Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 136 (2015) 802–807

Contents lists available at ScienceDirect

Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy

journal homepage: www.elsevier.com/locate/saa

Development of a cloud point extraction and spectrophotometry-based microplate method for the determination of nitrite in human urine and blood

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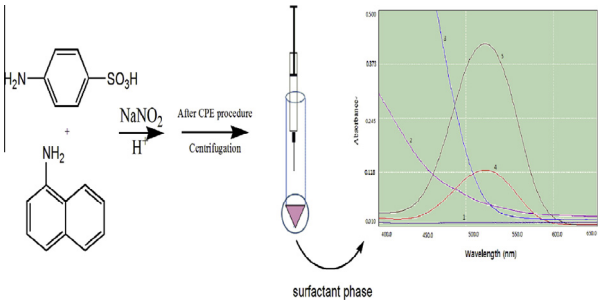
HIGHLIGHTS

- Decolorization treatment of urine and blood was applied to overcome the interference of other chromophores in the sample.
- A novel cloud point extraction method for nitrite was developed.
- Multi-sample can be simultaneously tested thanks to a 96-well microplate technique.
- The LOD is better than other methods based on spectrophotometric detection.
- Analysis of nitrite in human urine and blood samples was performed.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 11 June 2014
Received in revised form 2 September 2014
Accepted 22 September 2014
Available online 23 October 2014

Keywords:
Nitrite
Decolorization
Cloud point extraction
Microplate assay
Urine
Blood

ABSTRACT

A novel and simple method for the sensitive determination of trace amounts of nitrite in human urine and blood has been developed by combination of cloud point extraction (CPE) and microplate assay. The method is based on the Griess reaction and the reaction product is extracted into nonionic surfactant Triton-X114 using CPE technique. In this study, decolorization treatment of urine and blood was applied to overcome the interference of matrix and enhance the sensitivity of nitrite detection. Multi-sample can be simultaneously detected thanks to a 96-well microplate technique. The effects of different operating parameters such as type of decolorizing agent, concentration of surfactant (Triton X-114), addition of $(NH_4)_2SO_4$, extraction temperature and time, interfering elements were studied and optimum conditions were obtained. Under the optimum conditions, a linear calibration graph was obtained in the range of 10–400 ng mL^{-1} of nitrite with limit of detection (LOD) of 2.5 ng mL^{-1}. The relative standard deviation (RSD) for determination of 100 ng mL^{-1} of nitrite was 2.80%. The proposed method was successfully applied for the determination of nitrite in the urine and blood samples with recoveries of 92.6–101.2%.

Introduction

Nitric oxide (NO) is a highly reactive free radical and an important cellular signaling molecule regulating various physiological processes in human body, including blood vessel modulation, neural communication and immune response [1–3]. NO can be directly

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http://dx.doi.org/10.1016/j.saa.2014.09.097
1386-1425/

measured in the human circulation upon stimulation [4], however, endogenous NO at the basal state has not been reliably quantified in the urine and blood, thus far. Studies of NO are limited by its short half life, which it is 0.05–1.8 ms in biological fluids [5]. In plasma or other physiological fluids, NO is oxidized almost completely to nitrite and nitrate, where it remains stable for several hours [6,7]. Nitrite and nitrate are stable metabolites of NO, present both in blood and urine, and accessible to analysis. Quantitative determination of the concentration of nitrite and nitrate in biological fluids, notably plasma, serum and urine, is the most suitable method to assess NO synthesis in vivo. Studies in vivo in humans and mammals indicate that circulating nitrite rather than nitrate reflects endothelial-dependent NO synthesis in humans and mammals [8–10]. Nitrite is relatively low amounts comparing to nitrate in human plasma and urine and its concentration is under the detection limit. In addition, a large amount of nitrate in the in humans and laboratory animals (mouse, rat, rabbit, and guinea pig) come from alimentary sources. This means that nitrite concentration better describes the formation of nitric oxide than nitrate does [11].

In the past two decades, several methods have been used to measure the concentrations of nitrite in blood and urine. These methods include spectrofluorimetry [12–14], UV/VIS spectrophotometry [15–18] and ion chromatography [19], high performance liquid chromatography [11], and gas chromatography–mass spectrometry and liquid chromatography with fluorescence [20]. However, many published method are failure to detect nitrite in biological fluids because the nitrite in biological fluids is at a very low level and it is very susceptible to the substrate.

Spectrophotometry is probably the most commonly used method for nitrite measurement using colorimetric reaction. While a weakness of this method (and all other methods that rely on colorimetric detection) is interference of other chromophores in the sample. Based on considerations of simplicity, rapidity, colour stability, adherence to Beer's law and sensitivity, we propose decolorization processing to overcome the color interference from the sample matrix. In addition, trace nitrite in urine and blood is enriched into surfactant Triton-X114 phase using CPE technique. Cloud-point extraction (CPE) is an extraction technique based on the clouding phenomenon of surfactants and was first introduced by Watanabe and Tanaka to preconcentrate metal ions from aqueous samples [21]. At certain temperature, aqueous solution of a non-ionic surfactant becomes turbid. Under the impact of the centrifugal force, solution separates in two phases. The small volume of the surfactant-rich phase obtained by using the cloud point methodology permits to design an extraction strategy presenting low cost, good extraction efficiency and lower toxicity. Moreover, Triton-X114 does not exhibit high background absorption in visible region interfere with the determination of target analytes.

The other innovation of the present paper is the implementation of the whole analytical procedure into a microtiter plate, to take thus advantage of great possibilities offered by microtiter plate assays, namely (1) the small volume of the surfactant-rich phase obtained by using the cloud point methodology do not to be diluted; (2) automation possibilities, fast preparation of microplates and multi-sample analyses; (3) decrease the consumption of test materials and reagents; (4) absorbance value was recorded by microplate reader and directly stored in the computer, and error can be reduced from different experimenter. Despite these recognized advantages, microtiter plate assays have been seldom applied to chemical analysis [22–24] and we can reasonably assume the ability to selectively enhance the detection properties of a given tissue could have widespread clinical utility. Therefore, it can be a valuable instrument in experimental and clinical studies to determine the physiologic and pathophysiologic relevance of NO.

Experimental

Instrumentation

A UV–vis spectrophotometer model Tu-1810 (Beijing, China) was used to record the spectra. Recording the absorbance measurements was made by a Microplate reader model (Biotek Elx800, America) using 96-well microplate read modes and built-in 4 pieces of filter, 405, 450, 490, 630 nm, respectively. A water bath (Shanghai, China) was used to maintain solutions at certain temperatures. A vortex mixer (XW-80A, China) was used to blend fluids quickly. A centrifuge with calibrated centrifugal tubes (Shanghai, China) was used for the phase separation process.

Reagents

A stock solution of 200 μg/mL of nitrite was prepared by dissolving 300 mg of dried sodium nitrite (Aladdin Chemical Co., Shanghai) (at 110 °C for 2 h, to remove the water of crystallization) in double distilled water and diluting to 1000 mL in a volumetric flask. Working solutions were prepared by an appropriate dilution of the stock solutions on the day of use. A 10 mg/mL sulphanilic acid solution was prepared by dissolving 1.0 g of sulphanilic acid (Aladdin Chemical Co., Shanghai) in 100 mL hydrochloric acid (0.5 M) at 45 °C. A 1 mg/mL 1-naphtylamine solution was prepared by dissolving 100 mg 1-naphtylamine (Aladdin Chemical Co., Shanghai) into 100 mL methanol (50%). A Triton X-114 (10%) was prepared by diluting 11.1 mL of Triton X-114 (90%) (Sigma, USA) in a 100 mL volumetric flask.

All chemicals used were of analytical grade and double distilled water was used throughout.

Preparation of urine and blood samples

Whole blood samples

The whole blood samples (5 mL) of ten volunteers were obtained from the First People's Hospital of Yunnan Province (Kunming, China) at early morning time. Plasma in the upper layer was transferred to a new tube after each whole blood sample was centrifuged at 2000 rpm for 10 min. Plasma was either used for analysis of nitrite immediately or stored at −20 °C until analysis. The protein precipitant and decolorizer of 400 μL NaOH (2.0 M) and 0.4 g $ZnSO_4$ were added into a 10 mL centrifuge tube with 2 mL of plasma. The mixture was vortex-blended for 30 s, and deposited by centrifugation at 5000 rpm for 10 min. The clear and colorless supernatant was then transferred to a new tube, and 200 μL of sulphanilic acid solution (10 mg mL^{-1}) and 200 μL of 1-naphtylamine solution (1 mg mL^{-1}) were added. The mixture was mingled completely and diluted to the volume of 5 mL with distilled water for further analysis according to CPE procedure.

Urine samples

Urine samples were collected from the same ten volunteers using propene polymer (PP) one-time urine cup at early morning time. All samples were either used for analysis of nitrite immediately or stored at – 20 °C until analysis. 0.4 g active carbon used as decoloring agent was added into 10 mL centrifuge tube with 5 mL urine sample, followed by mixing for 30 s. The urine sample was filtered with one piece of filter paper and collected with a new tube then 200 μL of sulphanilic acid solution (mg mL^{-1}) and 200 μL of 1-naphtylamine solution (mg mL^{-1}) were added. The mixture was mingled completely and diluted to the volume of 5 mL with distilled water for further analysis according to CPE procedure.