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Determination of essential amino acids in human serum by a targeting method based on automated SPE–LC–MS/MS: Discrimination between artherosclerotic patients

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ABSTRACT

An automated method based on a hyphenated SPE–LC–MS/MS configuration has been optimized for the determination of essential amino acids (threonine, valine, methionine, leucine, isoleucine, lysine, tryptophan, and phenylalanine) in human serum, with the aim of discriminating between different states of coronary artery disease. Validation in terms of sensitivity (detection limits below 28.0 ng on column) and precision (repeatability expressed as relative standard deviation below 6.0%) supports the suitability of the method for application to a cohort of 122 atherosclerosis patients confirmed by a catheterization test. The cohort was composed by 80 individuals diagnosed with stable angina and 42 patients who suffered from acute myocardial infarction (AMI). Both groups of individuals are differentiated by the occurrence of ischemia in AMI patients due to the formation of thrombi. The chemometric treatment of the data obtained by multivariate analysis of variance (MANOVA) allowed comparison between both groups of diagnosed patients. Therefore, amino acids whose serum levels were affected by ischemia have been identified. The contribution of risk factors such as obesity and hypercholesterolemia as well as the individuals' gender to the concentration of essential amino acids has also been studied.

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1. Introduction

Amino acids are molecules with a common structure that contains an amine group, a carboxylic acid group and a characteristic side-chain that varies among amino acids. They have critical functions to life with a direct involvement in metabolism by serving as the building blocks of proteins. Apart from their functions in proteins synthesis, amino acids are key regulators of gene expression and the protein phosphorylation cascade, and act as precursors for synthesis of hormones and low-molecular weight nitrogenous substances with enormous biological importance such as nitric oxide, polyamines, glutathione, taurine, etc [1]. The biochemical relevance of amino acids, their levels and those of their metabolites may be of interest in the study of metabolic and nutritional disorders as well as in other pathologies related with oxidative stress.

The heart is one of the organs more affected by reactive oxygen species (ROS) [2], so one of the pathologies related with oxidative stress is atherosclerosis, a disease that represents a state of heightened oxidative stress characterized by lipid and protein oxidation in the vascular walls [3–5]. Furthermore, atherosclerosis is clearly related with the inflammatory cascade where ROS are key mediators starting from the initiation of fatty streak development, through lesion progression, to ultimate in plaque rupture and thrombi formation (resulting in myocardial infarction) [6].

Atherosclerosis causes are not clear, but there are certain traits, conditions or habits known as risk factors that may raise the risk of suffering atherosclerosis [7]. Most common atherosclerosis risk factors are age, hypercholesterolemia, hypertension, diabetes, obesity and smoking. Among the different factors, obesity is a major public health crisis worldwide considered a leading risk factor for different pathologies such as diabetes, atherosclerosis, stroke, hypertension and some types of cancer [1,8,9]. Gender also influences atherosclerosis development, as men are more likely than women to develop this disease; however, gender difference narrows as men and women grow older.

Due to their central role in biochemistry, amino acids are important in nutrition. Plasma amino acid concentrations represent the balance of protein turnover (protein synthesis and degradation), amino acids absorption from diet, and metabolism of individual amino acids [9]. In fact, there are essential amino acids

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(*viz.* leucine, isoleucine, lysine, methionine, phenylalanine, threonine, histidine, tryptophan and valine) that the human body cannot synthesize at the concentration demanded for normal development and may be obtained from diet. Some essential amino acids have been proposed as cardiovascular disease biomarkers. Thus, threonine and phenylalanine have been proposed as myocardial infarction biomarkers [10]. The explanations given to this behavior are either their metabolism pathways are obstructed by the ischemia and oxygen deficiency condition, or the catabolism of the injured myocardial proteins to amino acids is enhanced due to the ligation of the left anterior descending coronary artery. Also, leucine, isoleucine and threonine are compounds affected immediately (10 min) after the onset of myocardial infarction [11].

Different analytical platforms have been proposed to determine amino acids in biofluids such as serum [12,13] and urine [14]. Most of them involve an electrophoretic or chromatographic step with subsequent determination by fluorimetry (usually with laser as excitation source), UV absorption spectrophotometry or mass spectrometry. A derivatization step prior to separation to enhance detection of amino acids by increasing signal given by analytes or ionization properties can also be involved. In fact, different labeling reagents such as 9-fluorenylmethyl chloroformate have been proposed for derivatization of amino acids [15]. Methods without previous derivatization have also been reported for determination of amino acids in serum and urine [16,17]. Furthermore, a Hitachi analyzer based on ion exchange chromatography with a spectrophotometer detector specific for amino acids is available in the market [18]. This analyzer, which allows the determination of a wide range of amino acids in 70 min, has been used to study the levels of amino acids in both patients with heart failure and normal individuals.

Because of the lack of automated methods for the simultaneous determination of essential amino acids of interest in the clinical field, the aims of this research were as follows: (i) to develop a method for determination of these amino acids in serum by on-line coupling of an automated solid-phase extraction system (Symbiosis-Pharma system) with an LC–MS/MS device; (ii) to apply the resulting method to the determination of the target compounds in a cohort formed by individuals affected by atherosclerosis; (iii) to correlate levels of essential amino acids in atherosclerotic patients and different risk factors such as obesity, gender, hypercholesterolemia and smoking habit.

2. Materials and methods

2.1. Reagents

A multistandard solution of amino acids from Sigma–Aldrich (Madrid, Spain) with an individual concentration for each analyte of 0.5 mM ± 4% in a 0.2 N lithium citrate buffer (pH 2.2), 2% of thiodiglycol and 0.1% of phenol was used to optimize both chromatographic separation and detection. L-Valine, L-leucine, L-phenylalanine and L-lysine standards and phosphate buffer solution (PBS) were provided by Sigma–Aldrich. D-Tryptophan was provided by Fluka (Spain) and L-methionine and L-threonin were from Merck (Madrid, Spain). An aqueous stock solution containing 100 μg/mL of each target amino acid was used to prepare diluted working solutions.

LC–MS grade acetonitrile and ammonium formate were purchased from Scharlab (Barcelona, Spain). Deionized water (18 mΩ cm) from a Millipore Milli-Q water purification system was used for preparation of all aqueous solutions.

Table 1
Features of the cohort under study.

Characteristic	Patients (n = 122)
Age	65.5 ± 13.5
Male gender, n (%)	99 (81.1)
Obesity, n (%)	50 (41.0)
High cholesterol level, n (%)	61 (50.0)

2.2. Blood extraction and serum isolation

Venous blood was collected in evacuated sterile serum tubes without additives (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA) and incubated for 30 min at room temperature to allow coagulation. Then, the tubes were centrifuged at 2000 × *g* for 15 min at 4 °C to isolate the serum fraction (processing within 2 h after collection). Serum was placed in plastic ware tubes and stored at −80 °C until analysis.

2.3. Cohort selected for the study

A total of 122 patients clinically diagnosed with an episode of either stable angina (80 individuals) or myocardial infarction (42 individuals) formed the cohort. All patients were affected by atherosclerosis after evaluation through a cardiac catheterization. The main characteristics of the patients are shown in Table 1. The cohort was composed by individuals with an average age of 66 ± 14, 81% of them male individuals, 53% smokers, 26% diabetic, 41% obese, 58% with hypertension and 50% with a high cholesterol level.

All steps from blood extraction to analysis were performed in compliance with the guidelines dictated by the World Medical Association Declaration of Helsinki of 2004, which were supervised by specialized personnel from Miguel Servet Hospital (Zaragoza, Spain). Individuals selected for this study were previously informed to obtain consent.

2.4. Instruments

Hyphenated SPE was performed with an automated robotized workstation Symbiosis-Pharma (Spark Holland, Emmen, The Netherlands) equipped with an autosampler Reliance (Spark Holland) furnished with a 250-μL sample loop and a refrigerated stacker sample compartment. The SPE workstation is endowed with a unit for SPE cartridge exchange—automatic cartridge exchanger (ACE)—and two high-pressure dispensers (HPD) for SPE solvents delivery. Peek tubes of 0.25 mm i.d. (VICI, Houston, TX, USA) were used for all connections. The ACE unit included three switching valves, one of them equipped with a T-rotor, and two clamps. The Sparklink 3.10 SP#3 software was used to control the system. Hysphere MM anion cartridges (8 μm, 10 mm × 2.0 mm, Spark Holland) were used as sorbent material in the SPE step.

Chromatography was performed with an Agilent (Palo Alto, CA, USA) 1200 Series chromatograh composed by a binary pump and a Luna (3 μm, 100 mm × 4.6 mm) hydrophilic interaction chromatography column (HILIC) from Phenomenex (Torrance, CA, USA).

Detection was carried out by an Agilent 6410 Triple quadrupole mass spectrometer, furnished with an electrospray ionization (ESI) source.

2.5. Sample treatment

Serum samples (100 μL) immersed in an ice bath were treated for deproteinization with 600 μL methanol, the most common solvent used with this aim. The mixture was shaken for 1 min and the precipitate removed after centrifugation for 5 min at 6 °C

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