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Development of a new HPLC-based method for 3-nitrotyrosine quantification in different biological matrices

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ABSTRACT

Background: The nitration of tyrosine residues in proteins is associated with nitrosative stress, resulting in the formation of 3-nitrotyrosine (3-NT).[1] 3-NT levels in biological samples have been associated with numerous physiological and pathological conditions. Hence several attempts have been made in order to develop methods that accurately quantify 3-NT in these matrices. The aim of this study was to develop a simple, rapid, low-cost and sensitive high-performance liquid chromatography (HPLC)-based 3-NT quantification method.

Methods: All experiments were performed on an Hitachi LaChrom Elite® HPLC system. The method was validated according to International Conference on Harmonisation (ICH) guidelines for serum samples. Additionally, other biological matrices were tested, namely whole blood, urine, B16 F-10 melanoma cell line, growth medium conditioned with the same cell line, bacterial and yeast suspensions.

Results: From all the protocols tested, the best results were obtained using 0.5% CH_3COOH:MeOH:H_2O (15:15:70) as mobile phase, with detection at wavelengths 215, 276 and 356 nm, at 25 °C, and using a flow rate of 1 mL min^{-1}. By using this protocol, it was possible to obtain a linear calibration curve, limits of detection and quantification in the order of μg L^{-1}, and a short analysis time (<15 min *per* sample). The developed protocol allowed the successful detection and quantification of 3-NT in all biological matrices tested, with detection at 356 nm.

Conclusion: This method, successfully developed and validated for 3-NT quantification, is simple, cheap and fast. These features render this method a suitable option for analysis of a wide range of biological matrices, being a promising useful tool for both research and diagnosis activities.

1. Introduction

Molecules modified by interactions with reactive oxygen species (ROS) in the microenvironment, and those changed in response to increased redox stress, are considered biomarkers of oxidative stress [1]. The nitration of tyrosine (Tyr) residues in proteins is associated with nitrosative stress. L-Tyr and protein-associated Tyr are the target of various reactive-nitrogen species (RNS), resulting in the formation of free 3-nitrotyrosine (3-NT) [(2-amino-3-(4-hydroxy-3-nitrophenyl) propanoic acid)] and protein-associated 3-NT [2–4]. It is formed after the substitution of a hydrogen by a nitro group (NO_2) in the *ortho* position of the phenolic ring of the Tyr residues [2,4]. Recently, a study suggested that 3-NT is likely to have a deleterious effect on protein function and less likely to be important in normal cellular function [5].

The nitration of proteins is a common process that occurs under physiological conditions [3,6,7] and the concentration of 3-NT in plasma of healthy humans is on the threshold of the nM-to-pM range [3].

A significant increase in the extent of this process results in increased 3-NT levels in biological samples and has been associated with a wide range of diseases [3,6]. Among these there are cardiovascular diseases [8,9], diseases associated with immunological

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[1] 3-NT – 3-nitrotyrosine; ICH – International Conference on Harmonisation; ROS – reactive oxygen species; Try – tyrosine; RNS – reactive-nitrogen species; PYCC – Portuguese Yeast Culture Collection; MEM – Minimal Essential Medium; TSA – Trypticase Soy Agar; YEPD – yeast extract peptone dextrose.

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reactions [10,11], neurological diseases and psychiatric disorders [12,13]. Other diseases have also been associated with increased protein nitration, such as Fabry disease [14], diabetes mellitus [15,16], diabetic vascular dysfunction [17], Chagas disease [18], systemic lupus erythematosus [19], erectile dysfunction [20] among others. Measurement of 3-NT in biological samples can be used as a biomarker of nitrosative stress, since it is very stable and suitable for analysis.

Since 3-NT was suggested as a biomarker of nitrosative stress, a substantial effort has been made to develop analytical methods that can be applied to biological samples [21]. 3-NT has been detected in several biological matrices and fluids including plasma, serum, urine, cerebrospinal fluid, synovial fluid, tissue sample and other biological samples [22].

Among the techniques used for 3-NT detection and quantification, chromatographic methods have been shown to exhibit better performance. The different chromatographic methods described for the detection and quantification of this molecule include: (i) liquid chromatography, namely high-performance liquid chromatography (HPLC)-based methods that use electrochemical (ECD) and diode array (DAD) detection, liquid chromatography-mass spectrometry (LC–MS) and liquid chromatography-tandem mass spectrometry (LC–MS/MS); (ii) gas chromatography, such as gas chromatography-mass spectrometry (GC–MS) and gas chromatography-tandem mass spectrometry (GC–MS/MS) [23,24]. These methods have been developed during the last years, all of them presenting pros and cons, although it is evident that chromatography-based methods present good sensitivity and specificity. GC-based methods exhibit the highest sensibility in the quantification of 3-NT [25–27]. Nevertheless, and owing to 3-NT chemical properties, a derivatization step prior to analysis is required, which ends up being time-consuming for the analyst [28]. Moreover, derivatization reactions often induce artefacts formation, which may further influence the final analysis. Conversely, HPLC does not require such derivatization step and is cheaper, despite not being as accurate as GC [7,29].

Currently, 3-NT has raised great interest concerning its potential as biological tool for the therapeutic monitoring of various diseases involved in nitrosative stress. In this sense, our study aimed to develop a new chromatographic method, which is simple, cheap and user-friendly, without compromising the sensitivity and specificity levels required for quantification of 3-NT. The ultimate goal was to apply this method to a myriad of biological samples, so that it can be used both in research and medical laboratories. The results obtained were validated and interpreted in accordance with the International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use guidelines for serum samples [30]. Additionally, and in order to provide a proof-of-concept for our method, other biological matrices were tested, namely whole blood, urine, B16 F-10 melanoma cell line, growth medium conditioned with the same cell line, bacterial and yeast suspensions. In the future, validation for these matrices should be performed as well.

2. Material and methods

2.1. Instrument and software

All experiments were performed on a Hitachi LaChrom Elite® HPLC system (Hitachi High – Technologies Corporation, Tokyo, Japan) composed by HTA L-2130 LaChrom Elite quaternary pumps, L-2200 LaChrom Elite autosampler, L-2300 LaChrom Elite column heater and L-2455 LaChrom Elite photo DAD. EZChrom Elite Compact Software Version 3.3.2. (Agilent Technologies, Inc., Santa Clara, CA, United States) was used for data collection and analysis.

2.2. Chemicals, reagents and consumables

3-Nitro-L-tyrosine was purchased from Santa Cruz Biotechnology, Inc. (Bergheimer, Heidelberg, Germany). L-Tyrosine was purchased from AppliChem – BioChemia GmbH (Ottoweg, Darmastadt, Germany). Glacial acetic acid (100%) was purchased from Merck S.A. (Algés, Portugal). Methanol (HPLC GOLD Ultra Gradient) was purchased from Carlo Erba Reagents (Chaussée du Vexin, Val de Reuil, France). Ultrapure water was obtained from the Water Purification System TKA Barnstead™ GenPure™ capsule 0.2 μm (Thermo Fisher Scientific, Wilmington, DE, EUA). Trifluoroacetic acid was purchased from Biochem Chemopharma (Ligne, Cosne sur Loire, France). LiChrospher® 100 RP-18 (5 μm) LiChroCART® 250-4 was purchased from Merck S.A. (Algés, Portugal). Membrane filters 0.45 μm, 47 mm, were purchased from Advantec®, Toyo Roshi Kaisha, Ltd. (Tokyo, Japan). Puradisc™, 0.2 μm, 25 mm sterile and endotoxin free filters were purchased from Whatman™ (GE Healthcare UK Limited, Buckinghamshire, UK). 2 mL syringes were purchased from Terumo® Medical Corporation (Leuven, Belgium).

2.3. Biological fluids and cellular models

The biological fluids' samples tested in this study are described in Supporting information Table 1. B16 F10 murine (ATCC, No CRL®-6475) melanoma cell line was obtained from American Type Culture Collection (ATCC). Gram-positive *Staphylococcus aureus* (ATCC® 25923) and gram-negative *Escherichia coli* (ATCC® 25922) bacteria, as well as the yeast *Saccharomyces cerevisiae* [PYCC (Portuguese Yeast Culture Collection) 4072].

2.4. Analytical procedure

2.4.1. Mobile phase – 0.5% CH_3COOH:MeOH:H_2O

0.5% CH_3COOH:MeOH:H_2O solutions were prepared according to the following proportions: 30:0:70 and 15:15:70. All mobile phases were filtered through a 0.45 μm membrane.

2.4.2. Calibration standards

0.5 gL^{-1} 3-NT and Tyr stock solutions were prepared using the aforementioned mobile phases as solvents. All stock solutions were filtered through a filter membrane device. The first assays were performed using standard solutions containing either 3-NT or Tyr in the following concentrations (50,000; 25,000; 10,000; 5000.0 2500.0; 1250.0; 625.00 and 312.50 $\mu g\,L^{-1}$). Standard solutions were prepared by diluting the respective stock solution into the desired mobile phase. These standard solutions were used for calibration purposes.

2.4.3. Chromatographic conditions

The chromatographic conditions used in all assays were as follows: flow rate of 1 $mL\,min^{-1}$, detection in the range 190–400 nm, volume of injection of 25 μL and oven temperature of 25 °C.

2.5. Method optimization

2.5.1. Temperature optimization

In order to establish the best operating temperature, a wide range of temperatures (15, 20, 35, 45, 55 and 65 °C) were assayed using two standard solutions (50,000 $\mu g\,L^{-1}$ and 25,000 $\mu g\,L^{-1}$) and two serum samples.

2.6. Method validation

All the methods tested were validated according to ICH guidelines for validation of analytical procedures [30].