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Protein-bound tyrosine oxidation, nitration and chlorination by-products assessed by ultraperformance liquid chromatography coupled to tandem mass spectrometry

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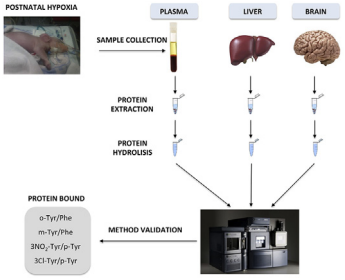
HIGHLIGHTS

- A validated liquid chromatographic method to determine protein-bound tyrosine oxidation, nitration and chlorination by-products.
- Plasma, Liver and Brain samples of hypoxic and normoxic animals from a postnatal hypoxia newborn piglets model.
- Small sample volume and suitable sample pre-treatment that includes protein extraction and subsequent enzymatic hydrolysis.
- High-throughput of sample analysis and high selectivity for Phe, p-Tyr, o-Tyr, m-Tyr, 3Cl-Tyr, $3NO_2$-Tyr phenylalanine, p-tyrosine, ortho-tyrosine, meta-tyrosine, 3-nitrotyrosine and 3-chlorotyrosine.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: Free radicals cause alterations in cellular protein structure and function. Oxidized, nitrated, and chlorinated modifications of aromatic amino acids including phenylalanine and tyrosine are reliable biomarkers of oxidative stress and inflammation in clinical conditions.
Objective: To develop, validate and apply a rapid method for the quantification of known hallmarks of tyrosine oxidation, nitration and chlorination in plasma and tissue proteins providing a snapshot of the oxidative stress and inflammatory status of the organism and of target organs respectively.
Material and Methods: The extraction and clean up procedure entailed protein precipitation, followed by protein re-suspension and enzymatic digestion with *pronase*. An Ultra Performance Liquid Chromatography–tandem Mass Spectrometry (UPLC-MS/MS) method was developed to quantify protein released

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Plasma
Tissue

ortho-tyrosine (o-Tyr), meta-tyrosine (m-Tyr), 3-nitrotyrosine ($3NO_2$-Tyr) and 3-chlorotyrosine (3Cl-Tyr) as well as native phenylalanine (Phe) and tyrosine (p-Tyr) in plasma and tissue from a validated hypoxic newborn piglet experimental model.

Results: In plasma there was a significant increase in the $3NO_2$-Tyr/p-Tyr ratio. On the other hand m-Tyr/Phe and 3Cl-Tyr/p-Tyr ratios were significantly increased in liver of hypoxic compared with normoxic animals. Although no significant differences were found in brain tissue, a clear tendency to increased ratios was observed under hypoxic conditions.

Conclusions: UPLC-MS/MS has proven suitable for the analysis of plasma and tissue samples from newborn piglets. The analysis of biomarkers of protein oxidation, nitration and chlorination will be applied in future studies aiming to provide a deeper insight into the mechanisms of oxidation-derived protein modification caused during neonatal asphyxia and resuscitation.

1. Introduction

Proteins play an essential role as functional and structural cell constituents. Both protein composition and structure are genetically determined. However, they may undergo post-translational modifications or may be damaged by various molecular mechanisms [1,2]. Oxidative stress (OS) and nitrosative stress (NS) play a physiologic role during physical exercise, fetal-to-neonatal transition or aging [3,4]. However, under certain circumstances OS/NS may significantly contribute to the genesis of severe medical conditions through the production of free radicals some of which are reactive oxygen or nitrogen species (ROS/RNS) [5]. ROS/RNS may attack proteins but also lipids, carbohydrates or nucleic acids. Moreover, protein oxidation and nitration will directly disturb the enzymatic redox system and subsequently ROS/RNS detoxification [6]. Furthermore, proteins have long half-life and may therefore, under unfavorable conditions, accumulate oxidative “hits” which directly correlate to the severity of diseases [7].

Oxidative and nitrosative modifications of phenylalanine (Phe) and tyrosine (p-Tyr) have been widely studied as biomarkers for protein oxidation and nitration [7]. Oxidation of Phe to p-Tyr is enzymatically mediated by phenylalanine hydroxylase (PheOH). However, upon reaction with hydroxyl radical (OH•), Phe may form two p-Tyr isomers such as ortho-Tyrosine (o-Tyr) and meta-Tyrosine (m-Tyr) [8,9]. Otherwise, in the presence of peroxynitrite ($ONOO^-$) p-Tyr can be converted into 3-nitrotyrosine ($3NO_2$-Tyr), a specific biomarker for protein nitration [10]. Protein nitration and RNS signaling play a role in cell functions such as inflammatory response and apoptosis. So, the excess of $3NO_2$-Tyr has been postulated as a marker of inflammation, caused by pathological conditions [11,12]. Likewise, 3-chlorotyrosine (3Cl-Tyr), formed by the attack of the hypochlorous acid (HClO) towards p-Tyr through the action of the enzyme myeloperoxidase (MPO), is considered a useful inflammatory biomarker [8,13,14]. On the whole, this panel of biomarkers might be considered as reliable assessment of ROS/RNS damage to proteins [7,8,15,16].

These biomarkers have been detected in numerous biological tissues and fluids, such as urine, plasma, cerebrospinal fluid (CSF), and exhaled breath condensate (EBC) in clinical and translational research [17–19]. Several methods have been developed for determining both in their free and protein-bound (released from proteins) forms. The latter methods are based on sample protein enrichment and hydrolysis and subsequent MS/MS detection. Some authors performed an acidic protein hydrolysis [20–22] while others conducted an enzymatic hydrolysis of protein bulks [23–25]. When the acidic hydrolysis is carried out, special care should be taken to avoid possible pitfalls [26]. On the other hand, several analytical methods can be found in literature to determine Phe, p-Tyr, o-Tyr, m-Tyr, $3NO_2$-Tyr and/or 3Cl-Tyr including immunohistochemistry [18,27], high performance liquid chromatography (HPLC) [23,27–29] with ultraviolet (UV) [30], electrochemical (ECD) [23] or mass spectrometry (MS) detection [28,29], as well as gas chromatography with MS detection (GC–MS) [31]. Among these methods, the sensitivity and specificity of immunodetection are limited by antibody quality and visualization methods [29]. The use of HPLC-UV or HPLC-ECD has drawbacks such as low accuracy and poor reproducibility, also these methods are expensive to perform as routine analysis [32]. GC–MS is characterized by tedious and time-consuming sample treatment [30]. From a clinical point of view, the simultaneous determination of several analytes becomes of particular importance, especially when the amount of sample is limited (eg. plasma samples derived from extremely low gestational age newborns). To our knowledge, only two methods have been developed to determine simultaneously the same panel of biomarkers proposed in this work, and they were applied to urine samples [33], cell culture, and tissue or blood samples [34]. Both methods are focused only on the detection of their free form and not in the protein-bound form. Thus, the measurement of the tyrosine by-products in their free form in tissue or plasma samples might be underestimating the true level of protein oxidation.

The aim of the present study was to develop a reliable and accurate analytical method to determine a panel of protein oxidation (o-Tyr/Phe and m-Tyr/Phe), nitration (3NO2-Tyr/p-Tyr) and chlorination (3Cl-Tyr/p-Tyr) biomarkers in tissue and plasma samples using Ultra Performance Liquid Chromatography coupled to tandem Mass Spectrometry (UPLC-MS/MS) and needing a small sample size that enhance its applicability in the perinatal period.

2. Material and methods

2.1. Reagents and material

Standards of Phe, p-Tyr, o-Tyr, m-Tyr, $3NO_2$-Tyr and 3Cl-Tyr, (96% w/w purity) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Deuterated phenylalanine (Phe-D_5) with a 98% atom D enrichment was purchased from CDN Isotopes (Pointe-Claire, Canada). Acetonitrile (ACN) (LC-MS grade), methanol (MeOH) (LC-MS grade), formic acid (analytical grade), trichloroacetic acid (TCA), sodium acetate and type XIV protease (*Pronase from Streptomyces griseus)* were purchased from Sigma Aldrich Química SA (Madrid, Spain) water was Milli-Q grade (18.2 MV) from a Millipore purification system.

2.2. Solutions

Three lysis solutions were prepared to optimize the tissue homogenization. Buffer A: Tris–HCl (20 mmol L^{-1}, pH 7.5), EDTA (1 mmol L^{-1}), NaCl (150 mmol L^{-1}), SDS (0.1%, w/v), Igepal (1%,v/v),