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Address: Molla Gürani Mah. Kaçamak Sk. No: 21/1 34093 İstanbul, Türkiye
Phone: +90 (530) 177 30 97 / +90 (539) 307 32 03
E-mail: info@galenos.com.tr • yayin@galenos.com.tr
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INHERITED METABOLIC DISORDERS AND NUTRITION

Editorial

Dear Colleagues,

We are delighted to announce the launch of the inaugural issue of Inherited Metabolic Disorders and Nutrition (IMDN), the official journal of the Child Nutrition and Metabolism Association. The journal provides a platform for academics to advance scientific research, report rare case studies, and share innovative ideas - all contributing to the growing body of knowledge in our field.

Inherited metabolic disorders represent a critical area of research, with an increasing number of researchers and publications dedicated to this field worldwide. IMDN was established in response to the ongoing need for additional avenues to publish high-quality research in this area. The journal aspires to make a lasting contribution to the fields of paediatric nutrition and inherited metabolic disorders. With the release of the first issue, a strong commitment is renewed to position IMDN as a leading resource within the international scientific community. Over time, we hope that IMDN will become an essential tool for sharing information and experiences related to metabolic disorders and nutrition.

IMDN will be published quarterly and will feature peer-reviewed research articles, case reports, reviews, original studies, and other relevant content. Our editorial board comprises experts from around the globe, and we welcome contributions from our international colleagues.

For more information about IMDN, please visit our website at www.imdn.org. I encourage you to read the first issue with great interest, and I extend my sincere thanks to the authors whose work is featured in it. As a newly established journal, IMDN will undoubtedly thrive with your continued support and contributions.

We look forward to receiving your submissions for future issues.

Best regards,
Nur Arslan
Editor-in-Chief, Inherited Metabolic Disorders and Nutrition (IMDN)

ORIGINAL ARTICLE

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Development and Validation of a Multiplex LC-MS/MS Assay for Lysosphingolipid Quantification in the Diagnosis of Gaucher and Fabry Diseases

Halide Akbaş[1], Ayşe Ergül Bozacı[2] , Bilge Karatoy Erdem[1], Nurullah Özsarı[3], Erdoğan Soyuçen[2]

[1]Akdeniz University Faculty of Medicine, Department of Biochemistry, Antalya, Türkiye

[2]Akdeniz University Faculty of Medicine, Department of Pediatric Metabolism, Antalya, Türkiye

[3]Bilecik Research and Training Hospital, Clinic of Biochemistry, Bilecik, Türkiye

Abstract

Objectives: Lysosphingolipids (LysoSLs), the N-deacylated forms of sphingolipids, are increasingly recognized as toxic metabolites that accumulate in various lysosomal storage disorders (LSDs), collectively known as sphingolipidoses. These compounds, due to their amphiphilic nature and bioactive properties, can contribute to cellular dysfunction and disease pathogenesis. Recent studies have demonstrated that specific LysoSLs, such as hexosylsphingosine (HexSph), globotriaosylsphingosine (LysoGb3), and lysosphingomyelin (LysoSM), are elevated in plasma, serum, or dried blood spots from patients with Gaucher, Fabry, Krabbe, or Niemann-Pick diseases. These findings have paved the way for the use of LysoSLs as sensitive and specific biomarkers in the diagnosis, prognosis, and therapeutic monitoring of sphingolipidoses. This study aimed to develop and validate a method for the simultaneous quantification of three LysoSLs—HexSph, LysoGb3, and LysoSM—using liquid chromatography–tandem mass spectrometry (LC-MS/MS), for use in the diagnosis and follow-up of sphingolipidoses.

Materials and Methods: A multiplex analytical method was optimized for quantifying HexSph, LysoGb3, and LysoSM by ultra-fast LC coupled to tandem MS (UFLC-MS/MS) in positive-ion mode with electrospray ionization (LC-20 AD UFLC XR; Shimadzu 8040, Japan), employing multiple reaction monitoring. The method was validated for linearity, accuracy, precision, limit of detection, limit of quantification, and recovery rates. Following method validation, plasma samples were collected from 14 patients diagnosed with Gaucher disease or Fabry disease at Akdeniz University Hospital and from 20 healthy adult volunteers. Sample preparation involved methanol/acetone/water extraction prior to analysis of LysoSL concentrations.

Results: The total analysis time for all three analytes was determined to be 10 minutes. Calibration curves demonstrated strong linearity, with coefficients of determination (r^2) of 0.995 for LysoSM, 0.982 for HexSph, and 0.961 for LysoGb3. Plasma concentrations of LysoSLs were measured in both the patient and control groups. Significantly elevated levels of LysoSM and HexSph were observed in Gaucher patients compared to healthy controls. While LysoGb3 levels were higher in Fabry patients than in controls, the difference did not reach statistical significance.

Conclusion: This validated multiplex LC-MS/MS assay enables rapid and reliable simultaneous quantification of key LysoSLs in plasma. The assay holds promise as a diagnostic and monitoring tool for sphingolipidoses and may be further expanded to include additional biomarkers relevant to other LSDs. Quantification of LysoSLs can support clinical decision-making, including therapeutic selection and monitoring of patients undergoing enzyme replacement therapy.

Keywords: Lysosphingolipid, Hexosylsphingosine, Globotriaosylsphingosine, Lysosphingomyelin, LC-MS/MS

Address for Correspondence: Ayşe Ergül Bozacı, Akdeniz University Faculty of Medicine, Department of Pediatric Metabolism, Antalya, Türkiye

E-mail: ergul.acar@yahoo.com.tr **ORCID ID:** orcid.org/0000-0002-9783-1016

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INTRODUCTION

Sphingolipidoses are a subgroup of lysosomal storage disorders (LSDs) caused by inherited defects in lysosomal degradation pathways of sphingolipids.[1,2] This group of inborn metabolic diseases includes primary sphingolipidoses, such as Niemann-Pick types A, B, and A/B (NPA/B), Fabry disease (FD), Krabbe disease (KD), Gaucher disease (GD), and Tay-Sachs disease, all of which are caused by mutations in genes encoding specific lysosomal enzymes. Secondary sphingolipidoses, such as NPC, as well as disorders related to saposin deficiencies, are included.

Due to overlapping clinical manifestations among various sphingolipidoses, accurate clinical diagnosis is often challenging. Therefore, identification and analysis of molecular biomarkers that can facilitate differential diagnoses have become increasingly important. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) is a widely used, highly sensitive, and highly specific analytical technique for biomarker quantification. One of its key advantages is the ability to simultaneously measure multiple analytes within a single run.

In recent years, the metabolism and biological functions of sphingolipids have been the focus of extensive research, and LC-MS/MS has become an essential tool in sphingolipid profiling. Although increased levels of glycosphingolipids have been reported in several sphingolipidoses, their relatively low sensitivity and specificity limit their utility as diagnostic biomarkers.[3] In contrast, lysosphingolipids (LysoSLs)—the N-deacylated derivatives of glycosphingolipids—have been found to accumulate markedly in the plasma and urine of patients with sphingolipidoses. These findings support their potential as sensitive and specific biomarkers for both diagnosis and therapeutic monitoring.[4]

Various forms of LysoSLs (LysoSLs) have been found to be selectively elevated in specific sphingolipidoses. For instance, significantly increased levels of globotriaosylsphingosine (LysoGb3) have been detected in both plasma and urine of male and female patients with FD.[5-7] In Krabbe disease, galactosylsphingosine (GalSph) is markedly elevated,[8,9] while glucosylsphingosine (GlcSph) shows a significant increase in GD.[3] Additionally, lysosphingomyelin (LysoSM), the N-deacylated derivative of sphingomyelin, has been reported to accumulate in patients with NPA, NPB, and NPC.[10,11]

The aim of this study was to establish and validate a robust analytical method for the simultaneous quantification of hexosylsphingosine (HexSph), LysoGb3, and LysoSM using LC-MS/MS. This method was optimized for use in clinical laboratories to support the diagnostic workflow for suspected sphingolipidoses and to monitor biochemical responses in patients receiving specific therapies, such as enzyme replacement therapy or substrate reduction therapy. The development of a multiplex assay for LysoSLs not only improves diagnostic efficiency but also facilitates the early detection and longitudinal assessment of disease activity, potentially leading to better patient outcomes. Our study introduces a locally optimized, rapid multiplex LC–MS/MS workflow for simultaneous quantification of LysoGb3, LysoSM, and HexSph with a 10-minute run time, which is shorter than that of comparable published methods.[12]

MATERIALS AND METHODS

Reagents and Chemicals

The following chemicals and reagents were used for LysoSL analyses: GlcSph (bovine buttermilk), N-omega-CD3-octadecanoyl-ceramide trihexoside (internal standard 1537; Matreya Chemicals), GalSph, LysoSM, LysoGb3, acetonitrile, methanol, acetone, chloroform, formic acid, and ultrapure water.

Sample Collection

Ethical approval was obtained from the Ethics Committee of Akdeniz University Faculty of Medicine (decision number: 373, dated: 30.05.2018). After obtaining informed consent, venous blood samples were collected into K2-EDTA tubes from 14 patients diagnosed with Fabry or GD and from 20 healthy adult volunteers at Akdeniz University Hospital. Plasma samples were separated and stored for analysis. Diagnoses were confirmed in all patients through demonstration of significant lysosomal enzyme deficiencies and/or mutation analysis.

Preparation of LysoSL Calibration Standards

Stock standard solutions were prepared by dissolving purified standards in a chloroform:methanol (2:1, v/v) mixture at the following concentrations: GlcSph, 0.5 mg/mL; LysoSM, 1 mg/mL; and LysoGb3, 0.1 mg/mL. GlcSph was used as the standard for HexSph. Working solutions at a concentration of 10 µmol/L for each analyte were prepared in the same solvent mixture and stored at -20 °C. These working solutions were diluted with methanol/acetone/water (45:45:10, v/v) to a final concentration of 10 nmol/L to prepare the precipitation solution containing the internal standard. A deuterium-labeled internal standard, N-omega-CD3-octadecanoyl-ceramide trihexoside (C18-CD3-Gb3), was used for quantification.

Calibration curves were constructed using six concentrations prepared by serial dilution of the stock solutions. The calibration ranges were 0-200 nmol/L for LysoGb3 and LysoSM, and 0-1000 nmol/L for HexSph. The accuracy and precision of the assay were evaluated using QC samples prepared at two concentration levels.

Sample Preparation

To 100 µL of plasma, calibrator, or QC sample, 500 µL of working precipitation solution was added. After vortexing for 30 seconds, the mixture was sonicated for 1 minute and subsequently centrifuged at 16,200 × g for 10 minutes. The clear supernatant was transferred to a new tube and evaporated under nitrogen. The dried residue was reconstituted in 100 µL of acetonitrile/water (1:1, v/v) containing 0.1% formic acid and centrifuged again at 16,200 × g for 10 minutes prior to LC-MS/MS analysis.

LC-MS/MS Method Optimization

Chromatographic separation of LysoGb3, HexSph, and LysoSM was achieved using a reverse-phase Raptor acid-resistant C18 column (2.1 × 100 mm, 2.7 µm particle size; Restek, USA). Because GlcSph and GalSph co-elute on this column, the two analytes were quantified together as HexSph (GlcSph + GalSph). Unlike most published studies focusing on a single analyte–disease pair, we provide a multiplexed approach applicable to multiple sphingolipidoses.

Mass spectrometric detection was performed on a UFLC-MS/MS system (LC-20 AD UFLC XR, Shimadzu 8040, Japan) equipped with a positive-mode electrospray ionization source. For each analyte, the precursor ions and optimized transitions were selected and monitored in multiple reaction monitoring mode. Detailed LC-MS/MS parameters are provided in Table 1.

Method Validation

The accuracy of the method was evaluated using quality control (QC) samples. Accuracy was calculated as the relative error (%), which was obtained by subtracting the nominal value from the mean measured value, dividing the difference by the nominal value, and multiplying by 100. Intra-day and inter-day precision were assessed by analyzing each standard in triplicate over consecutive days. Linearity was evaluated using six-point calibration curves for each biomarker. The limit of detection (LOD) and limit of quantification (LOQ) were determined for all analytes. Carryover effects were assessed by injecting blank samples after the highest calibration standard.

Table 1. Detailed LC-MS/MS parameters.

Parameter	Description
Mass spectrometer	Shimadzu 8040
Ionization mode	ESI, positive mode (+)
Column	Raptor ARC-18, 2.1 × 100 mm, 2.7 µm particle size
Column temperature	45 °C
Mobile phase A	H_2O + 0.1% FA
Mobile phase B	ACN + 0.1% FA
Gradient program (Pump B)	Time (min) %B 0–4 80 4–6 100 6–10 40
Flow rate	0.5 mL/min
Injection volume	2 µL
Total run time	10 minutes

H_2O 0.1% FA; water containing 0.1 percent FA, ACN 0.1% FA; acetonitrile containing 0.1 percent FA. ARC-18, acid-resistant C18; ESI, electrospray ionization; FA, formic acid.

Statistical Analysis

Statistical analyses were performed using SPSS version 20.0. Differences between groups were assessed using a two-tailed Student's t-test. A p-value ≤ 0.05 was considered statistically significant.

RESULTS

Chromatographic Analysis of LysoSLs

Chromatographic separation of LysoSLs was achieved with retention times of 1.57 minutes for LysoGb3, 1.72 minutes for LysoSM, and 1.98 minutes for HexSph. However, to ensure complete column elution and prevent carryover, the total run time was set to 10 minutes (Figure 1).

Linearity, Detection, and Quantification Limits

Repeated calibration curves were generated to assess linearity for LysoGb3, LysoSM, and HexSph, and the r^2 values were 0.995 for LysoSM, 0.982 for HexSph, and 0.961 for LysoGb3. A blank sample was injected after the highest calibration standard, and no carry-over was observed. The LOD and LOQ for LysoSLs were calculated from the mean and standard deviation of blank samples, which were run to obtain those statistics. The LOD and LOQ for LysoSLs were 0.012 and 0.036 nmol/L for LysoSM, 0.01 and 0.03 nmol/L for HexSph, and 7.18 and 21.54 nmol/L for LysoGb3, respectively (Table 2).

Precision and Accuracy

Precision and accuracy for each analyte were determined intraday (n = 5) and interday (n = 5). The % CV was below 20% for LysoSM and HexSph at all concentrations tested, whereas for LysoGb3 it was below 20% at 25 nmol/L and above.

LysoSL Levels in Healthy Controls and Lysosomal Disease (LD) Patients

Demographic characteristics of the study groups are summarized in Table 3. All Gaucher patients had Type 1 disease. Our study cohort consisted of seven male patients with the classic Fabry phenotype and three female patients with a stroke-associated Fabry phenotype. All patients were genetically confirmed. None of the patients were on enzyme replacement therapy at the time

of sampling. Plasma concentrations of LysoGb3, LysoSM, and HexSph in patients and controls are shown in Table 4.

Patients with GD exhibited significantly higher mean plasma levels of LysoSM and HexSph than those in healthy individuals. Although LysoGb3 levels were numerically higher in Fabry patients than in controls, the difference did not reach statistical significance ($p = 0.08$). The lack of statistical significance is likely due to the limited sample size (n=10) and the considerable

Table 2. Mass shifts and retention times of LysoSLs in LC-MS/MS.

Compound	Precursor ion (m/z)	Product ion (m/z)	Retention time (min)
LysoGb3	786.20	282.20	1.57
LysoSM	465.20	184.10	1.72
HexSph	462.20	282.30	1.98
LysoGb3-IS	843.20	264.30	1.40

Table 3. Demographic characteristics of patients and healthy controls.

Group	Controls	FD	GD
Sample size	20	10	4
Sex (M/F)	10/10	7/3	4/0
Age (mean, range)	37 (21–77)	37 (22–51)	40 (38–42)

Table 4. Plasma LysoSL levels in Gaucher and Fabry patients vs. healthy controls.

LysoSL	Group	Mean ± SEM (nmol/L)	p-value
LysoSM	Gaucher (n=4)	0.87 ± 0.28	0.001
	Control (n=20)	0.25 ± 0.02	
HexSph	Gaucher (n=4)	0.83 ± 0.39	0.001
	Control (n=20)	0.02 ± 0.002	
LysoGb3	Fabry (n=10)	35.63 ± 15.89	0.08
	Control (n=20)	10.03 ± 0.28	

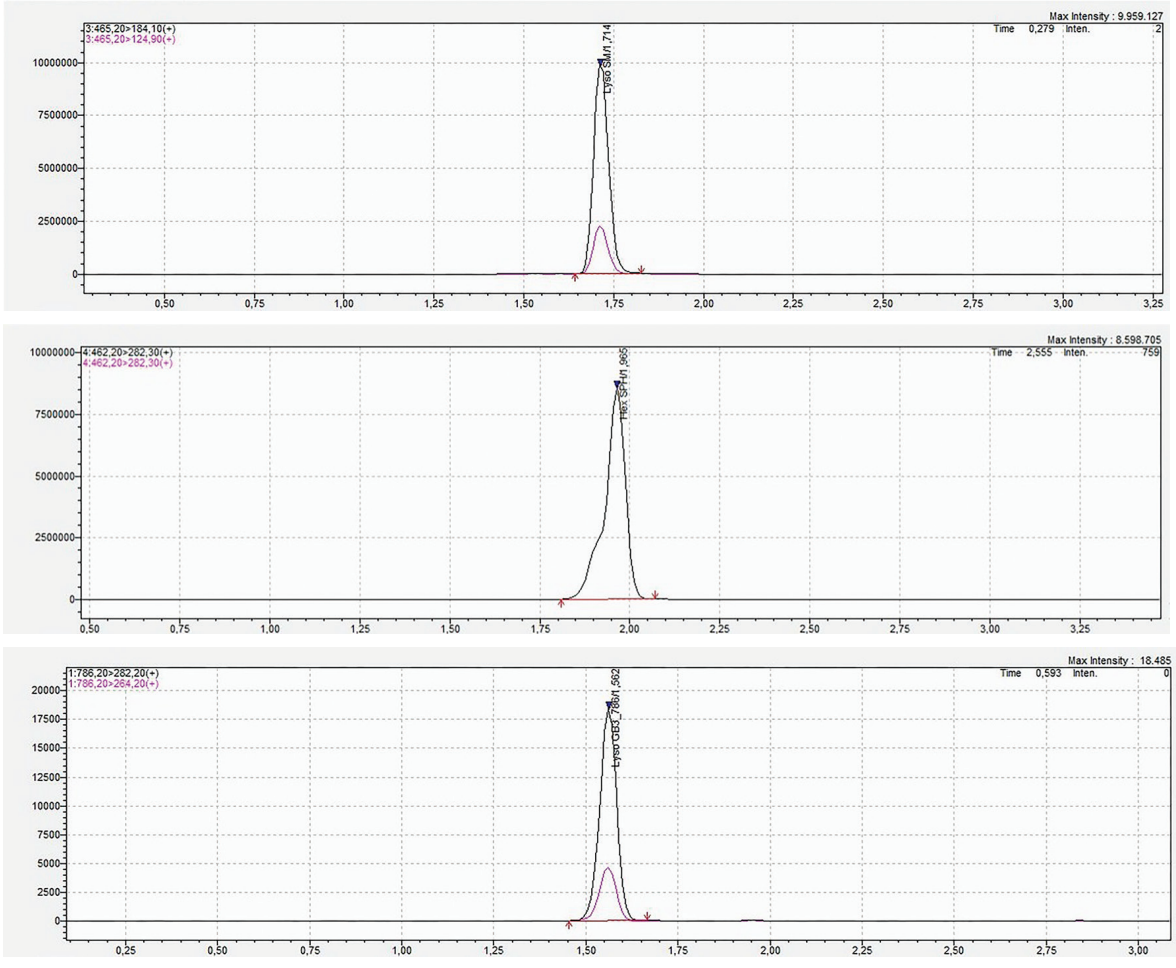

Figure 1. Chromatographic separation of LysoSLs with ARC-18 column in the 100 nmol/L calibrator (A. LysoSM 1.72, B. HexSph 1.98, C. LysoGb3 has an elution time (retention time) of 1.57 min.

variability between patients. In addition, differences in matrix and calibration, which affect absolute concentrations, may have masked numerical differences, even though they do exist.

DISCUSSION

LysoSLs have emerged as reliable and informative biomarkers for the diagnosis, prognosis, and therapeutic monitoring of sphingolipidoses, a group of inherited LSDs characterized by the accumulation of sphingolipid substrates due to enzyme deficiencies.[3,5] Numerous LC-MS/MS methods have been developed for the quantification of LysoSLs in various biological matrices, including plasma, urine, and dried blood spots.[6,9,11–15] However, a significant limitation of many previously published assays is their focus on a single analyte associated with a specific disease. While such approaches are effective for targeted diagnostics, they may fail to capture the broader metabolic alterations observed in LDs with overlapping or non-specific symptoms.

Recent studies have highlighted the value of multiplex panels that allow for the simultaneous measurement of multiple LysoSLs, suggesting that such panels can improve diagnostic yield, reduce turnaround time, and enhance cost-efficiency, particularly in high-throughput clinical settings.[14] The ability to evaluate multiple biomarkers from a single sample can also be particularly advantageous in early screening programs or when patient sample volume is limited. Moreover, multiplexing supports a more comprehensive understanding of disease pathology and may help differentiate between related disorders or phenotypic variants.

In this study, we developed and validated a rapid and accurate UFLC-MS/MS method for the simultaneous quantification of three key LysoSLs—HexSph (a combination of GlcSph and GalSph), LysoGb3, and LysoSM—in human plasma. The method is based on simple protein precipitation, avoiding the need for complex and time-consuming liquid–liquid extraction protocols. This streamlined sample preparation significantly reduces overall analysis time and enables high-throughput testing without compromising sensitivity or accuracy. The developed method is rapid, reproducible, and suitable for clinical laboratories. It simplifies sample preparation, reduces run time, and provides robust quantification.

Chromatographic separation was performed on a reversed-phase C18 column, which enabled efficient retention and resolution of LysoSLs. Due to the structural similarity between GlcSph and GalSph, these analytes were not resolved and were therefore reported collectively as HexSph. The use of a deuterated LysoGb3 internal standard ensured analytical robustness and reproducibility. The method demonstrated excellent linearity, precision, and accuracy, with detection and quantification limits comparable to or better than those reported in prior literature.

The analysis enabled the quantification of plasma LysoSL levels in healthy controls and in subjects with confirmed Gaucher and FD. Among Gaucher patients, LysoSM and HexSph were significantly elevated compared with controls, suggesting their relevance as disease-specific biomarkers. These findings are consistent with previous studies reporting elevated levels of LysoSM and GlcSph in GD, which is caused by β-glucocerebrosidase deficiency. In Fabry patients, plasma LysoGb3 levels were increased; however, this difference did not reach statistical significance, potentially owing to the limited sample size and the cross-sectional design of the study. Larger cohort studies may be needed to fully validate the diagnostic sensitivity and specificity of LysoGb3 in this population. During the study period (2018-2019), commercial control materials were not yet available in our laboratory.

Importantly, our method's short run time (10 minutes per sample) and compatibility with standard LC-MS/MS instrumentation make it well-suited for routine clinical application. The proposed panel could be expanded in future studies to include additional LysoSLs or disease-specific lipid species, thereby increasing its diagnostic utility. Furthermore, monitoring LysoSL levels over time may provide insights into disease progression and response to treatment, particularly in patients receiving enzyme replacement therapy or substrate reduction therapy.[16]

CONCLUSION

This study presents a robust, high-throughput UFLC-MS/MS method for the simultaneous quantification of clinically relevant LysoSLs in plasma. The approach holds promise for improving the efficiency and diagnostic accuracy of sphingolipidosis screening and may serve as a valuable tool in both clinical and research settings. Further validation in larger and more diverse patient populations is warranted to fully establish its diagnostic and prognostic utility.

Ethics

Ethics Committee Approval: Ethical approval was obtained from the Ethics Committee of Akdeniz University Faculty of Medicine (decision number: 373, dated: 30.05.2018).

Informed Consent: Informed consent was obtained from the parents of all patients included in this paper.

Footnotes

Authorship Contributions

Surgical and Medical Practices: H.A., E.S., Concept: H.A., Data Collection or Processing: H.A., B.K.E., N.Ö., Analysis or

Interpretation: H.A., E.S., Literature Search: H.A., A.E.B., B.K.E., Writing: H.A., A.E.B., B.K.E.

Conflict of Interest: The authors have no conflicts of interest to declare.

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Confirmation and Determination of Urinary Ethylmalonic Acid by Capillary Electrophoresis with Indirect UV Detection

Mehmet Şerif Cansever[1,2], Kağan Çalışgan[3], Ertuğrul Kıykım[3], Tanyel Zubarioğlu[3], Ayşe Çiğdem Aktuğlu Zeybek[1,3], Fatma Bedia Erim Berker[4]

[1]İstanbul University-Cerrahpaşa, Cerrahpaşa Faculty of Medicine, Research Laboratory of Metabolism, İstanbul, Türkiye
[2]İstanbul University-Cerrahpaşa, Vocational School of Health Services, Department of Medical Laboratory Techniques, İstanbul, Türkiye
[3]İstanbul University-Cerrahpaşa, Cerrahpaşa Faculty of Medicine, Department of Pediatric Nutrition and Metabolism, İstanbul, Türkiye
[4]İstanbul Technical University, Department of Chemistry, İstanbul, Türkiye

Abstract

Objectives: Ethylmalonic acid (EMA) is a key urinary biomarker of ethylmalonic encephalopathy (EE), but routine gas chromatography–mass spectrometry (GC–MS) is time- and resource-intensive. This study aimed to develop and validate a simple, rapid capillary electrophoresis (CE) method with indirect ultraviolet (UV) detection for the quantitative determination of EMA in urine.

Materials and Methods: A fused-silica capillary and a background electrolyte containing 5 mmol/L 2,6-pyridinedicarboxylic acid and 0.1 mmol/L cetyltrimethylammonium bromide (pH 5.74) were used. Separation was performed at 28 kV and monitored at 200 nm. Fresh human urine was diluted 1:20, filtered, and used to prepare matrix-matched calibrators (10–750 µmol/L). Linearity, precision, sensitivity, and accuracy were assessed according to International Council for Harmonisation validation guidelines, using spiked urine samples. Electropherograms from healthy control urine, EMA-spiked urine, and urine from a patient with EE illustrated the applicability.

Results: Under optimized conditions, EMA migrated at approximately 2.0 min with baseline resolution from endogenous components. Calibration was linear between 10 and 750 µmol/L (R^2 = 0.99920). Within-run precision, expressed as relative standard deviation (%), was 0.17% for migration time and 0.89% for corrected peak area, while between-day precision for peak area was 1.91%. The limits of detection and quantification were 11.78 and 39.3 µmol/L, respectively. Recovery in spiked urine ranged from 96 ± 2% to 98 ± 2%.

Conclusion: The CE–UV method enables rapid, reliable quantification of urinary EMA with minimal sample preparation. Its short analysis time and modest instrumentation requirements support its use for first-line screening and monitoring of EE, with GC–MS reserved for confirmatory analyses.

Keywords: Ethylmalonic Acid, Electrophoresis, Capillary, Urine, Biomarkers, Metabolism, Inborn Errors

INTRODUCTION

Ethylmalonic encephalopathy (EE) is an autosomal recessive mitochondrial disorder caused by pathogenic variants in the *ETHE1* gene.[1] Clinically, EE is characterized by developmental delay, relapsing petechiae, orthostatic acrocyanosis, and chronic diarrhea, with a heterogeneous phenotype that can vary widely among patients.[2] The prognosis is poor. Approximately half of affected children die before the age of two due to metabolic decompensation.[3] Biochemical findings typically include persistently elevated lactate and increased butyryl- and isovalerylcarnitine in blood, together with urinary excretion of ethylmalonic acid (EMA), methylsuccinic acid, butyrylglycine, and isovalerylglycine.[1] Combined treatment with coenzyme Q10, N-acetyl cysteine, and decontamination of bowel with metronidazole results in marked neurological improvement, reduction of acrocyanosis and petechial lesions, and disappearance of diarrhea, especially in early treatment.[4]

Address for Correspondence: Mehmet Şerif Cansever, İstanbul University-Cerrahpaşa, Cerrahpaşa Faculty of Medicine, Research Laboratory of Metabolism, İstanbul, Türkiye; İstanbul University-Cerrahpaşa, Vocational School of Health Services, Department of Medical Laboratory Techniques, İstanbul, Türkiye
E-mail: mehmet.cansever@iuc.edu.tr **ORCID ID:** orcid.org/0000-0003-3315-5124

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For disorders like EE, in which urinary organic acid analysis is integral to diagnosis, gas chromatography–mass spectrometry (GC–MS) remains the reference method for comprehensive analysis.[5] However, capillary electrophoresis (CE) offers an accessible and operationally simple alternative for targeted analytes. It enables rapid separations with minimal sample preparation and instrument time.[6,7] Indirect ultraviolet (UV) detection by CE has previously been applied to biologically relevant short-chain organic acids, including EMA.[8] More recently, urinary EMA has also been determined using CE with capacitively coupled contactless conductivity detection (CE–C^4D), illustrating detector-dependent trade-offs between sensitivity and instrumentation requirements.[9] These attributes make CE an attractive option for clinical laboratories performing high-throughput screening and follow-up quantification.

The objective of this study was to develop an simple, rapid, and accurate CE method to determine EMA in urine.

MATERIALS AND METHODS

Materials

EMA, cetyltrimethylammonium bromide (CTAB), and 2,6-pyridinedicarboxylic acid (PDCA) were obtained from Sigma-Aldrich, Merck (Darmstadt, Germany), and Fluka (Buchs, Switzerland), respectively. Solutions were prepared with purified, deionized water (Elgacam C114, Elga, England filtration system).

Urine samples were prepared daily by diluting fresh human urine in purified water. The dilution ratio was 1/20. EMA stock solution was dissolved in purified water and stored at +4 °C. Solutions prepared daily were filtered before analysis using a microfilter with a 0.45-µm pore size. The background electrolyte (BGE) used for separations consisted of 5 mmol/L PDCA and 0.1 mmol/L CTAB, adjusted to pH 5.74.

Apparatus and Operating Condition

Agilent CE system (Germany) equipped with a diode-array detector was used for EMA seperations. Agilent ChemStation software was used for data processing. 75-µm i.d. fused-silica capillaries for the separation experiment were purchased from Agilent. The total lengths of the capillary and the detector were 57 cm and 49 cm, respectively. The separations were performed at 28 kV with a wavelength set at 200 nm in indirect-UV mode.

The new fused-silica capillary adjustment was performed by rinsing with 1 mol/L NaOH for 30 min, followed by purified water for 10 min. The capillary was rinsed with 0.1 mmol/L NaOH and water for 3 min each, and was buffered for 3 min on each working day. A three-min flush with buffer was performed between runs. Injections were administered at 5 × 10−3 MPa for 5 s.

pH measurements were performed using Metrohm 654 Digital pH Meter (Metrohm, Switzerland) equipped with a pH glass electrode. The Addition of CTAB provided a positively charged inner surface, slowing or reversing electroosmotic flow (EOF) toward the anode, so that anionic analytes co-migrated with the bulk flow under the applied field.

Sample Preparation

Fresh urine was diluted 1/20 (urine/water), mixed, and filtered (0.45 µm) immediately prior to analysis. The 1:20 dilution was selected to minimize urine matrix effects (e.g., high ionic strength or conductivity and viscosity), which can compromise peak shape, current stability, and the migration-time repeatability in CE, while still enabling reliable detection and quantification of EMA within the validated range. This sample-handling approach is consistent with prior CE methods for urinary organic acids, where urine is commonly analyzed after centrifugation, with dilution as needed; a 1:20 final dilution has been reported for urine matrices in CE–based organic acid profiling.[10] Matrix-matched calibrators and recovery samples were prepared by spiking aliquots of 1/20-diluted pooled urine with EMA at specified concentrations (see Sections 2.4–2.5).

Calibration and Linearity

Method validation was performed according to the International Council for Harmonisation (ICH) Q2 (R^2) guideline.[11] Linearity was evaluated in 1:20-diluted human urine over the range 10–750 µmol/L EMA using matrix-matched external calibration. Calibration points across this range were injected ($n \geq 3$ per level), and peak quantification was performed using corrected peak areas (A/t). The calibration model was fitted using ordinary least squares linear regression.

Precision

Within-run precision was assessed by six successive injections ($n = 6$) of a 50 µmol/L EMA standard in urine diluted 1:20; precision was expressed as relative standard deviation (RSD%) for migration time and the corrected peak area (A/t). Between-day precision was determined as RSD% of peak areas from measurements obtained on three different days ($n = 3$).

Sensitivity Limit of Detection/Limit of Quantification (LOD/LOQ)

The LOD was defined as the EMA concentration producing a signal with peak height three times the baseline noise ($S/N \approx 3$), and the LOQ as the concentration corresponding to $S/N \approx 10$.

Accuracy (Recovery)

Accuracy was assessed by recovery experiments in urine diluted 1/20 at three EMA concentrations (25, 50, and 100 µmol/L). Each level was measured six times ($n = 6$), and recoveries were calculated as (measured/added) × 100%.

Data Processing

Electropherograms were integrated using ChemStation. Quantification employed corrected peak areas (A/t) to minimize run-to-run variability associated with migration time shifts. Quality metrics (linearity, precision, recovery, LOD/LOQ) were summarized according to ICH-style analytical validation conventions.

RESULTS

Validation Results

Method performance characteristics are summarized in Table 1. Linearity was demonstrated over 10–750 µmol/L using matrix-matched calibration in 1:20-diluted urine; the regression equation was $y = 2.25045 \times 10^{-3} x + 1.59091 \times 10^{-2}$ with $R^2 = 0.99920$ (Figure 1). Within-run precision, expressed as RSD%, was 0.17 RSD% for migration time and 0.89 RSD% for the corrected peak area (A/t) (n = 6). Between-day precision for corrected peak area was 1.91% RSD (three different days).

Sensitivity, assessed using signal-to-noise criteria, yielded an LOD of 11.78 µmol/L and an LOQ of 39.3 µmol/L. Accuracy assessed by recovery experiments at 25, 50, and 100 µmol/L (n = 6 each) showed recoveries ranging from 96 ± 2% to 98 ± 2% (Table 1).

EMA Electropherograms and Urinary EMA Levels

Under the separation conditions described above, EMA migrated at approximately 2.0 min. Electropherograms of a healthy control urine sample, a control urine sample spiked with EMA, and a urine sample from a patient diagnosed with EE are shown in Figure 2A–C, respectively. In the healthy control urine (Figure 1), no distinct peak was observed at the EMA migration time, indicating that EMA < LOQ (39.3 µmol/L) under the applied conditions. In the matrix-matched spiked urine (Figure 2B), pooled urine (1:20 dilution) fortified with EMA at 50 µmol/L produced a well-defined peak at the same migration time, confirming the peak identity and demonstrating baseline resolution from endogenous components. In the patient sample (Figure 2C), a prominent peak at ≈ 2.0 min was detected and quantified as 78.4 µmol/L EMA in the analyzed (1:20 diluted) urine, which is above the LOQ. Because indirect UV detection yields negative peaks, electropherograms were displayed after polarity inversion to facilitate visualization and integration. Urinary EMA levels for the illustrative samples are summarized in Table 2.

Table 1. Validation values.

	Parameter	Result
Precision	Within-run precision (corrected peak area, A/t), RSD% (n = 6)	0.89
	Within-run precision (migration time), RSD% (n = 6)	0.17
	Between-day precision (corrected peak area, A/t), RSD% (n = 3 days)	1.91
Sensitivity	LOD (µmol/L)	11.78
	LOQ (µmol/L)	39.30
Accuracy (recovery)	EMA-spiked urine samples (25 µmol/L), recovery (%)	96 ± 2
	EMA-spiked urine samples (50 µmol/L), recovery (%)	96 ± 2
	EMA-spiked urine samples (100 µmol/L), recovery (%)	98 ± 2

EMA, ethylmalonic acid; LOD, limit of detection; LOQ, limit of quantification; RSD, relative standard deviation.

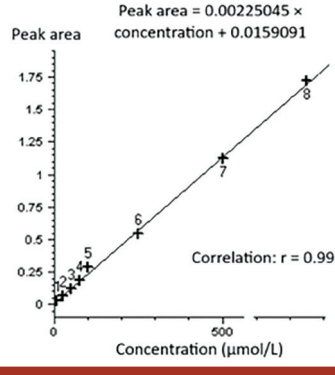

Figure 1. Calibration curve for EMA ethylmalonic acid.
EMA, ethylmalonic acid.

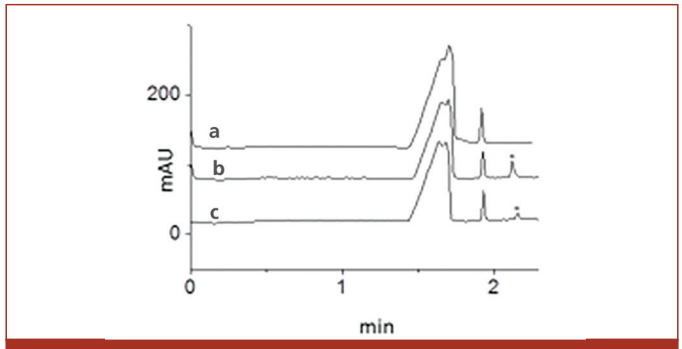

Figure 2. (a) Shows the electropherogram of healthy urine; (b) shows the electropherogram of a healthy urine spiked with EMA; (c) shows the electropherogram of a patient diagnosed with EE.
EE, ethylmalonic encephalopathy; EMA, ethylmalonic acid.

Table 2. Urinary EMA levels in illustrative human urine samples analyzed by CE–UV.

Sample (Figure 1)	Matrix/dilution	EMA status	Reported urinary EMA (µmol/L)	Interpretation
Healthy control	Urine, 1:20 diluted	Unspiked	<LOQ (39.3)*	Not quantifiable under conditions
Spiked control	Pooled urine, 1:20 diluted	Spiked	50	Peak identity confirmed at ≈ 2.0 min
EE patient	Urine, 1:20 diluted	Clinical sample	78.4	Quantifiable; above LOQ

*LOQ, 39.3 µmol/L. For the spiked sample, the concentration indicates the nominal added EMA level.

CE, capillary electrophoresis; EE, ethylmalonic encephalopathy; EMA, ethylmalonic acid; LOQ, limit of quantification; 2,6-pyridinedicarboxylic acid; UV, ultraviolet.

DISCUSSION

This study optimized a CE–UV method for the rapid quantification of urinary EMA. The method achieved a migration time of ~ 2 minutes in urine diluted 1:20, demonstrated excellent linearity across 10–750 µmol/L (r = 0.99920), exhibited tight intra- and interday precision, and showed high recovery. The LOD and LOQ were 11.78 and 39.3 µmol/L, respectively. EMA is a clinically significant urinary biomarker in EE and may be elevated in short-chain acyl-CoA dehydrogenase deficiency. Therefore, fast, reproducible first-line quantification of EMA has practical value for triage and monitoring.[1,12]

Traditionally, urine organic acid analysis is performed by GC–MS, which remains the confirmatory reference method. However, GC–MS requires derivatization and extraction as well as batch processing, which together extend turnaround times and increase resource use.[13] Faster preliminary results are clinically useful in disorders where EMA abnormalities inform acute and longitudinal management. This enables earlier decision-making while MS confirmation proceeds.[1] In this context, our CE–UV assay shortens the analytical step to minutes while maintaining the quantitative performance necessary for routine screening and monitoring. This enables a more efficient workflow in which cases that are clearly abnormal or ambiguous proceed to MS confirmation.[14]

The separation strategy employed in this study established principles for the separation of anionic analytes. Cationic surfactants, such as CTAB, dynamically modify the capillary wall, thereby reversing EOF. This promotes the rapid and reproducible migration of anions to the detector.[15,16] Because EMA lacks a strong native UV absorbance, indirect UV detection is appropriate. A Prior study has demonstrated indirect CE–UV of biologically relevant short-chain organic acids, including EMA, by using UV-absorbing co-ions and mobility matching.[8] The use of PDCA as the absorbing co-ion in the BGE during method implementations further supports robust indirect UV detection of carboxylic acids.[17] Our data expand upon existing literature by demonstrating the quantification of short-term EMA directly from minimally processed urine using a widely available UV/diode-array detector platform. Alternative CE implementations, such as CE–C^4D, have also achieved sensitive EMA detection, albeit with different detector trade-offs.[9]

Two methodological aspects merit emphasis. First, stable approximately 2-minute separations are achieved using low-level CTAB (0.1 mmol/L) with PDCA (5 mmol/L), a balanced EOF control, and indirect UV detection.[15] Second, our LOQ of 39.3 µmol/L is adequate for routine urine EMA screening within a reflex workflow, while CE–C^4D and MS methods achieve lower limits—an LOQ of ~ 3–4 µmol/L and an LOD of ~ 1 µmol/L—reflecting a trade-off between accessibility and sensitivity.[9] Given that EMA elevations in EE and related phenotypes are typically above normal, borderline results can be reflexed to MS for confirmation.[1]

From an interpretive standpoint, the method's analytical limitations should be considered when reporting results for healthy subjects and patients. In the healthy control (Figure 1), the absence of a distinct EMA peak at ≈ 2.0 min should be interpreted as EMA < LOQ (39.3 µmol/L) rather than complete absence. By contrast, the EE patient sample (Figure 1) showed an EMA concentration of 78.4 µmol/L in 1:20-diluted urine, which was readily quantifiable by CE–UV. Compared with indirect UV detection, CE–C^4D generally provides lower limits of detection for small ionic acids, which can improve interpretation of low-level or borderline EMA elevations (9).Therefore, samples near the LOQ or with strong clinical suspicion should be reflexed to CE–C^4D or MS-based confirmatory testing

This study has limitations. Indirect UV lacks the molecular specificity of MS; therefore, rare co-migrating matrix anions could interfere. This warrants orthogonal confirmation when measurements are near the LOQ or resolution is suboptimal. Also, the absolute concentrations in spot urine were reported without creatinine normalization. Laboratories that adopt this method should validate reporting in µmol/L with paired creatinine measurement or in mmol/mol creatinine, according to local practice standards. Finally, a head-to-head comparison against GC–MS and LC–MS in the same cohort was not performed to establish clinical concordance and decision thresholds for reflex testing.

In summary, we present a fast, precise, and accessible CE–UV assay for urinary EMA that can separate samples in approximately two minutes each with excellent linearity, low imprecision, and high recovery. In a reflex workflow, CE–UV can be used for same-

day screening. Samples with borderline concentrations can then be referred to GC–MS for confirmation or further analysis. This strategy could reduce turnaround time, allow GC–MS resources to focus on cases requiring greater specificity, reduce workload, and support longitudinal monitoring in EE.

CONCLUSION

A rapid and accessible CE–UV method for urinary EMA quantification was developed and validated. The assay provides an approximately 2-minute separation and demonstrates excellent linearity, low imprecision, and high recovery in urine diluted 1:20. This approach can support same-day screening and follow-up, with reflex MS confirmation recommended for borderline concentrations or when analytical specificity is required.

Ethics

Ethics Committee Approval: This study was approved by Clinical Research Ethical Committee of Cerrahpaşa Faculty of Medicine (approval number: E-29430533-604.01-01-273163, dated: 08.10.2015).

Informed Consent: All urine specimens were de-identified residual samples obtained during routine laboratory procedures, and no identifiable patient information was accessed. Therefore, the requirement for informed consent was waived by the Local Ethics Committee.

Footnotes

Authorship Contributions

Concept: M.Ş.C., E.K., T.Z., A.Ç.A.Z., F.B.E.B., Design: M.Ş.C., E.K., T.Z., A.Ç.A.Z., F.B.E.B., Data Collection or Processing: M.Ş.C., K.Ç., E.K., T.Z., A.Ç.A.Z., F.B.E.B., Analysis or Interpretation: M.Ş.C., K.Ç., E.K., T.Z., A.Ç.A.Z., F.B.E.B., Literature Search: M.Ş.C., K.Ç., E.K., T.Z., A.Ç.A.Z., F.B.E.B., Writing: M.Ş.C., K.Ç., E.K., T.Z., A.Ç.A.Z., F.B.E.B.

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