Orbital: The Electronic Journal of Chemistry
journal homepage: www.orbital.ufms.br
ISSN 1984-6428

| Vol 7 | | No. 2 | | April-June 2015 |

Full Paper

Determination of Ethylmalonic Acid in Human Urine by Ion Chromatography with Suppressed Conductivity

Orhan Destanoğlu[a], Mehmet Şerif Cansever[b], Gülçin Gümüş Yılmaz[a,*]

[a]*Istanbul Technical University, Faculty of Science and Letters, Department of Chemistry, 34469 Maslak, Istanbul, Turkey.*
[b]*Department of Pediatrics, Division of Metabolism, Cerrahpaşa Medical School, University of Istanbul, Cerrahpaşa 3430, Istanbul, Turkey.*

Article history: Received: 18 March 2014; revised: 04 June 2015; accepted: 17 June 2015. Available online: 26 June 2015.
DOI: http://dx.doi.org/10.17807/orbital.v7i2.574

Abstract: We present here a new method for the determination of EMA in urine by ion chromatography (IC) with suppressed conductivity detection. The separations on an anion exchange column have been accomplished with an optimized multi-step gradient eluent program (5 mM for 0-7 min, 5-50 mM from 7 to 30 min, 50 mM for 30-40 min, 50-5 mM from 40 to 45 min, 5mM for 45-50 min at flow-rate 0.2 mL/min) using NaOH as the eluent. The temperatures of both the column and suppressed conductivity detector were 40 °C. The suppressor current was 25 mA. The injection loop volume was 100 µL. Prior to injection, a clean-up procedure has been applied to urine samples for sulfate removal using $BaCl_2$ precipitation followed by elimination of cationic and some organic compounds using strongly acid cation-exchange resin and C18 solid phase extraction cartridge, respectively. Concentration range of EMA for linear external calibration curve was between 0.202 -30.278 µmol/L (r^2=0.999). The limit of detection and the limit of quantitation were 61 nmol/L and 202 nmol/L based on the signal-to-noise ratio equal to 3 and 10, respectively. The average recoveries of EMA for normal urine samples were between 98.2-99.3 % with less than 1.1 % relative standard deviation (RSD), and for pathological sample were between 97.2-107.8% with less than 1.9% RSD in both intra-day and inter-day assays. The average concentrations of EMA normalized against creatinine for healthy human urine samples and a pathological sample were 1.14 (0.88-1.50) and 128.95 (128.04-129.33) µmol $mmol^{-1}$ creatinine, respectively.

Keywords: ion chromatography; determination; ethylmalonic acid; ethylmalonic aciduria; urine

1. INTRODUCTION

Ethylmalonic aciduria is a biochemical finding in patients with inborn metabolic disorders including ethylmalonic encephalopathy (a genetic defect that affects fatty acid oxidation and thus metabolism) caused by deficiency of short-chain acyl-coenzyme A dehydrogenase [1-3]. This physiological condition is an autosomal recessively inherited disorder [1-5]. There are wide range of clinical manifestations of (i) ethylmalonic encephalopathy, (ii) SCADD, and (iii) GAII as follows: (i) developmental delay, acrocyanosis, petechiae, and chronic diarrhea [1]; (ii) hypotonia, congenital myopathy, joint contractures, growth and developmental retardation, seizures, neonatal metabolic acidosis, encephalopathy, axonal neuropathy, recurrent vomiting, progressive ophthalmoplegia with ptosis, hypoglycemia, cardiomyopathy, and muscular wasting and scoliosis [6]; (iii) recurrent episodes of lethargy, vomiting and hypoglycemia, metabolic acidosis, and hepatomegaly, or muscular involvement in the form of pain, weakness and lipid myopathy [5]. Ethylmalonic aciduria is a mortal disease that can result in death in first years of life [7-10]. Urinary excretion of ethylmalonic acid (EMA) concentration is markedly elevated in urine samples of patients with these metabolic diseases [11-23]. Thus, accurate determination of EMA in urine of patient has a vital importance to diagnose inborn errors of metabolism. Urinary EMA has been mostly analyzed in urine by gas chromatography (GC) or gas chromatography/mass spectrometry (GC-MS) [23-30], and by electrospray ionization tandem mass spectrometry (MS/MS) [17, 21, 31]. However, precolumn derivatization of EMA is an inevitable step prior to GC analysis. Silyl products and methyl esters are the common derivatives. Hence, *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA)

*Corresponding author. E-mail: ggumus@itu.edu.tr

[24], *N,O*-bis(trimethylsiliyl)trifluoroacetamide (BSTFA) [27], BSTFA + trimethylchlorosilane (TMCS) (10:1) [29], BSTFA+TMCS (99:1) [13, 21, 30] have been used as silylating reagents while trimethyloxonium tetrafluoroborate has been used as a methylating agent [25, 26]. Diazomethane, in addition, is an effective methylating agent, but its explosive and carcinogenic features render it a relatively-hard-to-handle reagent [26]. Besides, complicated derivatization operation in the sample preparation procedure can cause the total analysis time to significantly increase. The linear ranges of GC and GC-MS, also, are narrow. Liquid-liquid extraction has also some disadvantages such as time-consuming processes, excessive consumption of organic solvents and low recovery [23]. Sriboonvorakul et al. was utilized LC-MS for determination of EMA and some small organic acids in plasma and urine samples of metabolic acidosis in patients with severe malaria. In comparison, our proposed IC method is plausible in that both the detection limits and sample preparation [24]. Moreover, ion chromatography with suppressed conductivity detector is a cheaper system compared to GC-MS, MS/MS, and LC-MS. Furthermore, IC with suppressed conductivity detection is a very convenient technique for determining not only inorganic anions, but small organic acids as well. The present work describes a novel method for the determination of EMA in both normal urine samples and pathological urine samples by IC with suppressed conductivity detection. A clean-up procedure for urine sample, which is a typical requirement for IC analysis, has been utilized to obtain a clearer chromatogram and to extend the life-time of separation column. In our study, we have developed reproducible, reliable, simple, and applicable method for qualitative and quantitative analysis of EMA from urine samples. To the best of our knowledge, ion chromatography with suppressed conductivity detection method has not been reported to quantify ethylmalonic acid in urine samples. The method was successfully applied to urine sample from a volunteer patient with ethylmalonic aciduria.

2. MATERIAL AND METHODS

2.1. Chemicals and apparatus

All reagents were of analytical reagent grade. Ethylmalonic acid (97%) was purchased from Aldrich (Steinheim, Germany). $BaCl_2 \cdot 2H_2O$ (≥99%) and Na_2SO_4 (99%) were provided by Carlo Erba Reagenti (Milano, Italy). Sodium hydroxide, pellets pure (≥97%), hydrochloric acid fuming (37%), Ion-exchanger I (strong acid cation-exchange resin), and methanol (≥99.9%) LiChrosolv® Reag Ph Eur, gradient grade for liquid chromatography were purchased from by Merck (Darmstadt, Germany). SampliQ C18 ODS, 500 mg, 3 mL solid phase extraction (SPE) cartridges were supplied by Agilent Technologies (US). A high-performance micro centrifuge possessing a rotor with a radius of **7** cm (WiseSpin® CF-10 A) was purchased from DAIHAN Scientific (Seoul, Korea). PES (polyether sulfone) filters (pore size 0.2 µm, 17 mm) were provided by Analytical Columns (New Addington, Croydon, CR0 9UG, England).

2.2. Preparation of solutions

0.5 M $BaCl_2$ solution was prepared by dissolving 1.2214 g of $BaCl_2 \cdot 2H_2O$ in 10 mL of ultra pure water. A 1 M NaOH solution was prepared by dissolving 1.00 g of NaOH in 25 mL of ultra pure water which was immediately boiled once again to remove dissolved carbon dioxide before the preparation of the solution. 7.34 mmol/L stock standard solution of EMA (970 mg/L) was prepared by dissolving 0.100 g of EMA (97%) in 100 mL of ultra pure water which was stored in a refrigerator at 4 °C. Fresh working standard solutions have been prepared by appropriate dilutions of the stock solution at room temperature prior to use.

2.3. Instrumentation

Dionex ICS-3000 (Sunnyvale, CA, USA) ion chromatographic system equipped with a suppressed conductivity detector (ASRS 300 suppressor and conductivity cell) was used for separation and quantification of EMA in urine. Chromatographic separations were performed at 40ºC with a Dionex IonPac® AS20 analytical column (2x250mm) equipped with a Dionex IonPac® AG20 guard column (2x50mm). Analytical column resin composition is supermacroporous polyvinylbenzyl ammonium polymer cross-linked with divinylbenzene. Guard column resin was composed of microporous polyvinylbenzyl. Eluent gradients were generated on-line from ultra pure water using the Dionex EGC-NaOH EluGen II cartridge and then polished of contaminants using Continuously Regenerating Trap Columns CR-ATC. RFIC™ (Reagent-Free™ Ion Chromatography) system provides to avoid potential contamination compared to systems with manually prepared eluents. The instrument was also equipped with a pump and attached to an AS autosampler. Data acquisition and instrument control were performed via

Dionex Chromeleon® Client (Ver. 6.80) software. 100µL sample loop was used in all analyses. Ultrapure water of 18.2 MΩ cm resistivity or better was obtained from a New Human Power I Scholar UV system (Human Corporation, Seoul, Korea).

2.4. Eluent generation

Reagent-Free Ion Chromatography system with Eluent Generation (RFIC-EG) is advantageous in the sense that it offers ultra-pure NaOH eluent using solely DI water. Although OH-based eluents have some significant advantages such as higher sensitivity and linear response for analyte detection, manual eluent preparation remained a challenge as a result of CO_3^{2-} formation from atmospheric CO_2. It has been well established that carbonate contamination may alter the retention behaviors of analytes as a result of its being stronger eluent than hydroxide [32-34].

2.5. Self-regenerating suppressor

The suppressor is a principal component of the RFIC system. After separation, analyte with increased conductivity is delivered to conductivity cell. By decreasing eluent conductivity and noise whilst simultaneously increasing analyte conductivity, signal-to-noise ratio could be significantly improved by the use of the suppressor at autosuppression recycle mode. As shown in Figure 1, hydrogen gas and hydroxide ions are continuously formed in the cathodic chamber upon the electrolysis of water regenerant. At the same time, oxygen gas and hydronium ions are formed in the anodic chamber. The cation exchange membrane permits hydronium ions to transfer from the anodic chamber into eluent chamber. Hydroxide ions are neutralized herein by moved hydronium ions. The electric potential exerted pulls sodium ions from the eluent passing through the cathodic chamber. Thus, both electro-neutrality is sustained and sodium ions are combined with hydroxide ions [34, 35].

In keeping with this methodology, ethyl malonate is converted to a more conductive acid form with molecular structure illustrated in Figure 1 as eluent suppression is attained. Because of the relatively low first dissociation constant of ethylmalonic acid, which is equal to 2.99, it exhibited a good response on the conductivity detector [36]. The electric current of electrolysis in the suppressor has been adjusted in accordance with hydroxide concentration generated in EG module.

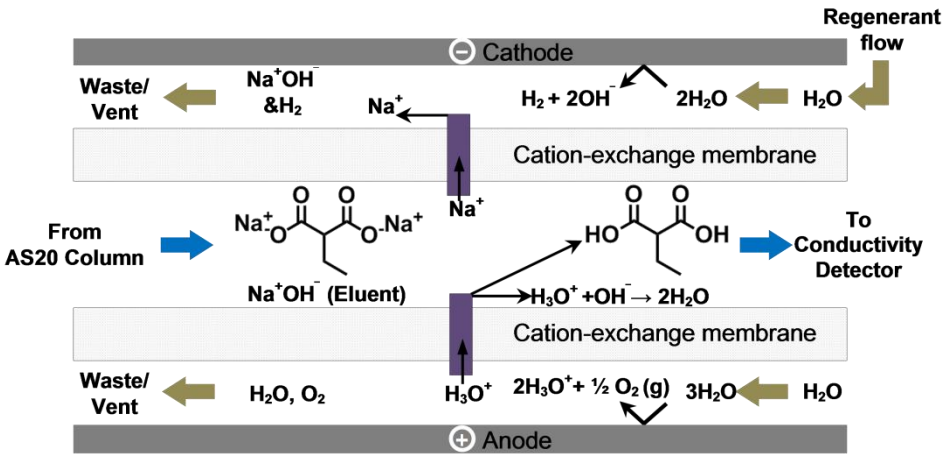

Figure 1. Autosuppression process.

2.6. Conditioning of C18 SPE cartridge and cation-exchanger

The SampliQ C18 SPE cartridges are for single use only. Prior to its conditioning, the cartridge was fitted into a vacuum manifold. Then, 5 mL of methanol and 5 mL of H_2O were applied into the cartridge respectively. The flow rate of each solvent was adjusted to 1-2 mL/min. The sorbent was not allowed to dryness at any point during conditioning after which urine samples were loaded.

The swollen cation-exchanger resin was conditioned according to the subsequent treatment steps: (i) six resin-bed volume of 1 M NaOH, (ii) DI water until neutral pH, (iii) six resin-bed volume of 1 M HCl, and (iv) DI water until complete removal of chloride ions in rinse water, which was done until no

white precipitate of silver chloride was observed with silver nitrate addition.

2.7. Urine samples

2.7.1. Urine sample collection and storage

The normal urine samples were obtained from three female healthy volunteers (5, 25 and 26 years old) who did not suffer from any systematic disease that could affect the content of urine. The urine sample of a volunteer patient (a seven-year-old female) affected by ethylmalonic aciduria was kindly provided by the Department of Metabolic Diseases in Children, Cerrahpaşa Medical Faculty (Istanbul University, Turkey).

All of the urine samples (patient urine and normal urine samples spiked/unspiked with EMA standard) were immediately frozen to −20 °C and stored at this temperature until analysis.

2.7.2. Pretreatment of urine samples

Prior to injection, a clean-up procedure was applied to each urine sample to minimize interference peaks, especially that of sulfate. A normal urine sample was divided into three different portions of two-milliliter volume. The urine samples were then spiked with three different concentrations (14.66 µmol/L, 43.79 µmol/L, and 87.06 µmol/L) by the addition of 4, 12, and 24 µL of the standard EMA solution, respectively.

The patient urine sample was spiked with two different EMA concentrations. To prepare 282.40 µmol/L and 543.88 µmol/L spike concentrations, 80 µL and 160 µL of stock EMA solutions were added to two-milliliter samples, respectively. Spiked and unspiked samples have been stored at −20 °C until analysis.

Frozen urine samples were kept in a water bath at 70 °C for 20 minutes. After 20 minutes, the samples were moved from the bath into a holder in the dark. When the temperature of the samples has reached room temperature, a 1.00 mL aliquot from the top of the samples, which was clearer than bottom of the sample, was transferred by an eppendorf micropipette to 1.5 mL eppendorf tubes. 20 µL of 0.5 M $BaCl_2$ was added to each 1.00 mL of the sample. After the samples had been shaken by vortex for 1 minute, the samples were centrifuged at 12.250xg for 15 minutes to completely precipitate $BaSO_4$ and suspended compounds in urine.

The supernatants were filtered through 0.2 µm pore size PES syringe filter to remove suspended and precipitated solids. Limpid solutions were then diluted by factors of 10 (for normal urine sample), 10, 100 and 500 (for spiked and unspiked patient urine samples). Noteworthy is that all dilution factors have been corrected according to additional volumes of the reagents.

In the next step, 1.6 g of swollen H^+-form cation-exchange resin was added to the diluted samples. The diluted samples with the resins were shaken by hand in 15 mL falcon tubes for 10-15 minutes. The pH of solutions was adjusted to minimum 10 by the addition of appropriate amount of 1 M NaOH to 5 mL of the diluted samples in 10 mL polypropylene tubes. Each 5 mL diluted sample was passed through at 1 mL per minute from preconditioned single-use SampliQ C18 SPE cartridges fitted into a vacuum manifold. The reason for adjusting pH of the diluted samples to ≥10 was to elute the EMA that was weakly retained on C18 SPE cartridge below pH 10. The eluates were then filtered through 0.2 µm pore size PES syringe filters before loading into 10 mL polystyrene sample vials of autosampler for IC analysis.

2.7.3. 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, Manheim, Germany). The concentration of EMA is expressed as a ratio with urinary creatinine concentration (µmol/mmol of creatinine) to take into account the variations of urinary volume among subjects, this procedure is commonly used in clinical biochemistry

3. RESULTS AND DISCUSSION

3.1. Removal of sulfate interference

The concentration of sulfate is high in urine sample [37], which made the accurate IC analysis of EMA rather impossible without any treatment. These anions showed close interactions on Dionex AS20 analytical column and sulfate gave broad peak that completely overlaps with EMA. Although 150 µmol/L of EMA is added into sample, it appears just as a shoulder. To remove its interferant effect, sulfate can be precipitated with barium cation at any pH value, specifically at acidic pH values. $BaCl_2$ was preferred as

barium salt because soared chloride has no effect on EMA analysis. On the contrary, elevated concentration of nitrate coming from $Ba(NO_3)_2$ could lead to interference.

In order to optimize required amount of $BaCl_2$, 10 µL and 100 µL of 0.5M $BaCl_2$ were added to five-milliliter of the aqueous solutions containing 104.1 µmol/L sulfate and 3.7 µmol/L EMA. Figure 2 shows the chromatograms of the solution and after addition of 10 µL and 100 µL of 0.5M $BaCl_2$.

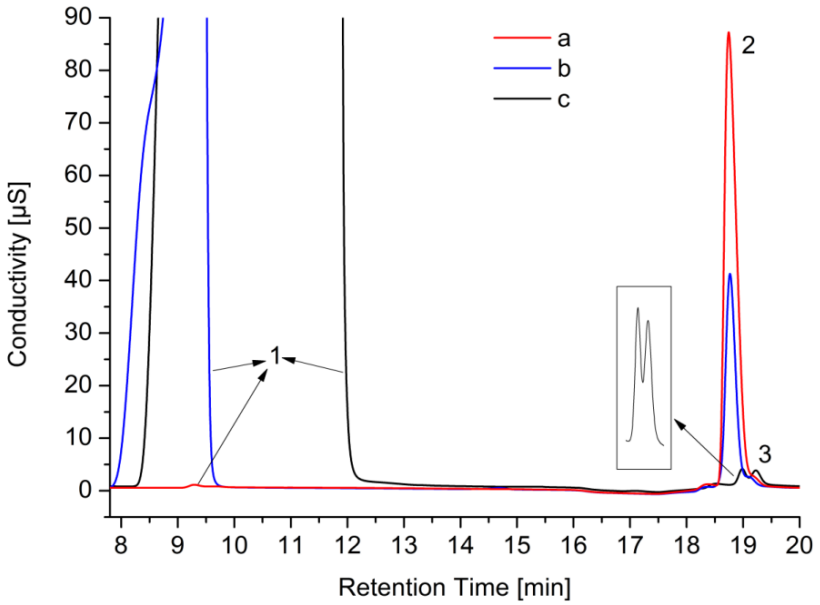

Figure 2. Overlaid chromatograms of (a) the aqueous solution containing 104.1 µmol/L sulfate and 3.7 µmol/L EMA; (b) treated with 10 µL 0.5 M $BaCl_2$; and (c) treated with 100 µl of 0.5 M $BaCl_2$. (1) chloride; (2) sulfate; (3) EMA. Rs=0.77 was found between peaks of sulfate and EMA on (c).

3.2. Chromatographic conditions

EMA is a dicarboxylic acid. Its anionic form (ethyl malonate) exhibited close retention time especially to sulfate anion on AS20 column. Although sulfate peak was dramatically declined after addition of 100 µL of 0.5 M $BaCl_2$ (see Figure 2c), adequate resolution between EMA (3.7 µmol/L) and remained sulfate peaks was not attained. Therefore, further parameters on the flow-rate (between 0.20 mL/min and 0.25 mL/min), column temperature (between 30 °C and 40 °C), and eluent concentration (various gradient modes) have been examined in detail. The parameters and values of the optimum conditions are shown in Table 1. As shown in Figure 3, the EMA was successively separated from sulfate under the optimum chromatographic conditions. In all urine samples, no signals from any interference peak have been observed on the chromatograms with retention time of EMA at optimum chromatographic conditions obtained after many experiment. Resolution between peaks of sulfate and EMA was found as 1.64.

Table 1. The optimum chromatographic conditions for analysis of the EMA.

Ion chromatographic parameter	Optimum value(s)
Eluent (NaOH) concentration	5 mmol/L from 0 to 7 min (isocratic),
(Multi-step gradient mode)	50 mmol/L from 7 to 30 min (gradient),
	50 mmol/L from 30 to 40 min (isocratic),
	5 mmol/L from 40 to 45 min (gradient),
	5 mmol/L from 45 to 50 min (isocratic, re-equilibration step)
Column temperature	40 °C
Detector cell temperature	40 °C
Eluent flow-rate	0.2 mL/min
Suppressor current	25 Ma

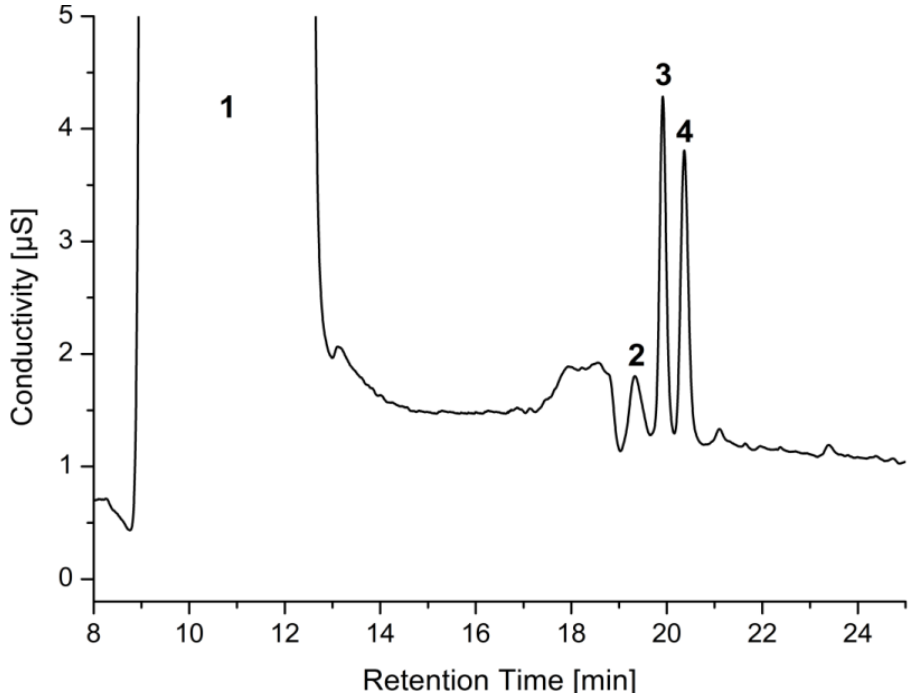

Figure 3. Chromatogram of the solution containing 104.1 µmol/L SO_4^{2-} and 3.7 µmol/L EMA treated with 100 µL of 0.5M $BaCl_2$. Optimum chromatographic conditions as for Table 1. (1) chloride; (2) carbonate; (3) sulfate; (4) EMA.

3.3. Performance characteristics of the IC system

External calibration method was used to determine the concentration of EMA in samples. Thus, nine aqueous calibration standards were injected five times into the IC system under the optimum conditions. The concentration range of EMA was from 0.202 µmol/L to 30.28 µmol/L. The linear relationship could be established using the linear regression equation y=0.1781x-0.0219, here y is peak area (µS min) and x is concentration (µmol/L), with a correlation coefficient of 0.999. Repeating five time injections of 0.76 µmol/L and 12.11 µmol/L EMA have been carried out for intra-day assay. Relative standard deviations (RSDs) of retention time, peak area, and peak height were calculated for method precision (see Table 2). Chromatographic characteristics related theoretical plates (77490 and 72832, respectively) and the symmetry factors (1.10 and 1.11, respectively) for 0.76 µmol/L and 12.11 µmol/L EMA are acquired. Detection limit (LOD), which was calculated from the peak height as the average concentration corresponding to the signal-to-noise ratio equal to 3, and quantification limit (LOQ), which was determined with the signal-to-noise ratio equal to 10, were 61 nmol/L (8.0 µg/L) and 202 nmol/L (26.7 µg/L), respectively. Consequently, under the optimum experiment conditions, EMA showed good linear relationship, sensitivity and repeatability.

3.4. Analysis of urine samples

3.4.1. Analysis of healthy human urine samples

The proposed clean-up procedure and optimized ion chromatographic conditions described in Table 1 were applied for the determination of EMA in urine samples gathered from both three female healthy volunteers and a patient with ethylmalonic aciduria. The whole method validation presented was performed with human urine. The urine samples have been spiked with different concentrations of EMA prior to clean-up. To evaluate the repeatability of the proposed method (e.g., precision), each sample was injected five times under the identical operating conditions over a course of one day. The spiked samples were also injected five times over three consecutive days for testing inter-day reproducibility of the proposed method. Table 3 presents the repeatability, reproducibility, accuracy and recovery (%) values for both one of the healthy urine and patient urine samples. The mean Relative Standard Deviations (RSDs) for intra-day assay and inter-day assay were also presented in Table 3. Figure 4 shows the overlaid chromatograms of unspiked and spiked with various concentration of EMA of a normal urine sample. Resolution values

between sulfate and EMA peaks were found between 1.51-1.89. The mean EMA concentrations normalized against creatinine in three normal urine samples range from 0.88 µmol $mmol^{-1}$ creatinine to 1.50 µmol $mmol^{-1}$ creatinine (see Table 4). These results are a clear indication that our proposed method can allow the quantification of EMA when its concentration is even at low µg/L levels in normal urine samples. Hence, we can conclude that validation of this proposed method was attained.

Table 2. Linear calibration curve parameters, RSD of the retention time, area, and peak height for 0.76 µmol/L and 12.11 µmol/L EMA, LOD (S/N=3) and LOQ (S/N=10). All the measurements have been performed by repeating five time injections.

Calibration Range (µmol/L)			**0.202-30.28**
Regression Equation (n=9)*		Area	y_a=0.1781x-0.0219
		Height	y_h=0.8206x+0.3032
Correlation coefficient (r^2)		Area	0.9999
		Height	0.9958
Retention time (min)	0.76 µmol/L	Average	20.16
		RSD (%) (n=5)	0.01
	12.11 µmol/L	Average	20.10
		RSD (%) (n=5)	0.01
Area (µS min)	0.76 µmol/L	Average	0.11
		RSD (%) (n=5)	2.03
	12.11 µmol/L	Average	2.15
		RSD (%) (n=5)	1.08
Height (µS min)	0.76 µmol/L	Average	0.63
		RSD (%) (n=5)	1.28
	12.11 µmol/L	Average	11.02
		RSD (%) (n=5)	0.86
LOD (µmol/L)			0.061
(µg/L)			8.0
LOQ (µmol/L)			0.202
(µg/L)			26.7

* y_a and y_h are the equations of area (µS min) and height (µS), respectively; x is the concentration (µmol/L).

Table 3. Assessment of recovery, precision and accuracy of proposed method for the determination of EMA in urine samples.

Sample	**Corrected Dilution Factor[a]**	**Amount found ± S.D. (µmol/L)**	**Amount added (µmol/L)**	**Total found ± S.D. (µmol/L)**	**$RSD_{intra-day}$ (%, n=5)**	**$RSD_{inter-day}$ (%,n=5x3 days)**	**Recovery (%)[b]**	**Average Recovery (%)**	**Accuracy[c]**
Healty	10.26	4.34±0.33	14.66	18.73±0.05	0.32	0.84	98.22±0.31		1.78
Urine	10.30	4.24±0.25	43.79	47.72±0.15	0.35	1.09	99.31±0.35	98.81 ±0.57	0.69
	10.36	4.34±0.33	87.06	90.43±0.36	0.41	0.85	98.89±0.41		1.11
Patient	106.42	601.33±2.31	282.40	875.77±4.30	0.49	-	97.18±1.52		2.82
Urine	532.12	600.00±10.55	282.40	896.11±5.41	0.60	-	104.85±1.92	103.94±4.67	4.85
	110.44	601.33±2.31	543.88	1187.83±8.90	0.75	-	107.84±1.64		7.84
	552.20	600.00±10.55	543.88	1235.34±20.02	1.62	-	105.89±1.72		5.89

[a] Dilution factors were calculated depending on both dilution amount and different additional volumes of 1 M NaOH. All concentration values were calculated by multiplying corrected dilution factors.

[b] Recovery (%) = [(total found – found in sample) / added] x 100.

[c] Accuracy = [(subtraction between total found and amount found) – (concentration added) / (concentration added)] x 100.

Table 4. The concentrations of EMA in urine samples normalized against creatinine in urine samples.

Sample	Corrected Dilution Factor	Concentration of EMA ± SD (µmol/mmol Creatinine)	RSD (%, n=5)
Healthy Urine 1	10.40	0.88±0.05	5.63
Healthy Urine 2	10.40	1.50±0.08	5.64
Healthy Urine 3	10.24	1.04±0.05	4.61
Patient Urine 1	104.14	129.32±0.50	0.38

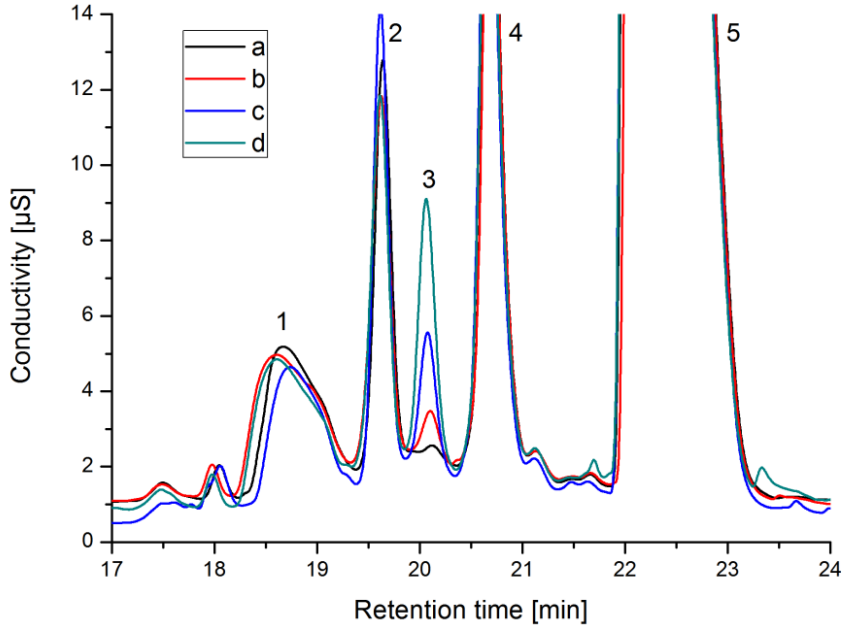

Figure 4. Chromatograms of healthy human urine sample. (a) unspiked urine; (b) spiked with 14.66 µmol/L EMA; (c) spiked with 43.79 µmol/L EMA; (d) spiked with 87.06 µmol/L EMA. (1) carbonate; (2) sulfate; (3) EMA; (4) and (5) were unidentified peaks. Optimum chromatographic conditions as for Table 1.

3.4.2. Analysis of urine from patient with ethylmalonic aciduria

As defined previously *(vide supra)*, ethylmalonic aciduria is a metabolic disease wherein the elevated excretion of EMA is encountered. Accordingly, we have analyzed urine samples from a patient with ethylmalonic aciduria. For this purpose, approximately 100 mL of first morning urine was collected from the patient. Upon the analysis under the optimum chromatographic conditions described in Table 1, our results are in keeping with the description of ethylmalonic aciduria disease, meaning that we had observed drastically increase EMA concentration in urine. The sample with 10-fold dilution has been exposed to the analogous pretreatments as those from normal samples. This dilution factor seemed insufficient for the separation, as the enormous peak of EMA is in interference with those of the matrix components. Hereafter, we have switched to 100- and 500- fold dilutions that seemed to be more suitable for these samples. Consequently, this approach has solved the interference behavior of EMA peak, as a result of which resolution values have been found to be 0.74, 1.64, and 1.90 after 10, 100 and 500-fold dilutions, respectively. Chromatograms of these samples are shown in Figure 5. The mean concentration of EMA has been found to be 129.18 µmol $mmol^{-1}$ creatinine. At this point, we feel compelled to underline that this concentration of EMA is well-above normal urinary concentration. Following this step, the sample from patient has been spiked with two different concentration of EMA (282.40 µmol/L and 543.88 µmol/L) with the purpose of method validation (see Table 3). After 100- and 500-fold dilutions, the spiked samples have been pretreated with the same procedure and analyzed with mean recovery of 103.94% and

mean accuracy of 5.35. The concentration of EMA in patient urine was given as a ratio with urinary creatinine concentration (µmol/mmol of creatinine) in Table 4. These results and the performance characteristic values mentioned above adequately support that the proposed method could be used for the quantification of EMA from patient's samples. Our statistical methods and values which we obtained from experiments were similar to many other studies in the literature. For instance, Li et al. satisfied with the similar statistical values at their work [35].

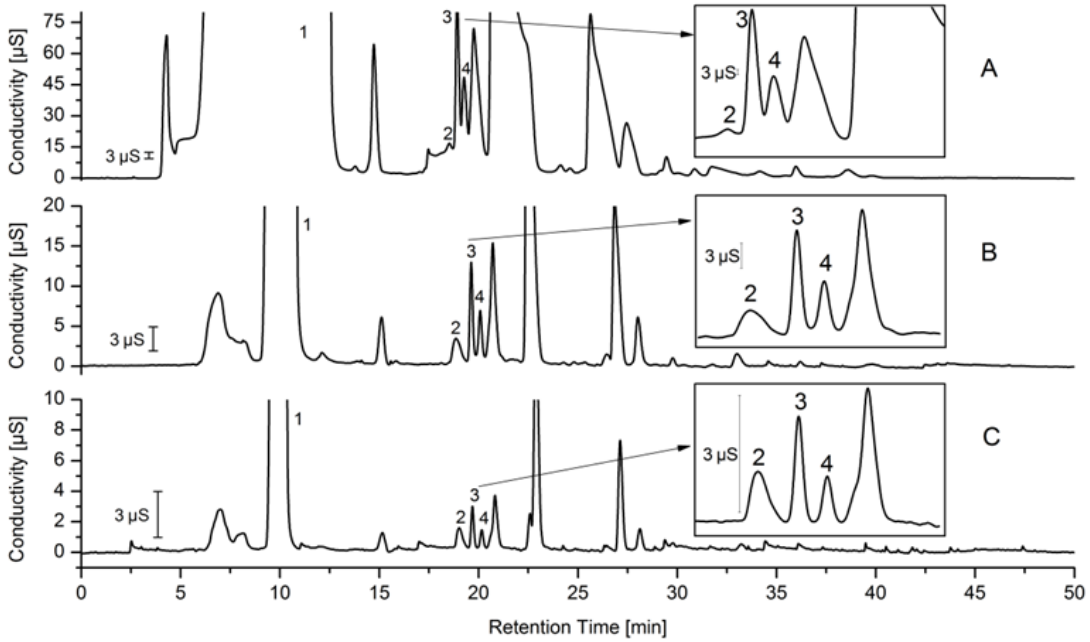

Figure 5. Chromatograms of pathological urine sample; (A) 10-fold diluted urine sample; (B) 100-fold diluted urine sample; (C) 500-fold diluted urine sample. (1) chloride; (2) carbonate; (3) sulfate; (4) EMA**.**

4. CONCLUSION

In this study, we propose a novel method for the determination of ethylmalonic acid (EMA) in urine samples by ion chromatography with suppressed conductivity detection. GC, GC-MS, and MS/MS, which are commonly utilized to detect EMA to date, present a significant disadvantage like the necessity of pre-derivatization. In contrast, proposed method presents more advantageous over the others in point of its simplicity, sensitivity, wide linear range and precision for EMA determinations in urine. In particular, the method has quite good recovery and accuracy. The proposed method was successfully applied to both a patient urine sample with ethylmalonic aciduria and healthy human urine samples. It is also well-suited for routine clinical analysis of EMA in urine samples

5. REFERENCE AND NOTES

[1] Burlina, A.B.; Zacchello, F.; Dionisi-Vici, C.; Bertini, E.; Sabetta, G.; Bennet, M.J.; Hale, D.E.; Schmidt-Sommerfeld, E.; Rinaldo, P. Lancet **1991,** 338, 1522. [CrossRef]

[2] Burlina, A.B.; Dionisi-Vici, C.; Bennett, M. J.; Gibson, K. M.; Servidei, S.; Bertini, E.; Hale, D. E.; Schmidt-Sommerfeld, E.; Sabetta, G.; Zacchello, F. *J. Pediatr* **1994**, 124, 79. [CrossRef]

[3] Ozand, P. T.; Rashed, M.; Millington, D. S.; Sakati, N.; Hazzaa, S.; Rahbeeni, Z.; Al Odaib, A.; Youssef, N.; Mazrou, A.; Gascon, G. *G. Brain Dev.* **1994**, 16, 12. [CrossRef]

[4] Jethva, R.; Bennett, M. J.; Vockley, J. *Mol. Genet. Metab.* **2008**, *95*, 195. [CrossRef]

[5] Beresford, M. W.; Pourfarzam, M.; Turnbull, D. M.; Davidson, J. E. *Neuromuscul. Disord.* **2006**, *16*, 269. [CrossRef]

[6] Okuyaz, C.; Ezgü, F. S.; Biberoglu, G.; Zeviani, M.; Tiranti, V.; Yilgör, E. *J. Child Neurol.* **2008**, *23*, 703. [CrossRef]

[7] Tiranti, V.; D'Adamo, P.; Briem, E.; Ferrari, G.; Mineri, R.; Lamantea, E.; Mandel, H.; Balestri, P.; Garcia-Silva, M.-T.; Vollmer, B.; Rinaldo, P.; Hahn, S. H.; Leonard, J.; Rahman, S.; Dionisi-Vici, C.; Garavaglia, B.; Gasparini, P.; Zeviani, M. *Am. J. Hum. Genet.* **2004**, *74*, 239. [CrossRef]

[8] Garcia-Silva, M.T.; Ribes, A.; Campos, Y.; Garavaglia, B.; Arenas, J. *Pediatr. Neurol.* **1997**, 17, 165. [CrossRef]

[9] Di Rocco, M.; Caruso, U.; Briem, E.; Rossi, A.; Allegri, A. E. M.; Buzzi, D.; Tiranti, V. *Mol. Genet. Metab.* **2006**, *89*, 395. [CrossRef]

[10] Kompare, M.; Rizzo, W. B. *Semin. Pediatr. Neurol.* **2008**, *15*, 140. [CrossRef]

[11] Lehnert, W.; Ruitenbeek, W. *J. Inherit. Metab. Dis.* **1993**, *16*, 557. [CrossRef]

[12] Corydon, M. J.; Gregersen, N.; Lehnert, W.; Ribes, a; Rinaldo, P.; Kmoch, S.; Christensen, E.; Kristensen, T. J.; Andresen, B. S.; Bross, P.; Winter, V.; Martinez, G.; Neve, S.; Jensen, T. G.; Bolund, L.; Kølvraa, S. *Pediatr. Res.* **1996**, *39*, 1059. [CrossRef]

[13] Nowaczyk, M. J.; Lehotay, D. C.; Platt, B. a; Fisher, L.; Tan, R.; Phillips, H.; Clarke, J. T. *Metabolism.* **1998**, *47*, 836. [CrossRef]

[14] Young, S. P.; Matern, D.; Gregersen, N.; Stevens, R. D.; Bali, D.; Liu, H.-M.; Koeberl, D. D.; Millington, D. S. *Clin. Chim. Acta* **2003**, *337*, 103. [CrossRef]

[15] Seidel, J.; Streck, S.; Bellstedt, K.; Vianey-Saban, C.; Pedersen, C. B.; Vockley, J.; Korall, H.; Roskos, M.; Deufel, T.; Trefz, K. F.; Sewell, a C.; Kauf, E.; Zintl, F.; Lehnert, W.; Gregersen, N. *J. Inherit. Metab. Dis.* **2003**, *26*, 37. [CrossRef]

[16] Mikati, M. A.; Chaaban, H. R.; Karam, P. E.; Krishnamoorthy, K. S. *Pediatr. Neurol.* **2007**, *36*, 48. [CrossRef]

[17] Kobayashi, H.; Hasegawa, Y.; Endo, M.; Purevsuren, J.; Yamaguchi, S. *J. Chromatogr. B.* **2007**, *855*, 80. [CrossRef]

[18] Haas, R. H.; Parikh, S.; Falk, M. J.; Saneto, R. P.; Wolf, N. I.; Darin, N.; Wong, L.-J.; Cohen, B. H.; Naviaux, R. K. *Mol. Genet. Metab.* **2008**, *94*, 16. [CrossRef]

[19] van Maldegem, B. T.; Duran, M.; Wanders, R. J. a; Waterham, H. R.; de Koning, T. J.; Rubio, E.; Wijburg, F. a. *J. Pediatr.* **2010**, *156*, 121. [CrossRef]

[20] Uziel, G.; Ghezzi, D.; Zeviani, M. *Semin. Fetal Neonatal Med.* **2011**, *16*, 205. [CrossRef]

[21] Sun, W.; Wang, Y.; Yang, Y.; Wang, J.; Cao, Y.; Luo, F.; Lu, W.; Peng, Y.; Yao, H.; Qiu, P. *Clin. Chim. Acta.* **2011**, *412*, 1270. [CrossRef]

[22] Gallant, N. M.; Leydiker, K.; Tang, H.; Feuchtbaum, L.; Lorey, F.; Puckett, R.; Deignan, J. L.; Neidich, J.; Dorrani, N.; Chang, E.; Barshop, B. A.; Cederbaum, S. D.; Abdenur, J. E.; Wang, R. Y. *Mol. Genet. Metab.* **2012**, *106*, 55. [CrossRef]

[23] Tanaka, K.; West-Dull, a; Hine, D. G.; Lynn, T. B.; Lowe, T. *Clin. Chem.* **1980**, *26*, 1847. PMID: 7438430.

[24] Sriboonvorakul, N., Leepipatpiboon, N., Dondorp, A. M., Pouplin, T., White, N. J., Tarning, J., & Lindegardh, N. *J. Chromatogr. B.*, **2013**, *941*, 116. [CrossRef]

[25] Kim, K. R.; Kim, J. H.; Jeong, D. H.; Paek, D. J.; Liebich, H. M. *J. Chromatogr. B.* **1997**, *701*, 1. [CrosRef]

[26] Liebich, H. M.; Gesele, E.; Wöll, J. *J. Chromatogr. B.* **1998**, *713*, 427. [CrossRef]

[27] Liebich, H. M.; Gesele, E. *J. Chromatogr. A* **1999**, *843*, 237. [CrossRef]

[28] Meda, H. A; Diallo, B.; Buchet, J. P.; Lison, D.; Barennes, H.; Ouangré, A.; Sanou, M.; Cousens, S.; Tall, F.; Van de Perre, P. *Lancet* **1999**, *353*, 536. [CrossRef]

[29] Birkebaek, N. H.; Simonsen, H.; Gregersen, N. *Acta Pædiatr.* **2002**, *91*, 480. [CrossRef]

[30] Kawana, S.; Nakagawa, K.; Hasegawa, Y.; Kobayashi, H.; Yamaguchi, S. *Clin. Chim. Acta.* **2008**, *392*, 34. [CrossRef]

[31] Nakagawa, K.; Kawana, S.; Hasegawa, Y.; Yamaguchi, S. *J. Chromatogr. B.* **2010**, *878*, 942. [CrossRef]

[32] Dooley, K. C. *Clin. Biochem.* **2003**, *36*, 471. [CrossRef]

[33] Dionex Corporation, Product manual for eluent generator cartridges, Document No: 065018, revision 03, 2011.

[34] Martens, D. A.; Loeffelmann, K. L. *J. Chromatogr. A* **2004**, *1039*, 33. [CrossRef]

[35] Li, J.; Chen, M.; Zhu, Y. *J. Chromatogr. A* **2007**, *1155*, 50. [CrossRef]

[36] Dionex Corporation, Product manual for anion self-regenerating suppressor 300, Document No. 031956, revision 07, 2009.

[37] Tanford, C.; *J. Am. Chem. Soc.* **1957**, *79*, 5348. [CrossRef]

[38] Wan, Q. J.; Kubáň, P.; Tanyanyiwa, J.; Rainelli, A.; Hauser, P. C. *Anal. Chim. Acta* **2004**, *525*, 11. [CrossRef]