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Article

Potential Diagnostic Assay for Cystinuria by Capillary Electrophoresis Coupled to Mass Spectrometry

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Cistinúria é uma alteração genética autossômica recessiva caracterizada por transporte intestinal e renal anormal tubular de L-cistina, assim como de L-lisina, L-arginina e L-ornitina. Esta alteração leva a excreção urinária excessiva destes aminoácidos com a formação de pedras nos rins provocados pela baixa solubilidade de L-cistina na urina. Neste trabalho, um método analítico para a determinação destes quatro aminoácidos por eletroforese capilar acoplada à espectrometria de massas com ionização por *electrospray* (CE-ESI-MS) foi desenvolvido e validado. Usando soluções padrão de L-cistina, L-lisina, L-arginina e L-ornitina, os limites de detecção dos aminoácidos por este método foram 114,2, 61,3, 72,7 e 86,7 μmol L^{-1}. Soluções padrão foram amostrados em um capilar de sílica (50 μm de diâmetro interno e 70 cm de comprimento total) e injeção de 2 psi de pressão por 10 s. A separação ocorreu a 300 V cm^{-1}, utilizando 1,0 mol L^{-1} de ácido fórmico em 10% de metanol em água como eletrólito de separação. Aplicação do método para a urina de um paciente diagnosticado clinicamente como portador de cistinúria revelou a presença de 900,5 ± 5, 600,0 ± 2, 700,2 ± 1 e 500,0 ± 3 μmol L^{-1} de aminoácidos, respectivamente, e 75,3 ± 1 μmol L^{-1} de creatinina. O método de CE-ESI-MS descrito neste trabalho para a análise de L-cistina e outros aminoácidos associados com cistinúria constitui uma ferramenta para diagnóstico sensível e confiável para caracterização e monitoramento desta doença.

Cystinuria is an autosomal recessive genetic disorder characterized by abnormal intestinal and renal tubular transport of L-cystine as well as of L-lysine, L-arginine and L-ornithine. This leads to excessive urinary excretion of amino acids, with the formation of kidney stones caused by the low solubility of L-cystine in the urine. In this study, an analytical method for simultaneous determination of these four amino acids in urine by capillary electrophoresis coupled to electrospray ionization mass spectrometry (CE-ESI-MS) was developed and validated. Using standard solutions of L-cystine, L-lysine, L-arginine and L-ornithine, the amino acid detection limits by this method were 114.2, 61.3, 72.7 and 86.7 μmol L^{-1}. Standard solutions were injected in a silica capillary column (50 μm i.d. and 70 cm length) under 2 psi of pressure by 10 s. The separation occurred at 300 V cm^{-1}, using 1.0 mol L^{-1} formic acid in 10% methanol in water as the background electrolyte. The method was applied to the urine of a patient clinically diagnosed as a cystinuria carrier, which revealed the presence of 900.5 ± 5, 600.0 ± 2, 700.2 ± 1 and 500.0 ± 3 μmol L^{-1} of amino acid, respectively, and 75.3 ± 1 μmol L^{-1} of creatinine. The CE-ESI-MS method described here for analyzing L-cystine and other cystinuria-related amino acids is a sensitive and reliable diagnostic tool for characterizing and monitoring this disease.

Keywords: cystinuria, CE/ESI-MS, inborn error of metabolism, clinical analysis

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Introduction

Capillary electrophoresis coupled to mass spectrometry (CE-MS) emerged more than two decades ago.[1-3] The success of this analytical system is due to the combination of its high resolution, universal application, efficiency and good sensitivity when compared to other detection systems such as spectrophotometry in the UV-Visible region, the most popular technique, and laser induced fluorescence (LIF) which provides high sensitivity. However, both techniques cannot unveil the structure of the analyte.[4,5] CE is one of the main techniques used in the separation of ionized and neutral analytes. The capillary electrophoresis coupled to electrospray ionization mass spectrometry (CE-ESI-MS) has rapidly developed as a powerful analytical tool for charged species analysis of small molecules such as carboxylic acids, phenols, metal species, tetramines, herbicides, drugs, metabolites, proteins, amino acids and peptides. While CE confers rapid analysis and efficient resolution, MS frequently allows analyte identification with high selectivity and sensitivity. ESI has proven to be versatile and relatively easy to use in combination with CE.[6,7]

There is a great interest to determine biochemically active metabolites in biological samples aiming at understanding the molecular bases of the clinical manifestations of human disorders. A large number of amino acid catabolites has been suggested as biomarkers of various diseases. Errors in metabolism (EM) are diseases that typically result from a change in the structure of an enzyme or a protein, leading to abnormal catabolism/anabolism of certain carbohydrates, amino acids, nucleobases or organic acids. The symptoms of many of these diseases are lethargy, poor appetite, sleep apnea, frequent vomiting, and if not detected early, it can lead to mental retardation or death.[8] Recently, with the development of MS-ESI, it is possible to use a single test to identify a wide range of rare metabolic illnesses.[9,10] Cystinuria is an inborn error of metabolism resulting from an autosomal recessive genetic disorder, which impairs intestinal and renal tubular transport involving L-cystine, L-lysine, L-arginine and L-ornithine, leading to excessive urinary excretion of these amino acids, with the consequent formation of kidney stones due to the low solubility of cystine in the urine.[11,12] Urinary obstruction causes discomfort, pain, infections and eventually renal failure.[13] In its heterozygous form, there is a moderate loss of these amino acids, but in its homozygous form, there is massive excretion of amino acids.[14] This genetic disorder is related to the three mutant alleles I, II and III present in chromosomes 2 or 19, which determine the intestinal absorption changes of L-cystine, L-lysine, L-arginine and L-orthinine. Cystinuria prevalence ranges from 1/60,000 for heterozygous and 1/20,000 for homozygous patients,[11] and its incidence in humans displays ethnic variations. Early and accurate detection and quantification of the cystinuria-related metabolites are needed for the successful prevention and effective long-term treatment of patients. In its homozygous form, the formation of kidney stones is recurrent, being difficult to be removed by surgery or drugs. Cystinuria should be suspected especially when the first stones are formed in the patient in the first two decades of life and in subjects with family history of this disorder, the highest incidence occurring in the first year of life and after puberty. The faster it is correctly diagnosed, the greater the success rate in treating the patient. Normal concentration of cystine in the urine is below 30 mg *per* day (0.13 mmol *per* day), homozygous concentration is greater than 400 mg *per* day (1.7 mmol *per* day) and heterozygous concentration varies from 200 to 400 mg *per* day (0.8 to 1.7 mmol *per* day).[15] Lindell *et al.*[16] reported an increased incidence of urinary stone formation when L-cystine concentration in urine is above 700 mmol L^{-1}.[17]

In the present work, it is studied and developed a method for the urinary determination of cystine and other pathologically related amino acids using CE-MS in order to rapidly and unnequivocally diagnose cystinuria and prevent urinary stones. The literature reports only methods for cystinuria using liquid chromatography-mass spectrometry (LC-MS)[18-20] and gas chromatography/mass spectrometry (GC-MS).[21,22] CE has already been successfully explored as a separation technique, but using other detectors instead of MS, which brings about important analytical limitations.[23,24] Methods based on HPLC separation consume more reagents and produce more solvent waste than CE, whereas CE-MS constitutes a more sustainable analytical process.

Currently in Brazil, only five diseases related to errors of metabolism are monitored by the public health system, cystinuria is not included. The diagnosis of metabolic disorders is frequently performed by neonatal screening, but many rare genetic diseases can only be detected by sophisticated and expensive methods that are only available in some private hospitals. Thus, our group proposes here the CE-MS technique to diagnose cystinuria through a method that was developed and validated to analyze urine samples from both healthy donors and cystinuria patients.

Experimental

Reagents and samples

All reagents used were of analytical grade. CE separation used formic acid and methanol purchased from Merck

(Darmstadt, Germany). Standard samples of L-cystine, L-lysine, L-ornithine, L-arginine and L-tryptophan $^{13}C^{15}N$ (internal standard), purchased from Sigma-Aldrich (Saint Louis, USA), were prepared in Milli-Q purified water (Billerica, USA, USA).

Preparation of standard solutions and electrolytes

Addition of 10% methanol to the electrolyte composition showed to be necessary. The solution was prepared by diluting the appropriate volume of formic acid with water purified by Milli-Q (Billerica, USA). The solutions were all filtered using 0.22 μm pore size Millipore disposable nylon filters prior to use. The stock standard solutions of 5.0 mg L^{-1} of L-cystine, L-lysine, L-ornithine and L-arginine were dissolved in 2.0 mol L^{-1} formic acid to achieve the desired concentration. The internal standard was prepared with 0.45 mg mL^{-1}. All solutions were stored at 4 °C.

Urine samples

Urine samples were randomly collected from normal individuals (n = 10) and cystinuria carriers (n = 1) under documented consent and stored at −4 °C until use. The ethical guideline number is 1545/10. Samples were thawed and centrifuged at 400 g (Eppendorf centrifuge 5402, USA) at 4 °C for 10 min. The supernatant was filtered through a Millipore membrane 0.22 μm and sonicated (Elma Ultrasonic Digital S, USA) for 20 min.

Capillary electrophoresis conditions

Urine analyses were carried out in a CE system model P/ACE MDQ from Beckman Coulter (USA), using silica capillaries from Polymicro Technologies (USA) of 50 μm i.d. and 70 cm length. All amino acid standards and urine samples were injected under 2 psi pressure for 10 s. The separation occurred at 300 V cm^{-1} and using 1.0 mol L^{-1} formic acid in 10% methanol in water as the background electrolyte in CE.

Mass spectrometry conditions

Identification and quantification of the target analytes were performed on a Thermo LCQ Advantage MAX ion trap mass spectrometer (USA) coupled to the CE apparatus through an orthogonal electrospray interface. The Sheath liquid used was 49.5% of methanol and 0.5% of acetic acid in water. Conditions for ESI-MS analysis were: 4.5 kV, ESI temperature 275 ºC, N_2 pressure 2 bars. Optimization of the MS analysis of the cystinuria-related amino acids was obtained by employing the following parameters: source current, 80.00 μA; capillary voltage, 3.00 V; tube lens voltage offset, −5.00 V; multipole RF amplifier (Vp-p), 400.00 Vp-p, multipole 1, voltage offset, −3.50 V; multipole 2 voltage offset, −6.50 V, inter multipole lens voltage, −20.00 V, trap DC off set voltage, −10.00 V, and zoom micro, 5.

FT-Raman studies

FT-Raman spectra were obtained by a Bruker RFS-100/S, using a Nd/YAG laser as exciting source at 1064 nm (Germany). Neat cystine was introduced into the sample holder as a pressed pellet and the laser power was set to 200 mW. Cystine microcrystals of the urine sample were transferred to a glass slide for examination under optical microscopy (Ramanscope, Germany) attached to the spectrometer, with the laser power set to 400 mW. In all cases, the spectral resolution employed was 4 cm^{-1} and 128 scans were co-added to trace each spectrum.

Determination of creatinine

CE baseline separation was attained with 25 mmol L^{-1} Tris buffer (pH 2.5) as electrolyte in a bare fused silica capillary (40 cm total length and 10 cm effective length, 50 μm i.d.) under 10 kV potential with short-end injection configuration using 0.3 psi pressure, and 0.5 s. Urine samples were diluted 40-fold with water prior to analysis by CE.[25,26] The creatinine determination used CE with UV detection (λ = 205 nm).

Statistical analysis

For statistical treatment of the data presented the software OriginPro 8.5.1 was used. Student *t*-test was used to check the deviations from linearity.

Results and Discussion

The optimized and validated methodology proposed here for the determination of L-cystine, L-arginine, L-ornithine and L-lysine in urine is supported by the analytical parameters (specificity, linearity, precision, accuracy, limits of detection (LOD) and of quantification (LOQ)) required by the Brazilian National Health Surveillance Agency (ANVISA)[27] and the US FDA (Food and Drug Administration Guidance for Industry: Bioanalytical Method Validation).[28]

Background electrolyte and organic solvent effects

Different buffering conditions suitable for CE-MS were tested to attain optimal conditions to separate all amino acids excreted in cystinuria patients. The signal-to-noise (S/N) ratios, peak resolution and migration times (7-9 min) were obtained by using 1.0 mol L^{-1} formic acid as electrolyte. The addition of organic solvents, such as methanol (10%), to the background electrolyte considerably improved resolution and migration times of the amino acid peaks (Figure 1). The migration time increased, once addition of organic solvents contributes to decrease the electroosmotic flow, thereby promoting better separation, and methanol improves the ionization process, consequently increasing the S/N ratio.

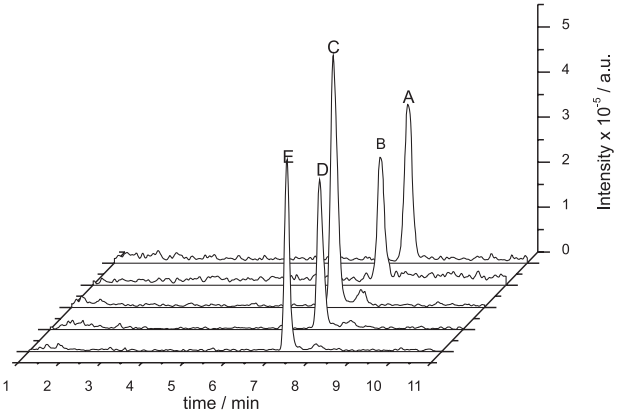

Figure 1. CE-MS base peak electropherograms of cystinuria-related amino acids at a concentration of 45 μg mL^{-1}. Peak identification, A: internal standard (L-tryptophan $^{13}C^{15}N$), B: L-cystine, C: L-lysine, D: L-arginine and E: L-ornithine. Peaks reconstructed according to the ratio *m/z* of analytes and their respective migration time obtained under the experimentally established conditions. Conditions for ESI-MS analysis were: 4.5 kV, temperature 275º C and N_2 pressure 20 bars. Silica capillary without internal coating, 50 μm i.d. and 70 cm length. Sample volume injected: 20 nL. The separation occurred at 300 V cm^{-1} and 2 psi pressure for 10 s. The background electrolyte CE was 1.0 mol L^{-1} formic acid in 10% of methanol in water. Mass full scan: *m/z* 100-300 ratio at the positive mode.

Pressure effect

Pressure was applied during the electrophoretic separation in order to reduce the analysis time. The pressures of 2, 3 and 6 psi were tested for 10 s each injection. The best running time (10 min) and peak resolution were reached with 2 psi. Other pressure values resulted in poorer separation resolution.

Specificity

The specificity of the method was evaluated by analyzing urine samples with the addition of authentic L-cystine, L-arginine, L-lysine and L-ornithine to assess the medium interference in the quantification and retention times of the amino acids and internal standards. The results were then compared with those obtained with an aqueous solution of the same standards at different concentrations. No changes were observed for retention times and analyte quantification.

Linearity

Calibration curves were obtained by tracing the electropherograms with MS spectrum detection of each amino acid. A linear dependence of peak area on the analyte concentration (3.5-225.0 μg mL^{-1}) was verified. The respective linear correlation coefficients (r^2) were all above 0.99, thus confirming a significant linearity of the method (Table 1). Standard amino acid and patient samples were supplemented with an internal standard solution containing 0.45 mg mL^{-1} of tryptophan $^{13}C^{15}N$, representing 10% of total sample volume. As the observed tabulated T values are smaller than the calculated ones (Table 1), one can rely on a statistically significant correlation between data and calibration curve with a confidence level of 95%.

Accuracy

The accuracy was evaluated by the repeatability and inter-day precision of the method by expressing the results as the coefficient of variation (CV in %). To determine repeatability, solutions containing 450.0, 112.5 and 28.1 μg mL^{-1} of each amino acid were prepared and the concentrations defined as 100% accurate. Five successive determinations were conducted for each solution in a

Table 1. Parameters of the calibration curves and student *t*-test

Amino acid	Equation: $y = bx + a$	r^2	N	Tc	Tt[a]
L-Cystine	$y = 0.7387x + 0.0170$	0.9993	5	65.4425	3.1824
L-Arginine	$y = 0.7993x + 0.0406$	0.9935	7	27.6662	2.5706
L-Lysine	$y = 0.4032x + 0.0080$	0.9997	8	134.818	2.4469
L-Ornithine	$y = 0.4026x + 0.0105$	0.9933	8	29.8249	2.4469

[a]Values obtained by student *t*-test distribution (n = 5); r^2: correlation coefficient; Tc: value calculated; Tt: value table.

Table 2. Accuracy, recovery and repeatability parameters for quantifications by CE-MS method developed for the quantification of amino acids in the urine of donors

Amino acid	Concentration[a] / (μg mL^{-1})	Accuracy / %	CV[b] / %	Recovery / %	CV[b] / %	Repeatability parameters for quantification[a] / %	CV[b] / %
L-Cystine	28.1	127.1	3.8	113.2	8.4	97.1	2.0
	112.5	154.8	2.7	109.2	7.2	99.0	3.8
	450.0	129.4	6.0	112.9	6.4	99.9	4.3
L-Arginine	28.1	108.8	4.1	110.7	5.0	101.0	4.2
	112.5	121.5	4.5	114.5	4.1	103.5	2.0
	450.0	114.4	5.0	105.0	5.0	98.0	0.9
L-Lysine	28.1	114.9	4.3	111.0	7.5	105.8	4.8
	112.5	103.4	4.1	106.2	6.3	108.1	4.6
	450.0	105.9	4.9	108.1	5.4	99.4	4.6
L-Ornithine	28.1	110.5	2.0	98.5	2.5	100.7	4.9
	112.5	97.0	4.4	110.5	5.3	106.4	4.1
	450.0	98.5	3.6	93.9	3.6	96.4	4.8

[a]Average of 5 determinations; [b]CV: coefficient of variation.

single day under the same conditions. Their respective mean values and coefficients of variation are expressed in Table 2.

Inter-day precision was studied by analyzing all amino acid solutions at a concentration of 112.5 μg mL^{-1} on two consecutive days, performing 5 determinations each day. The values of variation coefficients were below 1.5% (L-cystine = 0.81%, L-arginine = 0.48%, L-lysine = 0.83%, L-ornithine = 1.25%), thus conforming satisfactory repeatability values. The ANVISA regulation[27] considers a method accurate when its coefficient of variation is below 15%.

LOD and LOQ

The LOD and LOQ values were determined according to the ANVISA regulation,[27] which are based on three times the noise baseline for estimating LOD and ten times for LOQ, both values calculated from the slope of calibration curve of each amino acid. Thus, the method proved to be reliable and valuable to quantify the cystinuria related metabolites regarding sensitivity. The major contribution of a specific, sensitive and accurate method for cystinuria diagnostics is the prevention of urinary stones. Lindell *et al*[16] reported an increase in the incidence of urinary stone formation when the L-cystine concentration is above 700 mmol L^{-1}. Therefore, the LOQ data listed in Table 3 show that the method described here is efficient to quantify the amino acids involved in cystinuria for the diagnosis and treatment of patients.

Table 3. Limits of detection (LOD) and of quantification (LOQ) obtained from the calibration curves of amino acids

Amino acid	LOD / (μmol L^{-1})	LOQ / (μmol L^{-1})
L-Cystine	114.2	219.0
L-Arginine	71.3	234.5
L-Lysine	30.7	110.0
L-Ornithine	86.7	202.8

Accuracy

The accuracy of the method defined here as the closeness between experimental data and nominal values of amino acid concentrations related to cystinuria was evaluated from synthetic urine (inorganic solutions that simulated urine) using three analyte concentrations and performing 5 times each analysis. Synthetic and medication-free real urine samples display the same analytical parameters according to FDA[28] and ANVISA[27] parameters. Table 2 shows the data used for accuracy evaluation and respective coefficients of variation.

Analysis of real urine samples

The analysis of urine samples from healthy donors was performed by adding three different standard concentrations of cystinuria-related amino acids and a reference sample. Each sample was analyzed five times and the metabolites were quantified. The amino acid recovery values in the urine of healthy donors and respective coefficients of variation

are shown in Table 2. The accuracy of the method applied to synthetic urine and the recovery indices in the urine of donors were highly acceptable. In summary, the values found for the urine sample analysis were reproducible; the coefficients of variation were within the allowable limit for all biomarkers analyzed, there was no interference from other metabolites eventually present in the urine samples, and sample desalting was not required.

Analysis of patient samples by CE and CE-ESI-MS

Cystine and other cystinuria-related amino acids as well as creatinine were determined in the urine of a 2.8 years old male cystinuria patient. Total urine was collected randomly and the analyses were immediately performed. The amino acids were quantified by the same procedure used to analyze the standards. Creatinine was found to be 75.3 ± 1 μmol L^{-1} in the urine sample 40-fold diluted. The concentrations obtained for L-cystine, L-arginine, L-lysine and L-ornithine were 900.5 ± 5, 600.0 ± 2, 700.2 ± 1 and 500.0 ± 3 μmol L^{-1}, respectively.

Analysis by FT-Raman

The formation of crystals in the patient urine after collection and storage at 4 °C was a strong evidence of a high urinary concentration of cystine. The FT-Raman technique was used prior to CE-ESI-MS identification for qualitative characterization of the crystals precipitated in the patient urine. Figure 2 shows the close matching of the FT-Raman spectrum of authentic cystine crystal samples with that of the microcrystals collected from the urine of the cystinuria patient. The most prominent FT-Raman bands of cystine microcrystals are 498 cm^{-1} (assigned to the characteristic ν (S–S) stretching,[29] and other peaks at 105, 201, 457, 617, 678, 785, 1339, 1408, 2915 and 2966 cm^{-1}.

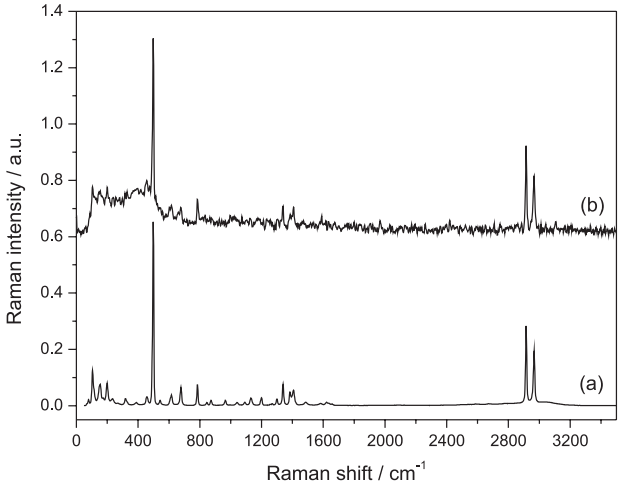

Figure 2. FT-Raman spectra of (a) authentic cystine crystals and (b) microcrystals collected from the urine sample. The intensity scale was adjusted for comparison.

Conclusion

Here, a specific, sensitive and reliable CE-ESI-MS method is optimized and validated for clinical analysis of cystinuria through the determination of L-cystine, L-lysine, L-arginine and L-ornithine in standard solutions prepared with urine and also in a patient urine sample.

Our results attest great potential to the method for use in the public health system. All analytical parameters of this method are in accordance with the regulations of the sanitary inspection agencies, with the LOD values much lower than the urinary concentration of amino acids found in healthy individuals. Another important advantage of the CE-MS method is the reduced use of solvent, which makes it environmentally more sustainable than those that use HPLC separation. Only patients with abnormally high levels of cystine are subjected to the formation of kidney stones, hence the need to monitor the levels of urinary cystinuria-related amino acid concentrations by rapid, sensitive and reliable methods such as the one described here to prevent pain discomfort and complications derived from surgery.

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