significantly increases the life expectancy of CF patients [2]. However, directions for nutrition in the group of pediatric patients with CF need to be verified as the currently available data revealed no significant differences between the children randomized for supplements and those given directions for nutrition [3,4].

Fat-soluble vitamins (vitamin A, D, E, K) are crucial for the adequate functioning of the body in physiological conditions, and suboptimal serum levels of these vitamins are considered as risk factors in degenerative diseases, such as diabetes mellitus, cardiovascular disease and cancer [5–9]. Moreover, fat-soluble vitamins prevent the progression of many pathological processes related to osteoporosis [5,10] and cystic fibrosis [3,11,12]. Deficiencies in fat-soluble vitamins in pediatric patients with CF are a result of pancreatic insufficiency and fat malabsorption, which may affect bone mineral status, and infectious and autoimmune diseases [3,12–15]. The literature data concerning blood levels of fat-soluble vitamins

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in pediatric patients with CF are not consistent. Congden et al. [3] demonstrated that deficiencies in D_3 and K_1 vitamins are infrequent in CF disease, although side-effects, such as rickets and life threatening bleeding, related with deficiencies of both vitamins have been proved. Moreover, the vitamin K level should be controlled regularly, since the currently available literature data do not indicate the most suitable dose of this vitamin for children with CF [16–18]. Due to the low circulating level of vitamin K_1 and 1,25-dixydroxyvitamin D_3 in comparison to other fat-soluble vitamins, the simultaneous determination of the serum concentration of these vitamins requires sensitive analytical instrumentation [17].

Several analytical methods have been proposed for the direct and simultaneous determination of fat-soluble vitamins in environmental and biological samples using normal-phase high-performance liquid chromatography (NP-HPLC) [19,20], and more frequently, reversed-phase liquid chromatography with fluorescence detection [21], diode-array detection (DAD) [22–25] or spectrophotometric detection (UV) [26,27] and the UHPLC method with UV detection [28,29]. Moreover, in recent years, hyphenated methods of liquid chromatography coupled to mass spectrometry (LC–MS) [24,30–34] and gas chromatography connected to mass spectrometry [35] have become popular for the quantitative determination of fat-soluble vitamins.

The aim of the present study was to develop a rapid and sensitive method for the simultaneous determination of fat-soluble vitamins including α-tocopherol (vitamin E), all-*trans*-retinol (vitamin A), phylloquinone (vitamin K_1) and two active metabolites of vitamin D_3: 25-hydroxyvitamin D_3 and 1,25-dihydroxyvitamin D_3 in serum samples using an elaborated LC–MS method. A comparative study between three groups of pediatric patients, including patients at the moment of diagnosis of cystic fibrosis, CF patients after one year of fat-soluble vitamin supplementation therapy, and healthy controls has been performed with the use of complex statistical analysis (PCA).

2. Materials and methods

2.1. Chemicals and reagents

Ammonium formate, formic acid, acetonitrile, *n*-hexane and methanol were purchased from Merck (Darmstadt, Germany). Vitamin A (all-*trans*-retinol), vitamin E (α-tocopherol), vitamin K_1 (phylloquinone), 25-hydroxyvitamin D_3, 1,25-dihydroxyvitamin D_3 and retinol acetate (internal standard – IS) were provided by Sigma (St. Louis, MO, USA) and were of a minimum purity of 99%. Deionized water was obtained from Milli-Q system (Millipore, Bedford, MA, USA) and used for samples and mobile phase preparation.

A stock standard solution of each analyte and IS containing 1 mg mL^{-1} were prepared in methanol and stored at −20 °C in dark bottles no longer than one month due to its susceptibility to oxidation and degradation processes. The working standard solutions were prepared daily by diluting the stock solutions in methanol to obtain appropriate concentrations.

2.2. Sample collection and preparation

Blood samples were collected in vacutainer tubes with no anticoagulant added to obtain serum samples and then centrifuged at 2683 × *g* for 15 min. Next, the serum was transferred to Eppendorf tubes and stored at −80 °C until the sample preparation step. A volume of 200 μL of serum sample was placed in an Eppendorf tube and spiked with 30 μL of retinol acetate. In order to ballast precipitation, a volume of 800 μL of methanol was added to the serum, vortex-mixed for 30 s and centrifuged at 16770 × *g* for 10 min at 4 °C. Then the supernatant was collected and 800 μL of *n*-hexane was added. The *n*-hexane layer was collected and evaporated to dryness at 45 °C in vacuum conditions. The procedure with the addition of *n*-hexane was repeated two times. The residue was dissolved in 100 μL of methanol and finally 10 μL of the solution was injected into the LC–MS system.

2.3. LC–MS conditions

The HPLC analysis of fat-soluble vitamins was carried out with an Agilent 1260 Infinity system (Agilent Technologies, Santa Clara, CA, USA) equipped with a binary pump, an autosampler, and a UV variable wavelength detector, and coupled to an Agilent 6120 mass detector with a quadrupole analyzer (Single Quad, Agilent Technologies, Santa Clara, CA, USA) equipped with an electrospray (ESI) ion source (ESI positive ion mode). The separation of all analyzed vitamins was achieved on a core-shell column – Poroshell 120 EC - C 18 column (100 × 3.0 mm, 2.7 μm particle size) (Agilent Technologies, Santa Clara, CA, USA). The chromatographic data were collected and processed by the data acquisition system ChemStation software (v. B.04.03, Agilent Technologies, Santa Clara, CA, USA). The mobile phase was composed of two constituents, A and B, where solvent A: acetonitrile mixed with 0.1% formic acid and 5 mM ammonium formate (90:10, v/v), and solvent B: methanol mixed with 0.1% formic acid and 5 mM ammonium formate. The flow rate was maintained at 0.5 mL min^{-1}, the injection volume was 10 μL. The optimized gradient elution program used for the analysis of fat-soluble vitamins was as follows: 0–2 min – 0% B, 2–7 min – 0–100% B, 7–18 min – 100% B, 18–18.1 min – 100–0% B and the equilibration process: 18.1–25 min – 0% B. The column and autosampler compartments were thermostated at 40 °C and 8 °C, respectively. The detailed working conditions of the MS tune were as follows: spray voltage and capillary temperature were set at 6000 V and 300 °C, respectively. The temperature of the ion source was kept at 300 °C to obtain the highest signal for the tested compounds. Nitrogen (NM32LA Nitrogen Generator, Peak Scientific Instruments, Billerica, MA, USA) was used as the drying gas, with flow rates of 11 L min^{-1}, the fragmentor voltage was set individually for the compound.

Data acquisition in SIM was performed by monitoring the characteristic ions for the subsequent analyzed compounds. The precursor ions (*m*/*z*) for the analytes were as follows: 1,25-dihydroxyvitamin D_3 *m*/*z* 399; 25-hydroxyvitamin D_3 *m*/*z* 383; vitamin A *m*/*z* 269; vitamin E *m*/*z* 431; vitamin K_1 *m*/*z* 451, IS *m*/*z* 473.

2.4. Study cohort

The study cohort consisted of 161 human serum samples collected in the Department of Pediatrics, Pediatric Gastroenterology, Hepatology and Nutrition of the Medical University of Gdańsk. Blood samples were taken by venepuncture from 75 pediatric patients with already diagnosed cystic fibrosis, from 53 pediatric patients after one year of vitamin supplementary therapy and from a group of 33 pediatric controls after an overnight fasting period. The pediatric patients with cystic fibrosis were under the supervision of the Cystic Fibrosis Outpatient Clinic of the Maciej Płażyński Children's Hospital in Gdańsk, Poland. The healthy control group comprised pediatric subjects residing in the Department and Clinic of Orthopedics and Traumatology of the Locomotor System for Children at the Medical University of Gdańsk. The research protocol was endorsed by the Ethical Committee from the Medical University of Gdańsk. Informed consent was obtained from each subject's parents or legal guardians before the collection of samples.