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Ion chromatographic method for the determination of orotic acid in urine

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ABSTRACT

Excess urinary orotic acid excretion occurs in patients with some inborn errors of metabolic pathways such as pyrimidine synthesis and urea cycle. Thus, rapid diagnosis of orotic aciduria has a vital importance for patients. In this paper, a novel method for determination of orotic acid in urine samples by ion chromatography with suppressed conductivity detection was investigated. The separation of orotic acid from urine matrix was accomplished by using an anion exchange column with optimized isocratic eluent program which utilized 50 mM NaOH. The other chromatographic conditions were as follows: the suppressor current was 31 mA; the flow rate of mobile phase was 0.25 mL min^{-1}; the column temperature was 30 °C; sample loop volume was 10 µL. Under optimized conditions, the limit of detection was 0.2 µmol L^{-1}. Dramatically elevated orotic acid concentration was observed on pathological urine samples comparing to healthy urines, as expected. There are a good many advantages of the proposed method, but using an environmentally friendly reagent free system, no organic solvent employment and its quick nature, and being a sensitive and reliable method are the most obvious ones. The proposed method, therefore, may be utilized as an alternative technique for clinical laboratories.

1. Introduction

The pyrimidine balance in humans is controlled by the *de novo* synthetic, salvage and catabolic pathways. Orotic acid (OA) is the fourth intermediate in the *de novo* pyrimidine synthetic pathway. The pathway starts with the formation of carbamoyl phosphate from glutamine, carbon dioxide and two molecules of ATP, by cytosolic enzyme carbamoyl phosphate synthetase II (CPS II) and continues with aspartate transcarbamylase and dihydroorotase enzymes [1]. Dihydroorotic acid dehydrogenase catalyzes the oxidation of dihydroorotic acid to orotic acid on the outer surface of the mitochondria [2]. Intracellular orotic acid is converted to uridine monophosphate (UMP) by UMP synthase. CPS I, on the other hand, is a part of the urea cycle, is activated by N-acetyl-glutamate (NAG) and utilizes ammonia in the synthesis of carbamoyl phosphate [3,4].

UMP synthase deficiency causes a rare inborn error of metabolism called hereditary orotic aciduria with a gross increase of orotic acid accumulation [5,6] while deficiencies in urea cycle enzymes such as ornithine transcarbamoylase and argininosuccinate synthetase or patients with lysinuric protein intolerance and mitochondrial ornithine transporter deficiency HHH (hyperornithinemia, hyperammonemia and homocitrullinuria) syndrome result in increases in the rate of *de novo* synthesis of pyrimidine synthesis and orotic acid due to accumulation of carbamoylphosphate [7–9]. Elevation of OA excretion can also be detected in patients which are treated by drugs such as allopurinol and 6-azauridine [10].

Urinary orotic acid measurements are described to be more relevant than that of plasma due to efficient urinary excretion [11]. In the literature, several analytical methods have been published for analysis of OA in urine so far. Quantification of OA in biological samples are difficult and generally require sophisticated instrumentation [11,12]. Briefly, the analytical methods for determination of OA in the literature are as follows: High Performance Liquid Chromatography with UV [13] and with Diode Array Detection (DAD) [14], Liquid Chromatography – Electrospray Tandem Mass Spectrometry (LC-ESI-MS/MS) [15,16], Hydrophilic Interaction Liquid Chromatography – Tandem Mass Spectroscopy (HILIC-MS/MS) [17], Gas Chromatography – Mass Spectroscopy (GC – MS) [18], Capillary Zone Electrophoresis (CZE) with UV [19,20] and with fiber-coupled DAD [21], Spectrophotometric [22] and Spectrofluorometric [12] methods. Even though these analytical

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instruments are advanced technology products, they have some handicaps such as being expensive and time-consuming, applying clean-up and derivatization procedures [13,18,22], and use/waste of toxic organic solvents. One of the powerful methods for analysis of inorganic anions and small organic acids is Ion Chromatography (IC).

In literature, an IC with activated pulsed amperometric detection (APAD) method was used for determination of OA in healthy urine samples [23]. This developed IC-APAD study had showed that the linear working range was 0.2–30 μmol L^{-1} which was almost close to ours (0.64–30 μmol L^{-1}). It was clear that LOQ value was approximately two times less than our value. To achieve successful separation, they manually prepared a strong eluent mixture (100 mM NaOH + 40 mM $NaNO_3$) because OA strongly retained on an anion exchange column (AEC). Besides, they used anion-exchange solid phase extraction (SPE) cartridge in the process of sample preparation. Although urine samples were diluted only two-fold in their work, long preparation time and use of SPE and chemicals told us that our method had a sample preparation procedure that was faster, easier, and cheaper.

Operation of electrochemical detector (ED) is a little more challenging compared to conductivity detector (CD) since conditioning of ED takes more time and more parts exists in ED. Yet, it is well known that ED is not as complicated as MS. LC-MS/MS systems are able to selectively and sensitively analyze targeted ions, but the major drawback of these systems is high costs of operation. Further, every-day-calibration and their complicated nature make it disadvantageous over IC in routine analysis.

It was reported that since orotic acid was weakly adsorbed on the reverse-phase C18 column, it was derivatized to methylated form to increase retention time on C18 column. The newly formed orotic acid methyl ester had a retention time only 2 min more than the previous form, by using 5% acetonitrile mobile phase [13].

Here comes the advantages of reagent free IC (RFIC). Eluent generation (EG) cartridge electrolytically produces high-purity hydroxide in RFIC systems. Therefore, both contamination risks and man-made errors are eliminated by using RFIC systems. What is more, RFIC systems maintain stability during long periods even if the systems are switched off.

After anionic forms of small organic acids and/or inorganic anions separated on AECs they are converted into H-form in the suppressor part prior to reaching to CD [24]. At the same time, Na^+ ion from NaOH in the mobile phase is exchanged with H^+ in the suppressor and water (H-OH) is formed. Therefore, the mobile phase's background conductivity is lowered and the analyte passes through the conductivity detector in the H-form and the sensitiveness is increased. In this case, H-form of a targeted analyte must always be dissociated to respond to CD. Since the pK_{a1} value of OA is 2.8, it can be detected by CD. The molecular structure of OA is demonstrated in Fig. 1. On the other hand, in contrast to orotate, the other compounds in the urine matrix can be early removed from AEC. These properties made the method more selective and advantageous, so we decided to develope an IC-CD method for determination of OA in urine samples. To the best of our knowledge, this is the first method of using RFIC with suppressed conductivity detection to measure orotic acid concentrations in both pathological urine samples and healthy subjects. As a result, this paper describes a novel IC method with advantages in many ways which are simple and rapid sample preparation (dilute-filter-run), no toxic organic solvent use, no derivatization step, and also thanks to the RFIC system excellent precision, high sensitivity, and long term stability are gained. It is recommended that the proposed method should be performed by clinical laboratories especially for fast diagnosis of orotic aciduria on patients and so start treatment if necessary.

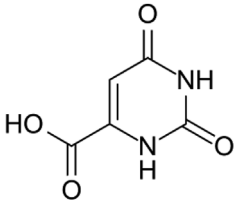

Fig. 1. Molecular structure of uracil-6-carboxylic acid (orotic acid).

2. Materials and methods

2.1. Chemicals and apparatus

All reagents were of analytical reagent grade. Orotic acid (6-Carboxy-2,4-dihydroxypyrimidine) ≥ 98% was purchased from Sigma Chemical Co. (St. Louis, MO 63178 USA).

Ultra pure water (min. 18.2 MΩ/cm resistivity) was received from a New Human Power I Scholar UV system (Human Corporation, Seoul, Korea). Standard aqueous solutions and urine solutions were prepared with this ultra pure water.

PES (polyether sulfone) filter (pore size 0.2 μm, 17 mm) was purchased from Analytical Columns (New Addington, Croydon, CR0 9UG, England).

2.2. Instrumentation

Chromatographic separations and analyses were conducted by using Dionex ICS-3000 (Sunnyvale, CA, USA) ion chromatography system. A suppressed conductivity detector (ASRS 300 suppressor and conductivity cell) was employed to collect signals after separation in the system. OA separation was accomplished by utilizing Dionex IonPac® AS20 analytical column (2 × 250 mm) with a Dionex IonPac® AG20 guard column (2 × 50 mm) as stationary phases and NaOH as mobile phase generated from the Dionex EGC-NaOH EluGen II cartridge using solely ultra pure water. Possible contaminants were eliminated using Continuously Regenerating Trap Columns (CR-ATC). The RFIC systems is superior compared to systems with manually prepared eluents in terms of stability, reproducibility, minimum contamination risk thanks to the EG module. Sample loop volume was 10 μL. Data acquisition and instrument control were performed via Dionex Chromeleon® Client (Ver. 6.80) software.

2.3. Urine sample collection and storage

Thirty normal urine samples were obtained from healthy volunteers who did not suffer from any systematic disease that could affect the content of urine. The urine sample of two volunteer patients who are a two-year-old female and a one-year-old male affected by orotic aciduria was kindly provided by the Department of Metabolic Diseases in Children, Cerrahpaşa Medical Faculty (Istanbul University, Turkey). The ethical permission of this study was approved by Istanbul University, Cerrahpaşa Medicine Faculty, Clinical Research Ethics Committee.

All of the urine samples were immediately frozen to −20 °C and stored at this temperature until analysis.

2.4. Creatinine measurements of urine samples

Creatinine concentrations of all urine samples were measured by a commercial kit, based on the Folin's method with the Jaffé reaction, on the automated instrument Roche-Hitachi P 800 Modular (Roche Dragnostic, Mannheim, Germany). The concentration of OA is expressed as a ratio with urinary creatinine concentration (μmol $mmol^{-1}$ of creatinine) to take into account the variations of urinary volume among subjects, this procedure is commonly used in clinical biochemistry.