Determination of Nitrite and Nitrate in Cell Culture Medium by Reversed-Phase High-Performance Liquid Chromatography with Simultaneous UV-VIS and Fluorescence Detection

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Abstract
A reliable method for determination of nitrite and nitrate in cell culture medium has been developed. The method is based on pre-column derivatization of nitrite with 2,3-diaminonaphthalene (DAN) under acidic medium to yield 2,3-naphthotriazole (NAT). For determination of nitrate, it was converted to nitrite by nitrate reductase before the derivatization step. NAT was separated on a 5 µm C18 column (250x4.6 mm, I.D) guarded by a 10 µm C18 column (20x4.6 mm, I.D), and eluted with 15 mM phosphate buffer (pH 7.5) containing 35% acetonitrile (flow rate 1.0 mL/min). Average retention time of NAT was 12.4 min. Absorbance of NAT was monitored at 369 nm and its fluorescence was monitored at 375 nm for excitation and 415 nm for emission. Calibration curves of nitrite or nitrate were linear in the range 0.03-2.0 µM for fluorescence detection and 0.5-50 µM for UV-VIS detection. Correlation coefficients were better than 0.994 and mean recoveries were 96±7 %.

Keywords: Nitrite; Nitrate; Cell culture medium.

Introduction
Nitrite (NO$_2^-$) is a major oxidation product derived from nitrogen monoxide (NO) that is produced by a wide variety of cell types by nitric oxide synthases [1, 2]. NO is a free radical and has selective reactivity with other paramagnetic species, including ferrous or ferric iron in heme proteins and other radical species such as superoxide anion (O$_2^-$) resulting in production of nitrite [3]. In vascular system, NO is rapidly oxidized by reaction with oxyhemoglobin (HbO$_2$) resulting in the formation of methemoglobin and nitrate (NO$_3^-$) [4].

In healthy human subjects, NO$_2^-$ can be detected at levels of 0.50-3.6 µM in plasma, ~15 µM in respiratory tract lining fluids, 30-210 µM in saliva, and 0.40-60 µM in gastric juice. Oral nitrite levels increased dramatically to near mM levels after ingestion of nitrate, because of nitrate reduction by the oral microflora. Although nitrite is a major end product of nitrogen monoxide metabolism, it doesn’t accumulate in vivo and rapidly oxidized to nitrate by oxyhemoglobin or oxymyoglobin to form methemoglobin or metmyoglobin and nitrate [4].

Numerous methods were reported for nitrite and nitrate analysis in cell culture medium extracts and biological fluids [5]. Methods based on HPLC separations with UV-VIS, electrochemical and fluorometric detections are more accepted due to

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elimination of matrix effects [8-10]. The most accepted method is based on pre-column derivatization of nitrite by 2,3-diaminonaphthalene (DAN) under acidic conditions to quantitatively yield the highly fluorescent 2,3-naphthotriazole (NAT) [9,10] (Fig. 1).

![Figure 1: Reaction of nitrite with 2,3-diaminonaphthalene (DAN) to form 2,3-naphthotriazole (NAT) under acidic conditions.](image)

Nitrate is usually reduced to nitrite before derivatization. The method was applied successfully for measurements of as low as 10 nM nitrite or nitrate up to 2.0 µM by monitoring fluorescence intensity of NAT [10]. Cell culture medium containing elevated concentrations are usually subjected to large dilutions, resulting in consumption of time and significant errors coming from backgrounds of nitrite or nitrate in distilled water (up to 0.10 µM) and cell culture medium (up to 0.30 µM) [9]. Thus, a more reliable method is required for determination of nitrite and nitrate in cell culture medium that extends the linear dynamic range, at least 10 folds, without affecting the sensitivity. By taking advantage of less sensitive UV-VIS absorbance of NAT, the linear dynamic range could be extended up to 50 µM.

The objective of this work therefore, was to develop a reliable analytical method for determination of nitrite and nitrate in cell culture medium extracts that could measure from nano-molar (nM) to tenth of micro-molar (µM) concentrations. Conditions were optimized to separate NAT from DAN and simultaneously monitor its UV-VIS absorbance and fluorescence intensity.

**Experimental**

The Shimadzu HPLC system (Columbia, Maryland, USA) consisted from a SCL-10AVP sample controller, two LC-ADVP solvent-delivery units, SIL10ADVP autosampler, SPD10AVP photodiode array detector (PDA), RF-10AXL fluorometric detector and a CLASS-VR® workstation. The software configures the HPLC system for complete system control and data handling.

Standard Dulbecco's modified Eagle's medium (DMEM) (SH30019.01) was obtained from Hyclone (Logan, Utah, USA). Modified DMEM containing 25 mM Hepes, 100 mM D-glucose, 100 units/mL penicilline, 100 µg/mL streptomycin, 0.25 µg/mL amphotericine and 10% FBS was prepared from standard DMEM in our laboratory before culture. HPLC-grade acetonitrile was obtained from Fisher Scientific (Houston, TX, USA). DAN, nitrate reductase, NADPH and other reagents were obtained from Sigma (St. Louis, MO, USA). Water was double distilled. Separations were performed.
by a 5- μm reversed phase C18 column (250X4.6 mm, I.D) guarded by a 10 μM reversed-phase C18 column (20X4.6 mm, I.D) both obtained from Supelco (Bellefonte, PA, USA). The mobile phase consisted of 65% of 15 mM potassium phosphate buffer (pH= 7.5) and 35% actonitrile and pumped isocratically at a flow rate of 1.00 mL/min. 30 μL of the derivatized nitrite-DAN solution was injected at ambient temperature. The fluorometric detector was set at λ (excitation)= 375 nm and λ(emission)= 415 nm. The PDA was set in the multi- wavelength mode, 300-400 nm.

Stock solutions of nitrite and nitrate were prepared by dissolving measured amounts from sodium nitrite and sodium nitrate in modified DMEM. Eight calibration standards 0.03-2.0 μM nitrite or nitrate and 0.5-50 μM nitrite or nitrate were prepared by spiking measured volumes of stock standards into modified DMEM. Calibration standards were employed for construction of calibration curves and validation studies. Calibration curves were generated by fitting absolute peak areas obtained from each detector versus concentration to linear least squares method.

For HPLC analysis of nitrite, 100 μL of nitrite calibration standard in DMEM were incubated with 10 μL of freshly prepared DAN (316 μM in 0.62 M HCl) at room temperature for ten minutes followed by addition of 30 μL distilled water and 5 μL of 2.8 M NaOH. Then 30 μL of the resulting mixture were injected into the HPLC.

For HPLC analysis of nitrate, 100 μL of nitrate calibration standard in DMEM were incubated with 10 μL of 1 unit/mL nitrate reductase and 10 μL of 120 μM NADPH for 1 h to quantitatively convert nitrate to nitrite. Afterwards the same procedure of nitrite was followed.

**Results and Discussion**

**Method optimization**

Small variations of mobile phase properties did not have significant changes on retention times or peak areas, so the optimized reported conditions were employed [9].

Firstly, for UV-VIS detection, the multi-wavelength mode (300-400 nm) was employed. Neither DAN nor NAT has interfering peaks in blank culture medium. When a calibration standard (20 μM nitrite) was analyzed, DAN and NAT peaks were not baseline separated (Figure 2A). When this multi-wavelength chromatogram was processed at 369 nm (Figure 2B) both peaks were baseline separated with much higher intensity of NAT over DAN. Thus, this wavelength was selected for further analysis. The importance of the multi-wavelength chromatogram is to check the freshness of DAN solution and in correlating some endogenous peaks eluting before DAN and NAT to composition of cell culture medium. Additionally, if a sudden interference occurred at 369 nm, the multi-wavelength chromatogram could be processed at another wavelength without the need to repeat the whole experiment.
Figure 2: Typical HPLC chromatograms for: A) nitrite analysis (20.0 µM) in cell culture medium, UV-VIS multi-wavelength detection (300-400 nm); B) chromatogram A processed at 369 nm. Retention times: DAN (11.1 min) and NAT (12.4 min).

Figure 3 shows typical chromatograms for analysis of nitrite and nitrate by the developed method. Figure 3A presents a typical chromatogram for nitrite in blank cell culture medium by fluorescence detection (left) and by UV-VIS detection (right). Nitrite was not detected by either method. Figure 3B shows a typical chromatogram for nitrate in blank cell culture medium by fluorescence detection (left) and by UV-VIS detection (right). Blank cell culture medium contains about 0.30 µM nitrate which corresponds to about 15 % of the linear dynamic range of fluorescence detection method. However, this background concentration is just detected by UV-VIS detection method. Figure 3C presents a typical chromatogram for 1.0 µM nitrite in cell culture medium by fluorescence method (left) and for 5.0 µM nitrate in cell culture medium by UV-VIS detection method (right). All chromatograms show baseline resolution between DAN and NAT.
Figure 3: Typical HPLC chromatograms for A) nitrite analysis in blank cell culture medium; A (left): with fluorescence detection and A (right): with UV-VIS detection. B) Nitrate analysis in blank cell culture medium; B (left): with fluorescence detection and B (right): with UV-VIS detection. C) Same as B, but C (left): 1.00 µM nitrate in cell culture medium and C (right): 5.0 µM nitrate in cell culture medium. Retention times: DAN (11.1 min) and NAT (12.4 min).

For analysis of nitrite and nitrate in cell culture extracts (cell culture medium after the end of experiment and removal of cells); two injections are required. The first injection is from nitrite procedure and the second one is from nitrate procedure. From each injection two chromatograms are simultaneously obtained; the UV-VIS and fluorescence chromatograms. NAT signals from first injection correspond to nitrite concentration, while NAT signals from second injection correspond to total nitrite and nitrate concentrations. If total nitrite and nitrate concentrations are less than 2.0 µM, both fluorescence chromatograms are employed for quantitation. If nitrite is higher than 2.0 µM both UV-VIS chromatograms are employed for quantitation. Otherwise,
fluorescence chromatogram from the first injection is employed for nitrite quantitation and UV-VIS chromatogram from second run is employed for nitrate quantitation.

**Method Validation**

Nitrite and nitrate standards in cell culture medium were employed in constructing calibration curves, while fortified standards in aqueous solutions and cell culture medium were employed in recovery studies. The fluorescence detection method was linear over the concentration range 0.03-2.0 µM for both nitrite and nitrate and the UV-VIS detection method was linear over the range 0.5-50.0 µM. Statistical results are presented in table 1. To make fair comparison between the two detection methods, we set the peak area of the highest concentration in each detection method to 100. The fluorescence method is more sensitive; however the UV-VIS method has larger dynamic range. For each method there is no significant difference between nitrite and nitrate statistical results, implying a quantitative conversion of nitrite to nitrate by nitrate reductase. Uncertainties of slopes are lower than 5% and correlation coefficients better than 0.994. Intercepts ± S.D for nitrite for both methods include zero implying that blank cell culture medium does not contain significant amounts of nitrite. Uncertainties of intercepts for nitrate by both methods are less than 13% (blank cell culture medium contains about 0.30 µM nitrate). Lower limits of quantitation (LOQs) were 0.03 µM for both nitrite and nitrate by the fluorescence method and 0.50 µM for the UV methods. Recovery results for three fortified concentrations are presented in table 2. Recoveries vary between 93% and 104% with average uncertainty of 7%.

**Table 1. Regression results for nitrite and nitrate by fluorescence and UV-Vis Detection**

<table>
<thead>
<tr>
<th>Detection</th>
<th>Slope (S.D)</th>
<th>Intercept (S.D)</th>
<th>Correlation Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescence a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrite</td>
<td>42.5 (1.8)</td>
<td>1.2 (1.4)</td>
<td>0.998</td>
</tr>
<tr>
<td>Nitrate</td>
<td>41.9 (1.7)</td>
<td>15.2 (1.8)</td>
<td>0.996</td>
</tr>
<tr>
<td>UV-VIS b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrite</td>
<td>0.85 (0.02)</td>
<td>0.02 (0.03)</td>
<td>0.995</td>
</tr>
<tr>
<td>Nitrate</td>
<td>0.84 (0.04)</td>
<td>0.24 (0.03)</td>
<td>0.994</td>
</tr>
</tbody>
</table>

*Based on three replicates of seven concentrations in the range: a. 0.03-2.00 µM and b. 0.5-50 µM

**Table 2. Recovery results for nitrite and nitrate by fluorescence and UV-VIS detection a**

<table>
<thead>
<tr>
<th>Nitrite added (µM)</th>
<th>% Recovery (S.D)</th>
<th>Nitrate added (µM)</th>
<th>% Recovery</th>
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<tr>
<td>Fluorescence</td>
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<tr>
<td>0.05</td>
<td>93.6 (8.7)</td>
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<td>98.3 (11.3)</td>
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<tr>
<td>0.50</td>
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<tr>
<td>1.50</td>
<td>97.9 (3.8)</td>
<td>1.50</td>
<td>98.5 (4.8)</td>
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<tr>
<td>UV-VIS</td>
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<tr>
<td>1.0</td>
<td>106.2 (7.8)</td>
<td>1.0</td>
<td>104.3 (12.3)</td>
</tr>
</tbody>
</table>
Conclusions

Cell culture studies, dealing with nitric oxide production and consumption, involve culturing various types of cells and evaluating various types of drugs, enzymes, regulators and inhibitors. The cell culture extracts are unpredictable mixtures that may contain from few nano-molar (nM) to tenth micro-molar (µM) of nitrite and nitrate. Thus, the applied method should have adequate sensitivity and large dynamic range. The developed method is based on simultaneous fluorescence and UV-VIS monitoring of NAT. Fluorescence chromatograms are employed for low concentrations and UV chromatograms for high concentrations, without the need for further dilutions and background corrections.

This method was applied successfully in various cell culture studies \cite{11} and has the potential to be applied for measurements of nitrite and nitrate in biological fluids with minimum modifications.

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References

\cite{1} Tiskas, D., \textit{Free Rad. Res.}, 2005, 39, 797-781.