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RAPID DETERMINATION OF L-2-HYDOXYGLUTARIC ACID IN URINE SAMPLES BY CAPILLARY ELECTROPHORESIS WITH INDIRECT UV DETECTION

İdrar Örneklerinde L-2-Hidroksiglutarik Asitin Kapiler Elektroforez-İndirekt UV Dedeksiyon ile Hızlı Tayini

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Abstract

Aim: L-2-hydroxyglutaric aciduria (L2HGA) which is autosomal recessive and characterized by psychomotor retardation, cerebellar ataxia, variable macrocephaly, and epilepsy is a rarely seen neurometabolic disease. The disease is biochemically identified by slightly increased L-2HGA levels in urine, cerebrospinal fluid, and plasma.

Materials and Methods: A sensitive and rapid capillary electrophoretic technique was used for the determination of L2HGA in urine. Since the L2HGA lacks of chromophore, an indirect UV detection method was applied.

Results: The running electrolyte consisted of 10 mmol/L 2,6-pyridinedicarboxylic acid (PDC) at pH 5.6 containing 0.1 mmol/L of cetyltrimethylammonium bromide (CTAB). PDC was chosen because of its good chromophoric property for indirect UV detection. CTAB was used as electro-osmotic flow (EOF) reversal. The method was well-validated in the selected conditions. The detection limit of the method L2HGA was 1.16 µmol/L. The migration time of L2HGA was less than 2.0 min.

Conclusion: The developed method was applied to the urine samples collected from 16 patients who suffer from L-2-hydroxyglutaric aciduria and 4 healthy volunteers.

Keywords: Capillary electrophoresis, indirect UV detection, L-2-hydroxyglutaric acid, L-2-hydroxyglutaric aciduria, urine.

Öz

Amaç: L-2 hidroksiglutarik asidüri (L2HGA) otozomal resesif geçişli, psikomotor gerilik, serebellar ataksi ve değişken makrosefali veya epilepsi ile karakterize edilen nadir görülen nörometabolik bir hastalıktır. Hastalık biyokimyasal olarak idrarda, beyin omurilik sıvısında ve çok az miktarda plazmada artmış L-2HGA seviyeleri ile tanımlanır.

Materyal ve Metot: Bu çalışmada, idrarda L-2-hidroksiglutarik asitin tayini için hızlı ve hassas bir kapiler elektroforez yöntemi geliştirilmiştir. L-2-hidroksiglutarik asit kromofor gruba sahip olmadığı için, indirekt UV deteksiyon metodu uygulanmıştır.

Bulgular: Çalışma tamponu pH 5.6'da 0.1 mmol/L setiltrimetilamonyum bromür (CTAB) içeren 10 mmol/L 2,6-piridindikarboksilik asitten (PDC) oluşmaktadır. PDC iyi bir kromoforik özellik göstermesinden dolayı indirekt deteksiyon için uygundur. CTAB elektro-osmotik akışı (EOF) ters çevirmek için kullanılmıştır. L-2-hidroksiglutarik asit için tayin limiti 1.16 µmol/L'dir. Geliş zamanı ise 2.0 dakikadan daha az bir süredir.

Sonuç: Geliştirilen metod 16 L-2-hidroksiglutarik asidüri hastasından ve 4 sağlıklı gönüllüden toplanan idrar örneklerine uygulanmıştır.

Anahtar Kelimeler: Kapiler elektroforez; indirekt UV deteksiyon, L-2-hidroksiglutarik asit, L-2-hidroksiglutarik asidüri, idrar.

INTRODUCTION

L-2-hydroxyglutaric aciduria (L2HGA) is a slowly progressive neurometabolic disease that is caused by defective 2-hydroxyglutarate dehydrogenase enzyme activity [1]. Developmental delay, mental

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retardation,epilepsy, cerebellar findings, pyramidal and/or extrapyramidal signs can be listed as major manifestations of the disease [2].

Brain magnetic resonance imaging (MRI) findings of L2HGA are highly spesific to disease and consists of subcortical white matter and basal ganglia alterations, cerebral and/or cerebellar atrophy [3]. There is not any specific therapy for L-2-hydroxyglutaric aciduria. However, riboflavin, carnitine and flavin adenine dinucleotide sodium (FAD) are treatment options that were reported to be beneficial and provided partial improvement in a limited data [4, 5].

The beneficial treatment is impossible without a proper diagnosis**.** Definite diagnosis can be made by quantitative analysis of L-2-hydroxyglutaric acid (L2GA) accumulation in urine, cerebrospinal fluid [2] and plasma or by molecular anaysis of *L2HGDH* gene [3]. Therefore**,** highly sensitive and fast methods are required in clinical analysis of L-2-hydroxyglutaric aciduria.

Several methods have been reported for the quantitative determination of L-2-hydroxyglutaric acid in the human body fluids by liquid chromatography/mass spectrometry [6], gas chromatography/mass spectrometry [7], capillary gas chromatography [8], NMR [9], and magnetic resonance imaging [10].

In this work, a capillary electrophoresis method was applied for the determination of L-2-hydroxyglutaric acid in the urine samples. A very low or no UV absorbance because of the lack of chromophore groups in a compound makes photometric detection a difficult task. In order to achieve this challenge, indirect UV detection is generally a good choice since it presents sensitive detection. In the indirect detection, the background electrolyte (BGE) selection is essentially important. The BGE must have a high extinction coefficient at the detection wavelength chosen. The mobility of the BGE is another important issue. It has to match the mobility of analytes in order to obtain signal peaks with neither fronting nor tailing.

2,6-pyridinedicarboxylic acid (PDC)/cetyltrimethylammonium bromide (CTAB) system was applied for the indirect-UV detection of L-2-hydroxyglutaric acid. This combination as background electrolyte was used in the the analysis of inorganic and organic anions in beer [11]; organic acids in honey and honeybee pollens [12, 13], beverages [14], and pomegranate juices [15] before.

In the present study, L-2-hydroxyglutaric acid was detected in the urine samples using this technique for the first time in the literature.

MATERIAL AND METHOD

Chemicals

L-2-hydroxyglutaric acid (sodium salt) was from Sigma-Aldrich (Steinheim, Germany). Sodium hydroxide, 2,6-pyridinedicarboxylic acid (PDC), and *N*-cetyl- *N,N,N*-trimethylammonium bromide (CTAB) were purchased from Merck (Darmstadt, Germany).

Preparation of standard solutions

The stock solution of L-2-hydoxyglutaric acid (sodium salt) was prepared in deionized water at 1000 µg/mL concentration and stored at −21°C until the analysis. Deionized water purified by Elga Purelab Option-7-15 model system was used for all solution preparation.

Urine samples

The urine samples were obtained from 16 patients who suffer from 2-hydroxyglutaric aciduria and 4 healthy volunteers. All the samples were stored in deep freeze at −21°C until analysis. The samples were centrifuged at 3500 rpm for 5 min and then filtered through a 0.45 µm microfilter. The filtered urine samples were diluted appropriately with deionized water, vortexed for 1 min.

Instrumentation

The analyses were performed with an Agilent 1600 capillary electrophoresis with Agilent ChemStation software. The detection was carried out with a diode array detector (UV-DAD). The signal wavelength was set at 350 nm. A reference wavelength at 200 nm was also set. The separations were performed in a 50 µm i.d. silica capillary column purchased from Polymicro Technologies, Phoenix, AZ, USA. The total length of the column was 65 cm and the effective length was 57 cm. The temperature was set at 25 °C. Injections to the capillary column were performed at 50 mbar for 6 s from cathodic side. The separation voltage was at -25 kV. In order to condition of the capillary column, it was washed with 1 mol/L NaOH solution for 30 min an deionized water for 15 min, respectively. Before work, the column was rinsed with the working buffer solution for min. The capillary column was rinsed with deinoized water for 2 min and the working buffer solution for 5 min between each run.

RESULTS AND DISCUSSION

Optimization of the electrolyte composition

The capillary electrophoretic separation of L-2-hydoxyglutaric acid was performed by indirect UV detection method because of the lack of chromophores in this compound. Indirect UV detection is used to visualize ions which have little or no UV chromophore group. Thus a carrier electrolyte with chromophore which has high molar absorptivity coefficient is used in this method.

For the indirect detection of L-2-hydoxyglutaricacid, PDC was selecte as carrier electrolyte. PDC has a high molar absorptivity coefficient (E=43680 L/mol.cm at 192 nm) [11]. The concentration of PDC was studied between 5-20 mM. Considering the peak symmetry and intensity 10 mM of PDC was chosen. The pH of PDC was adjusted to 5.6 using NaOH. In order to speed up the analysis, the electroosmotic flow is needed to be reversed. CTAB at 0.1 mM concentration was selected as the flow reversal. The polarity was reversed.

The key principle in indirect UV detection is that the migrating analytes displace a UV-absorbing component, the background electrolyte (BGE). This displacement results in a negative signal, which can be reversed by monitoring a signal wavelength outside the absorption area of the BGE in addition to the reference wavelength, which are chosen to match the absorption peak for the BGE. When the signal wavelength is subtracted from the reference wavelength, the negative signal is converted to a positive signal, which can be integrated directly for quantitative analyses. Several factors have to be

considered before the BGE is chosen for indirect detection systems. Signal wavelength of 350 nm was employed in this study. A reference at 200 nm was also set. Absorbance at 200 nm was decreased when L-2-hydoxyglutaric acid was occured. It is recorded as a relative increase of the signal at 350 nm. Fig. 1a presents an electropherogram of standard L-2-hydoxyglutaricacid at the optimized conditions.

Method validation

The method was well-validated as seen in Table 1. The validation of the proposed method was performed according to the Eurachem guidelines 16. The calibration curve was constructed between 5-100 µg/mL concentration of L-2-hydoxyglutaric acid (sodium salt). The correlation coefficient (R2) was found as 0.999. The LOD and LOQ values were calculated as three times and ten times the average noise taken for three different baseline areas, respectively. The LOQ was calculated as ten times the average noise taken for three different baseline areas. The LOD and LOQ values were 1.16 and 3.87 µg/mL, respectively. The method precision was performed according to the intra- and inter-day repeatabilities of both peak area and migration time. Intra-day and inter-day analyses were determined by injecting the L-2-hydoxyglutaric acid seven times a day and twenty-one times on different three days, respectively. The relative standard deviations were below 2.70 for each parameter as seen in Table 1. For the recovery studies, three urine samples were spiked with L-2-hydoxyglutaric acidat three different concentrations corresponding to 50, 100 and 200 % of the analyte concentration of the urine samples. The percentage of recovery was calculated with the formula 1:

$$\text{Recovery}(\%)=[(C1-C2]/C3]\times 100 \quad (1)$$

where C1 is the concentration determined in fortified urine sample, C2 is the concentration determined in unfortified urine sample and C3 is the concentration of added L-2-hydoxyglutaric acid (sodium salt) standard. Good recovery results were obtained, with values ranging between 92.0 and 104 % as seen in Table 1.

Table 1. Analytical performance of the method

Analytical parameter	
Intra-day precision (**n** = 7)	
Corrected peak area (RSD, %)	1.82
Migration time (RSD, %)	2.63
Inter-day precision (**n** = 21)	
Corrected peak area (RSD, %)	2.38
Migration time (RSD, %)	1.45
Linearity	
Linear range (µmol/L)	5.00-100
Regression equation	y=0.0131x – 0.0297
Correlation coefficient (R^2)	0.999
LOD (µmol/L)	1.16
LOQ (µmol/L)	3.87
Recovery(%)	92.0-104%

Real sample analyses

L-2-hydoxyglutaric acid concentration was found between 48.9 – 1,15 µg/mL in 16 patients. In all healthy volunteers, L-2-hydoxyglutaric acid was not detected. The results are shown in Table 2. Fig. 1b shows a representative electropherogram of a patient urine diluted 200 times. An electrophoregram of the urine sample from a healthy volunteer is shown in Fig. 1c.

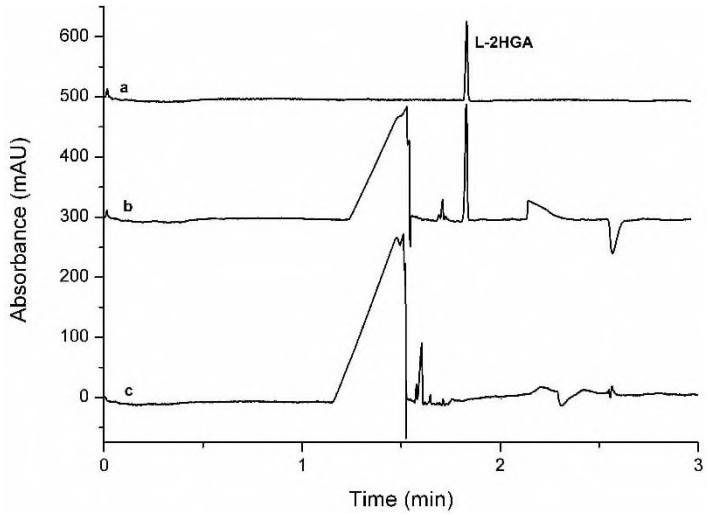

Figure 1: Electrophoregram of (a) 25 µg/mL standard L-2-hydoxyglutaricacid, (b) a patient urine diluted 200 times, (c) urine sample from a healthy volunteer. Separation buffer: 10 mmol/L of PDC + 0.1 mmol/L CTAB pH: 5.6. Detection wavelength: 350 nm with a reference at 200 nm. Injection: 50 mbar, 6 s. Voltage: -25 kV. Temperature: 25 °C.

Table 2. L-2-hyroxyglutaric acid concentrations in the urine samples

Patients	L-2-hyroxyglutaric acid (µg/mL ± SD*)
Patient 1	188 ± 8
Patient 2	584 ± 12
Patient 3	996 ± 26
Patient 4	55.3 ± 2.6
Patient 5	184 ± 4
Patient 6	906 ± 11
Patient 7	211 ± 6
Patient 8	894 ± 9
Patient 9	330 ± 5
Patient 10	87.7 ± 2.2
Patient 11	1,10 ± 23
Patient 12	290 ± 5
Patient 13	415 ± 8
Patient 14	1,15 ± 18
Patient 15	48.9 ± 3.3
Patient 16	50.7 ± 4.6

*n=2

CONCLUSION

A simple, inexpensive, and rapid capillary electrophoresis method was decribed and applied to urine samples. It requires no sample preparation or derivatization step. L-2-hydoxyglutaric acid was well-separated and detected only in 2 minutes. The proposed method immediately allows to recognize abnormal urine profiles related to L-2-hydroxyglutaric aciduria. Therefore, it could be used as a valuable routine diagnostic system.

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The local ethics committee of Diyarbakır Gazi Yaşargil Training and Research Hospital approved the study (Number: 08.11.2019/371).