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OBSEVATION REPORT

MEASURE OF ANTIOXIDANT POTENTIAL OF PLASMA BY LC-MS/MS

Raffaele Conte[1], Giusy De Rosa[1], Andrea Cavallo[1], Antonio Fico[1]

[1] *AMES GROUP Poly-diagnostic Center, Via Padre Carmine Fico 24, Casalnuovo di Napoli, Italy*

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ABSTRACT: Liquid chromatography-triple quadrupole mass spectrometry is a technique suited for rapid analysis of multiple analytes of similar and different structures and physicochemical properties. Antioxidants are a diverse group of biologically active compounds that can be present in plasma with the aim to contrast oxidative events related with atherosclerosis, arterial hypertension, stroke, myocardial infarction, diabetes, arthritis, dementia, colitis, pancreatitis, respiratory diseases, cancer, infections, etc . Current measures of antioxidant potential of plasma are based on spectrophotometry and are not able to identify the typology of antioxidants. Then, a sensitive and specific LC-MS/MS analytical method was developed for the identification and quantification of plasma antioxidants in order to assess the biological antioxidant potential of this matrix

KEYWORDS: Antioxidants, Liquid chromatography-mass spectrometry, Biological antioxidant potential

INTRODUCTION:

The measure of plasma antioxidant status is a clinical analysis with increasing use due to its importance in understanding the role of oxidative events in the initiation and progression of numerous diseases such as atherosclerosis, arterial hypertension, stroke, myocardial infarction, diabetes, arthritis, dementia, colitis, pancreatitis, respiratory diseases, cancer, infections, etc.[1] Human blood contains several compounds which oppose the oxidant potential of oxidant chemical species (also called ROS).[2] These are endogenous compounds like albumin, transferrin, ceruloplasmin, bilirubin, uric acid, reduced glutathione and substances deriving from food such as tocopherols, carotens, ubiquinol, ascorbate, methionine, flavonoids and polyphenols. All these molecules act giving equivalent reducing units (electrons) to block the potential damage of ROS. [3]

Corresponding Author:
Raffaele Conte,
AMES Polydiagnostic center, Via Padre Carmine Fico 24,
Casalnuovo di Napoli, Italy
Email- affaele.conte86@tiscali.it

An indirect method to have information about blood antioxidant status monitors oxidative stress in plasma through the detection of the derivatives of reactive oxygen metabolites (d-ROMs). In particular, hydroperoxides are converted into radicals that oxidize *N*,*N*-diethyl-*para*-phenylendiamine in order to be detected through spectrophotometric procedures and expressed as carratelli units (U.CARR.). One U.CARR. corresponds to 0.8 mg/L hydrogen peroxide. In normal subjects U.CARR. values range from 250 to 300.[4 Unfortunately, also if the peroxyl radicals are the most common free radicals in human blood, exists other radical sources that can have fairly dramatic effects on the observed antioxidant capacity.[5] Then, there is the need to develop additional assays that utilize other radical and oxidant sources, such as the hydroxyl radical, singlet oxygen, superoxide radical and reactive nitrogen species, to measure relevant antioxidants activity.[6] Because of this limit, is currently utilized a direct measure of the antioxidant potential obtained through a test that measures the ability of a blood sample to reduce ferric ions to ferrous ions.[7] On the other hand, also using this procedure no indications are obtained on the identity of the antioxidants. Consequently, most recent developed diagnostic techniques aim to asses antioxidant activity evaluating the levels of low-molecular weight antioxidants in plasma like ascorbic acid and tocopherol, also if great attention must be given in order to avoid the exclusion of a number of other compounds that influence in vivo antioxidant activity such as flavonoids and polyphenol. On this aim, great help derives from the increasing use of high throughput diagnostic techniques such as liquid-chromatography mass spectrometry that permit to develop screening analysis able to detect in a fast and accurate way a wide range of compounds.

LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

Liquid chromatography–mass spectrometry is a powerful analytical technique that combines the physical separation capabilities of liquid chromatography with the detection specificity of mass spectrometry.[8] briefly, liquid chromatography separates the sample components and then introduces them to the mass spectrometer that creates and detects charged ions. The LC/MS data give information about the molecular weight and quantity of specific sample components. Such technique has very high sensitivity and is used for the detection, identification or purification of chemicals in complex mixtures.[8] LC/MS significantly expands the effective analytical use of mass spectrometry to a much larger number of organic compounds. In fact, it is suitable for the analysis of large, polar, ionic, thermally unstable and nonvolatile compounds.[9] Mass spectrometers can operate in either scan mode or a selected ion monitoring mode. In the scan mode, the instrument detects signals over a mass range (e.g. from 50–2000 m/z) during a short period of time. During this scan period, the MS electronics sequentially read the signals detected within narrower mass intervals until the full mass range is covered. This mode of operation is typically selected for qualitative analysis or for quantification when analytes masses are not known in advance. In the SIM mode, mass spectrometers are set to monitor a single mass to-charge ratio (m/z). This technique is used for target compound analysis. Moreover, a more accurate SIM analysis is accomplished by a process called collision-induced dissociation (CID) in which ions break apart as a result of collisions with other molecules. Are then produced spectra of fragments derived from the selected ions that lead to a more specific detection of the analyte. LC-MS\MS is currently used for the identification of unknown compounds, the determination of the isotopic composition of elements in a molecule, and the study of the

structure of a compound by observing its fragmentation. Then, it is of very common use in analytical laboratories that work on physical, chemical or biological properties of molecules. [10]

BIOLOGICAL ANTIOXIDANT POTENTIAL BY LC-MS/MS

The assay routinely designed to assess the Biological Antioxidant Potential of plasma is the "BAP" test, a spectrophotometric test developed by the Italian Chemist Mauro Carratelli[7]. This assay is based on the principle that when a ferric salt is dissolved in a uncolored solution containing a particular thiocyanate derivative, the resulting solution becomes red, as a function of the ferric ions concentration. This phenomenon is ascribable to the formation of a complex between ferric salt and thiocyanate. The addition of a small amount of plasma will reduce ferric ions to ferrous ions thus making uncolored the initial red solution. This chromatic change can be read through a photometer set at the wavelength of the chromogen. The entity of absorbance change will directly correlate with the antioxidant "potential" of plasma against the specific substrate.[7] However, to address the need for identification and quantitative analysis of specific antioxidants in biomedical specimens, we developed a new assay based on LC-tandem mass spectrometry (LC-MS/MS). This approach utilizes the selectivity of HPLC separation and high resolution tandem mass spectrometric analysis for the characterization, identification and the quantitative analysis of antioxidant species in complex samples. In particular, the developed method quantifies the concentrations of uric acid, glutathione, tocopherol, ascorbate, and the most available flavonoids and poly-phenols in foods in order to give a reliable value of biological antioxidant potential.

MATERIALS AND METHODS:

Chemicals

Ascorbic acid (Vit C), Alpha-tocopherol (Vit E), Uric acid, Glutathione, Thiamine (Vit B1), Pyridoxine (Vit B6) and all the other standards were purchased from Sigma-Aldrich (Sigma-Aldrich Chemical Co, Milan). LCMS grade acetonitrile and water were purchased from Carlo Erba (Carlo Erba reagents, Milan). All other chemicals and solvents are ACS reagent grade, unless stated otherwise.

Sample preparation

100 µL of plasma or plasma with standards were added to 1.5 ml eppendorf tubes and vortexed briefly. Then, 400 µL of Acetonitrile was added to each tube and vortexed for 1 min prior to centrifugation for 10 minutes at 15000 rpm. The supernatant was transferred to an MS vial and capped prior to injection. The calibration curves range from 1 ng/mL to 1000 ng/mL.

LC-MS/MS Conditions

LC-MS/MS analyses were carried out using a Shimadzu (Shimadzu Italy, Milan) LC-20A UHPLC system interfaced to a Shimadzu LCMS-8060 (Shimadzu Italy, Milan) triple quadruple mass spectrometer. Substances were extracted with online extractor Shim-pack MAYI-ODS (G) (Shimadzu Italy, Milan) using water containing 0.01% formic acid as eluting phase and separated on a Kinetex 2.6 µm Polar C18 100 mm × 3 mm i.d. analytical column (Phenomenex, Milan) with a linear gradient from 5–95% acetonitrile in water containing 0.01% formic acid at a flow rate of 0.3 mL/min. The injection volume was 15 µL. Positive ion electro spray tandem mass spectrometric analysis for quantitative analysis of each antioxidant was carried out at unit resolution using collision-induced dissociation and selected reaction monitoring (SRM). The ion source temperature was 350 °C, the ion spray voltage was

−4200 V and the dwell time was 0.5 s/ion. During SRM the antioxidants were measured by recording the signals for the transitions of the deprotonated molecules shown in **Table 1.**

Table 1. SRM transitions of antioxidants

ANTIOXIDANT	TRANSITIONS
Ascorbic acid (Vit C)	175.00>115.00
Alpha-tocopherol (Vit E)	277.80>277.80
Gallic acid	169.00>125.00
Hesperetin	301.30>164.10
Trimethoxyflavone	312.00>312.00
Arbutin	271.20>107.80
Rosmarinic acid	359.00>359.00
Ursolic acid	455.00>455.00
Apigenin	269.00>150.90
Amentoflavone	537.10>374.90
Luteolin	284.90>133.00
Quercetin-3-O-glucoside	463.10>300.00
Quercetin-7-O-glucuronic acid	477.00>301.00
Kaempferol-3-O-glucose	609.10>284.00
Quercetin-3-O-hexose-deoxyhexose	609.10>300.00
Isorhamnetin-3-O-rutinoside	623.10>314.00
Isorhamnetin-7-O-pentose	447.10>315.00
Luteolin-7-O-glucoside	447.10>285.00
Kaempferol-3-0-glucuronic acid (7-O-glucuronic acid; 4'-O-gluvuronic acid)	461.10>284.00
Kaempferol-3-0-pentose	417.10>284.00
Kaempferol-3-0-hexose-deoxyhexose	593.10>284.00
Oleuropein	539.00>539.00
Tyrosol	153.40>123.00
Hydroxytyrosol	137.40>119.00
Protocathecoic Acid	153.00>109.00
Vanillic Acid	167.00>123.00
Syringic Acid	197.00>182.00
p-Hydroxybenzoic Acid\ Salicilic Acid	137.00>93.00
Gentisic Acid	153.00>109.00
Caffeic Acid	179.00>135.00
p-Coumaric Acid	163.00>119.00
Sinapic Acid	223.00>208.00
Ferulic Acid	193.00>134.00
Trans-Cinnamic Acid	147.00>147.00
Chlorogenic Acid	353.00>190.00
Catechin\Epicathechin	289.00>245.00
Gallocathechin\Epigallocathechin Gallate	457.00>169.00
Epicathechin Gallate	441.00>289.00
Gallocathechin\Epigallocathechin	305.00>125.00
Procianidin	577.00>407.00
Quercetin	301.00>151.00
Myricetin	317.00>151.00
Kamepferol	285.00>257.00
Rutin	609.00>301.00
Narigin	579.00>459.00
Uric Acid	169.00>141.00
Glutathione	308.01>76.02
Thiamine (Vit B1)	265.40>81.00
Pyridoxine (Vit B6)	170.00>152.00

Data were acquired and analyzed using Lab Solutions software by Shimadzu.

RESULTS:

The optimized method is routinely used in the industrial and forensic toxicology laboratory of AMES Group (Casalnuovo di Napoli, Italy).

Linearity/Sensitivity

The assay was linear over the calibration curve for the substances in plasma as shown in **Figure 1**.

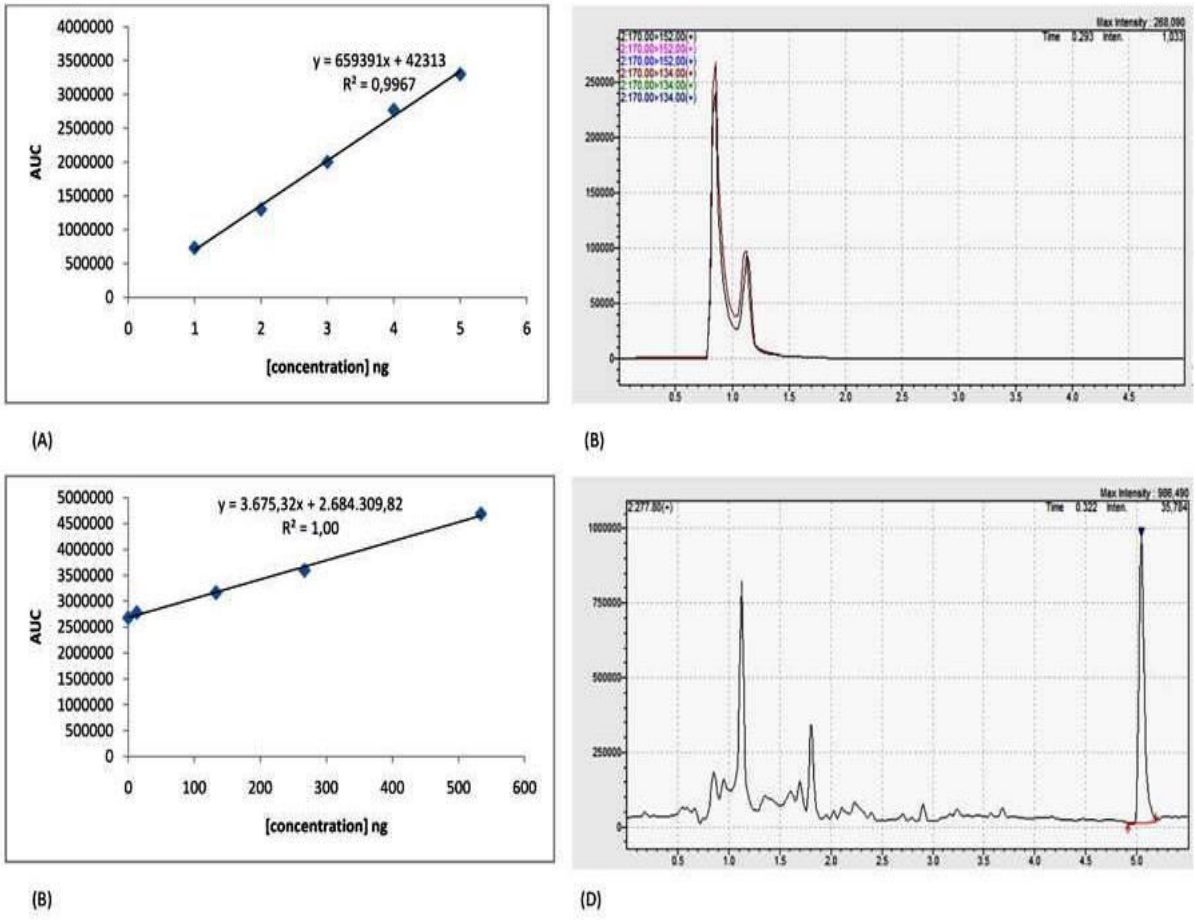

Figure 1. (A). Example of calibration curve of Pyridoxine (Vit B6) in plasma; (B). Chromatogram of Pyridoxine (Vit B6) in plasma at 144 ngml; (C). Example of calibration curve of alpha-tocopherol (Vit E) in plasma; (D). Chromatogram of alpha-tocopherol (Vit E) in plasma at 133 ngml

Note: Such graphs are given as example. Please contact the corresponding author for calibration curves and chromatograms of the other antioxidants

The linearity was determined in triplicate over 3 days and the results are shown with the LOQ being determined as 10:1 of signal to noise. The mean coefficient of determination (R^2) > 98 for each sample extraction technique and the %CV for each calibration point were all <10% in order to be accepted.

CONCLUSIONS:

The described method is an accurate estimation of the biological antioxidant potential of plasma, characterized by the baseline separation of the large number of compounds analyzed by LC-MS/MS in positive mode and with good LOQ. The use of the online extractor is aimed to obtain a clean plasma matrix, required to achieve the desired calibration curve and LOQ. The increasing diffusion of this method will permit to collect data in order to better correlate the plasma concentration of the analyzed antioxidants with the biological antioxidant potential.

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