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Short communication

Evaluation of an LC–MS/MS assay for ^{15}N-nitrite for cellular studies of L-arginine action

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ABSTRACT

The utility of an LC–MS/MS assay for nitrite determination in studying L-arginine (ARG) cellular action was examined in vitro. EA.hy926 human endothelial cells or cellular fractions (membrane and cytosol) were exposed to 0–500 μM of $^{15}N_4$-ARG for 2 h. ^{14}N-nitrite and ^{15}N-nitrite in the cell lysate and in the incubation medium were derivatized with 2,3-diaminonaphthalene (DAN) to form ^{14}N- and ^{15}N-naphthotriazole (i.e., ^{14}N-NAT and ^{15}N-NAT). Peak responses of ^{14}N-NAT and ^{15}N-NAT were analyzed by LC–MS/MS with 1H-naphth[2,3-d]imidazole as an internal standard. The calibration curves of DAN-derivatized ^{14}N-NAT and ^{15}N-NAT from ^{14}N-nitrite and ^{15}N-nitrite were linear. Intra- and inter-day variability of the quantification was below 14.2% in quality control samples. Following incubation of EA.hy926 cells with $^{15}N_4$-ARG, saturable increases of ^{15}N-nitrite accumulation with increasing $^{15}N_4$-ARG exposure were observed clearly. This increase however could not be detected by the classical fluorescence method, nor were changes in ^{14}N-nitrite level observed. When cellular fractions were exposed to $^{15}N_4$-ARG, ^{15}N-nitrite formation was only observed in the membrane fragments. The sensitive and selective LC–MS/MS method reported here can be applied to quantify accumulated nitrite levels in human endothelial cells. The selectivity of this stable-isotope labeled LC–MS/MS method offers an advantage over other traditional methods for elucidating cellular ARG action when its stable isotope is employed as a substrate.

1. Introduction

Due to the pivotal role of nitric oxide (NO) in physiology, numerous analytical methods have been developed and used to quantify NO generation from the enzymatic reaction between nitric oxide synthase (NOS) and L-arginine (ARG). Direct measurement of NO in biological fluid is extremely difficult because NO is a short-lived molecule with an estimated in vivo half-life in human blood of 3–5 s [1]. Therefore, the oxidation end-products of NO, viz., inorganic nitrite and nitrate ions are measured in blood and urine, and their accumulation has been extensively used as an index of NO production. Various analytical approaches have been used for measuring the concentration of nitrite. These include a colorimetric assay based on the Griess reaction [2], chemiluminescence [3,4], fluorometric assay [5], high performance liquid chromatography [6] and gas chromatography–mass spectrometry [7]. The nitrate ion, which is a potential oxidation product of nitrite, is converted to nitrite either by reducing metals such as cadmium or by nitrate reductase prior to applying these methods. Despite the availability of these methods, reliable quantitative analysis of nitrite and nitrate as an index of NO production in biological samples remains an analytical challenge because of a number of confounding factors. First, the extent to which these ion concentrations represent quantitative NO production under diverse experimental conditions is unknown [8]. In addition, endogenous nitrite concentration, e.g., from dietary sources, are quite high (e.g., ~0.3 μM in human plasma [9,10]) which would make the measurement of nanomolar increases in additional nitrite production from NO impossible.

Some of these methodological difficulties may be alleviated, in principle, by measuring the stable isotope-labeled analogues of these ions, i.e., ^{15}N-nitrite and ^{15}N-nitrate, which are formed from ^{15}NO when ^{15}N-labeled ARG is used as an added substrate. With this approach, background correction is not necessary, and assay sensitivity can be greatly improved. ^{15}N-nitrite and ^{15}N-nitrate can be measured by GC–MS as their pentafluorobenzyl derivatives [11], or by a liquid chromatography–tandem mass spectrometric (LC–MS/MS) assay as a fluorescent 2,3-diaminonaphthalene (DAN) derivative [12]. These methods are anticipated to be superior because of their higher specificity and sensitivity in biological samples. However, the advantages of these methods over unlabeled nitrite determination in biological studies, if they exist, have not been examined. Here, we validated the LC–MS/MS method of ^{14}N-nitrite and ^{15}N-nitrite determination in cell samples, and examined

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their utility in estimating their production when exogenous ^{15}N-labeled ARG, i.e., $^{15}N_4$-ARG, was employed in studies with human endothelial cells.

2. Materials and methods

2.1. Chemicals and reagents

^{14}N-nitrite [as $NaNO_2$] and 2,3-diaminonaphthalene (DAN) were purchased from Sigma. ^{15}N-nitrite [as $NaNO_2$ (^{15}N, 98%+)] was purchased from Cambridge Isotope Laboratories, Inc. The internal standard, 1H-naphth[2,3-d]imidazole, was provided as a gift from Pfizer. These compounds were used without further purification. Cell culture reagents were purchased from Invitrogen.

2.2. In vitro cell study

EA.hy926 human vascular endothelial cells [13] were grown in a further modified Dulbecco's modified Eagle's medium (DMEM) containing 0.9 g/L of glucose and 21 mg/L of ARG supplemented with 10% fetal bovine serum, and 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a 5% CO_2 incubator. After cells were grown to confluence in 6-well plates for 7 days, they were washed twice with phosphate-buffered saline and equilibrated in Locke's solution (LS; 154 mM NaCl, 5.6 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM HEPES, 3.6 mM $NaHCO_3$ and 5.6 mM glucose) for 1 h. To prepare subcellular fractions, cells were harvested by adding 0.25% trypsin and centrifuged. The cell pellet was resuspended in LS and then sonicated for 2 min. After centrifugation at 300 × *g* for 5 min, the supernatant containing cytosol fraction was separated from the pellet containing membrane fraction. Different concentrations of $^{15}N_4$-ARG (0, 20, 50, 100, 200, and 500 μM) were added to the cells or the isolated cell fractions (i.e., membrane or cytosol). After 2 h, the cell incubation medium was collected and cells were lysed with lysis buffer. Protein concentrations in the cell lysates and cellular fractions were determined by Lowry assay [14].

2.3. Sample preparation

Stock solutions containing ^{14}N-nitrite and ^{15}N-nitrite were prepared in milli-Q water (Milli-Q Purification System, Millipore Corporation, Billerica, MA) and added to the cell lysate or the incubation medium, i.e., Locke's solution, to produce calibration standards. Quality control samples at 3 concentration levels were also prepared with calibration standards. ^{14}N-nitrite and ^{15}N-nitrite in the calibration standards or samples were first converted to ^{14}N-2,3-naphthotriazole (^{14}N-NAT) and ^{15}N-2,3-naphthotriazole (^{15}N-NAT), respectively, by using the procedure reported by Misko et al. [5]. A 100 μL aliquot of cell lysates and cell medium sample was incubated with 10 μL of 0.5 mM DAN in 0.5 M HCl for 10 min at room temperature, and the reaction was stopped by adding 10 μL of 1.4 M KOH. After proteins were precipitated by the addition of 100 μL acetonitrile, the samples were centrifuged at 13,000 × *g* for 5 min. An aliquot of 20 μL of the supernatant was then mixed with 10 μL of the internal standard, 5 ng/mL 1H-naphth[2,3-d]imidazole, and diluted with 170 μL of water and then 10 μL was injected to the LC–MS/MS.

2.4. LC–MS/MS conditions

The liquid chromatography consisted of a Shimadzu LC-20AD delivery pump, SIL-20AC autosampler, and CBM-20A system controller (Shimadzu Scientific Instruments, Columbia, MD). Chromatographic separation was accomplished on Agilent XDB-C18, 2 mm × 50 mm, 5 μm column, maintained at ambient temperature by a gradient elution. Mobile phase A consisted of 0.1% (v/v) formic acid in water and mobile phase B consisted of 0.1% (v/v) formic acid in acetonitrile. Elution was carried out at a flow rate of 0.3 mL/min, with a gradient ratio of 5% mobile phase B in mobile phase A, rising linearly to 95% mobile phase B in 4 min. $[M+H]^+$ ions were analyzed in the multiple reaction monitoring (MRM) mode of the ABI/Sciex *API3000* triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) equipped with an electrospray ion (ESI) source. The ion spray voltage was 5.5 kV, and the temperature was set at 350 °C. Nebulizer and curtain gas flow rates were 10 mL/min and 7.0 mL/min, respectively. Fragmentation took place at collision gas pressure of 5.0 mTorr. Each transition was monitored with 200 ms dwell time. The optimized declustering potential, focusing potential, collision energy, and collision cell exit potential were 40.0 V, 200 V, 35.0 eV, and 8.0 V, respectively for ^{14}N-NAT and ^{15}N-NAT. A declustering potential of 81.0 V, and a collision energy of 45.0 eV were selected for the IS. The observed MRM transitions were m/z 170.1 → 115.0 for ^{14}N-NAT, m/z 171.1 → 115.0 for ^{15}N-NAT, and m/z 169.2 → 115.0 for the IS.

2.5. Fluorometric assay

The Nitrate/Nitrite Fluorometric Assay Kit (Cayman, MI) was used to determine nitrite concentration in the incubation media and in the cell lysate samples following the method of Misko et al. [5].

2.6. Statistical analysis

Comparisons among different groups were performed using one-way ANOVA, followed by Tukey post hoc analysis. Differences with $p < 0.05$ were denoted as statistically significant.

3. Results

3.1. Assay development and validation

Fig. 1 shows the representative ion chromatograms of nitrite–DAN derivatives, i.e., ^{14}N-NAT and ^{15}N-NAT, and the internal standard. The retention times for ^{14}N-NAT, ^{15}N-NAT, and the internal standard were 3.42, 3.42, and 2.52 min, respectively. A complete chromatographic run took 7 min. The calibration curves of ^{14}N-nitrite and ^{15}N-nitrite were linear between 0.04–2 μM and 0.004–1 μM, respectively, with correlation coefficients >0.99 both in the incubation medium and in the cell lysates. While the slopes of the calibration curves were relatively similar, there were substantial differences of the *y*-intercept values between ^{14}N-NAT and ^{15}N-NAT. The negligible *y*-intercept values of ^{15}N-nitrite indicate the absence of ^{15}N-nitrite in these assay samples relative to ^{14}N-nitrite, which is present endogenously (Supplementary Fig. 1).

In the present study, the lower limit of quantification (LLOQ) was defined as the lowest standard on the calibration curve at which the analyte peak was at least 5 times the response relative to blank response, with a precision of ≤20% and an accuracy of 80–120% [15]. The LLOQ of ^{14}N-nitrite and ^{15}N-nitrite was determined as 0.04 and 0.004 μM, respectively, independent of the two matrices studied, i.e., EA.hy926 cell lysates and the incubation medium. The LLOQ of ^{15}N-nitrite observed here was comparable to the 0.005 μM reported for a matrix of rat liver S9 fraction, using LC–MS/MS [12]. The intra- and inter-day variability of the method was evaluated by using quality control samples and the results are summarized in Table 1. Satisfactory accuracies were obtained for ^{14}N-nitrite and ^{15}N-nitrite with intra- and inter-day bias less than 8.30% and 13.2%, respectively, over the concentration ranges studied. The intra- and inter-day precision for ^{14}N-nitrite ranged from 3.60 to 14.2% in the incubation medium and from 1.17 to 13.7% in the cell lysate.

Table 1
Intra- and inter-day variability of quality control samples for ^{14}N-nitrite and ^{15}N-nitrite. Accuracy is determined as the percentage deviation of calculated concentrations from the nominal concentrations. Precision is determined as the coefficient of variation (CV) of the 5 measurements.

Analyte	Matrix	Concentration (M)	Intraday ($n=5$)		Interday ($n=5$)	
			Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
^{14}N-nitrite	Incubation medium	1.6	−6.13	3.60	6.00	7.60
		0.8	2.22	8.30	−8.30	8.90
		0.1	−1.20	8.44	−5.26	14.2
	Cell lysate	1.6	−3.38	9.39	−0.50	7.86
		0.8	−3.16	1.17	−4.70	9.34
		0.1	−0.50	6.89	−5.98	13.7
^{15}N-nitrite	Incubation medium	0.8	−7.35	4.67	−1.38	9.49
		0.5	−3.28	9.22	−12.2	12.8
		0.01	−3.86	7.71	−4.88	6.88
	Cell lysate	0.8	−13.2	5.98	2.40	8.53
		0.5	3.52	6.24	−5.28	12.4
		0.01	−1.66	5.47	1.20	6.95

Correspondingly, these values for ^{15}N-nitrite were 4.67–12.8% and 5.47–12.4%, respectively. These ranges are within the criteria stated in the FDA guidance on bioanalytical method validation [15]. Ion suppression was assessed by the ratio of the analyte peak response in the presence of matrix ions to that in the absence of matrix ions. The peak responses of ^{14}N-nitrite and ^{15}N-nitrite in the presence of the incubation medium were 90.4 ± 7.4 and 92.1 ± 7.1%, respectively, compared to those in water ($n=4$). In the presence of cell lysate, the peak responses of ^{14}N-nitrite and ^{15}N-nitrite were 95.0 ± 4.7 and 96.9 ± 4.6% when compared to those in the water ($n=4$).

3.2. Application of the assay in cellular studies

Fig. 2 compares the total nitrite (^{14}N-nitrite + ^{15}N-nitrite) concentration measured by fluorometric assay vs. the ^{14}N-nitrite and ^{15}N-nitrite concentrations measured by the present LC–MS/MS assay in the incubation medium (panel A) and in the cell lysate (panel B) after EA.hy926 cells were incubated with 0–500 μM of $^{15}N_4$-ARG for 2 h. In the incubation medium, the total nitrite (^{14}N-nitrite + ^{15}N-nitrite) concentration was significantly increased with increasing $^{15}N_4$-ARG exposure concentration from 50 μM. When ^{14}N-nitrite and ^{15}N-nitrite concentrations were determined individually by LC–MS/MS assay, substantial variability was found in ^{14}N-nitrite levels and no significant changes in ^{14}N-nitrite was

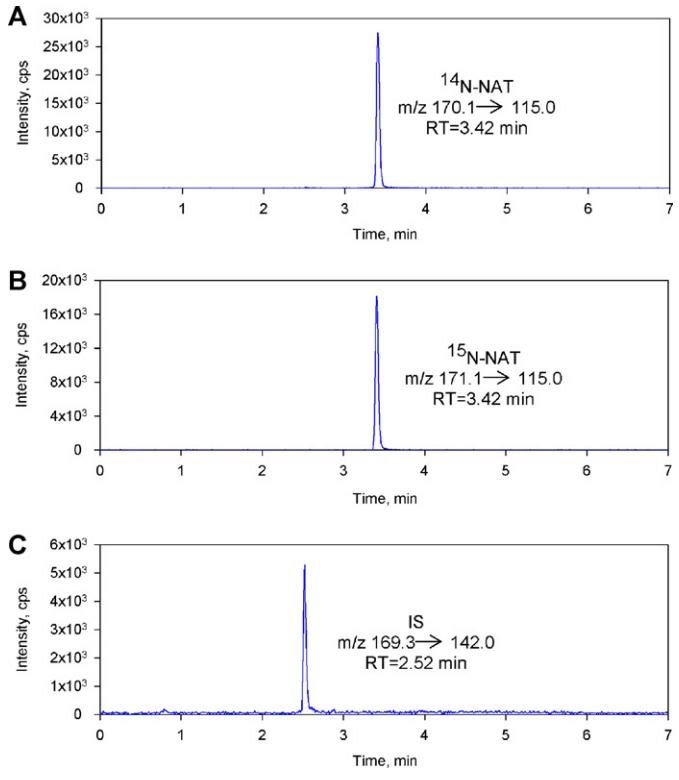

Fig. 1. Representative ion chromatograms of (A) ^{14}N-naphthotriazole (^{14}N-NAT), (B) ^{15}N-naphthotriazole (^{15}N-NAT), and (C) 1H-naphth[2,3-d]imidazole (IS).

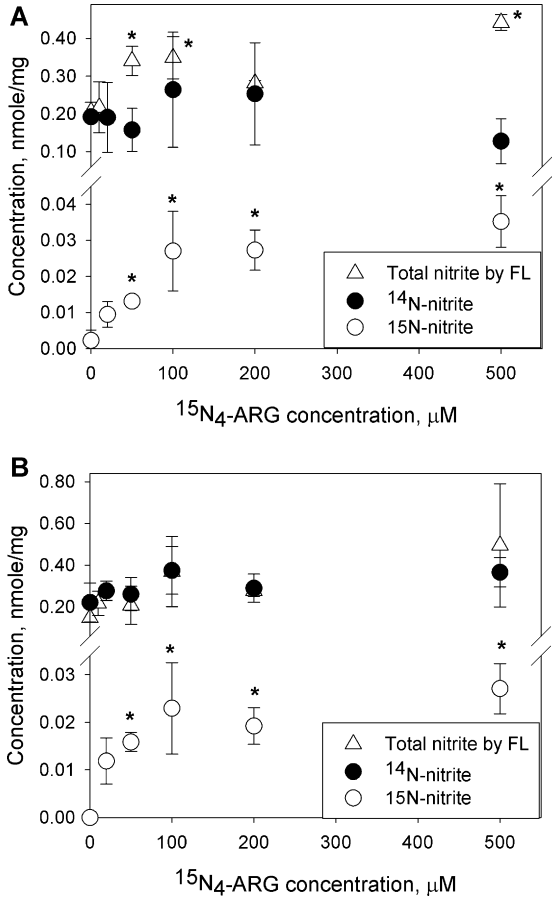

Fig. 2. ^{14}N-nitrite, ^{15}N-nitrite, and total nitrite (^{14}N-nitrite + ^{15}N-nitrite) accumulation in the incubation medium (panel A) and in the cell lysate (panel B) after EA.hy926 cells were exposed to $^{15}N_4$-ARG for 2 h. After determination by fluorescence assay, total nitrite was converted to concentration (in nmole/mg) based on the observed protein contents in the samples. Data are presented as mean ± SD ($n=6$ for ^{14}N-nitrite and ^{15}N-nitrite, $n=3$ for total nitrite by FL). *$p<0.05$ vs. control, i.e., $^{15}N_4$-ARG = 0 μM.

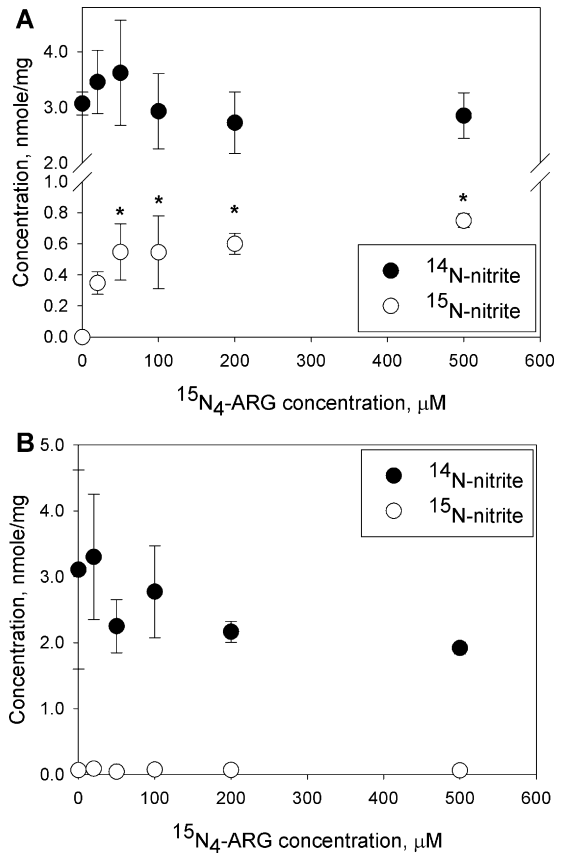

Fig. 3. ^{14}N-nitrite and ^{15}N-nitrite production after membrane fractions (panel A) or cytosol fractions (panel B) were exposed to $^{15}N_4$-ARG for 1 h. Nitrite was normalized to concentration unit as described. Data are presented as mean ± SD ($n = 3$). *$p < 0.05$ vs. control, i.e., $^{15}N_4$-ARG = 0 μM.

found. On the other hand, ^{15}N-nitrite concentration was approximately 10-folds lower than ^{14}N-nitrite concentration and showed a clear saturable increase with $^{15}N_4$-ARG exposure concentration. The maximum accumulated ^{15}N-nitrite concentration was observed from $^{15}N_4$-ARG exposure concentration above 100 μM (Fig. 2A). In the cell lysate, total nitrite and ^{14}N-nitrite concentration showed substantial variability and no significant changes were observed with increasing $^{15}N_4$-ARG exposure. Similar to that in the incubation medium, however, ^{15}N-nitrite concentration in the cell lysate showed a clear saturable increase with increasing $^{15}N_4$-ARG exposure concentration and much lower background level compared to those of total nitrite and ^{14}N-nitrite (Fig. 2B).

Fig. 3 shows the accumulation of ^{14}N-nitrite and ^{15}N-nitrite after cell membrane fragments or the cytosol fraction were incubated with 0–500 μM of $^{15}N_4$-ARG for 1 h. Similar to the whole cell study, no significant changes of the ^{14}N-nitrite in either membrane or cytosol fractions were observed after $^{15}N_4$-ARG exposure. On the other hand, ^{15}N-nitrite concentration was significantly elevated in the membrane fraction with increasing $^{15}N_4$-ARG exposure concentration (Fig. 3A). The maximum accumulated ^{15}N-nitrite concentration was observed from $^{15}N_4$-ARG exposure concentration above 50 μM. When cytosol fractions were analyzed, negligible levels of ^{15}N-nitrite were detected over the entire range of $^{15}N_4$-ARG exposure concentrations (Fig. 3B).

4. Discussion

Nitrite ion accumulation as a result of NO production through NOS enzymes has been traditionally measured by its reaction with DAN, to produce a highly fluorescent product, NAT. This method is capable of a detection limit of 0.01–0.02 μM for nitrite. The ability of this assay to detect minute changes in accumulated nitrite in cell systems however is questionable, particularly in view of the relatively high endogenous concentrations of nitrite ions in cells. Additionally, fluorescence quenching and interference by biological components may also confound nitrite quantification. In this assay, we used an LC–MS/MS method instead of fluorescence to quantify NAT. Besides having the obvious advantage of avoiding the problems associated with fluorescence detection (e.g., quenching), this approach allows the potential monitoring of all isotope forms of nitrite, e.g., ^{14}N-nitrite vs. ^{15}N-nitrite. Because ^{15}N-nitrite is absent endogenously, background contribution from ^{14}N-nitrite does not interfere with its determination, thus allowing a much higher sensitivity when ^{15}N-ARG is used as a substrate. The LLOQ of ^{15}N-nitrite found in this study was 0.004 μM, which is 10 times lower than that for ^{14}N-nitrite in the cell lysate as well as in the incubation medium. In comparison, the detection limits of ^{14}N-nitrite were 0.01 μM by an HPLC method [6], 0.01–0.02 μM by fluorometric assay [5], and 1–2 μM by the Griess colorimetric assay.

As an application of this method, we showed that it was possible to monitor $^{15}N_4$-ARG derived ^{15}N-nitrite accumulation selectively in the presence of high background level of ^{14}N-nitrite (Fig. 2). We observed that ^{15}N-nitrite accumulation occurs in the lower nanomolar ranges after $^{15}N_4$-ARG exposure, in the presence of approximately 10-fold higher concentrations of ^{14}N-nitrite in both the incubation medium and cell lysate samples. When total nitrite (^{14}N-nitrite + ^{15}N-nitrite) was measured by the fluorometric assay under the same experimental conditions, the changes in total nitrite concentration in the endothelial cells have been largely masked by high background levels. Our results showed that the estimated Km of NOS activity for $^{15}N_4$-ARG exposure concentration in EA.hy926 cells was 36.2 ± 9.8 μM, which is consistent with the reported value of 29 ± 6 μM in bovine aortic endothelial cells obtained by assaying radioactive conversion of ^{3}H-ARG to ^{3}H-citrulline [16]. Our results also indicated that NOS activity in the endothelial cells is associated with the cell membrane rather than with cytosol, consistent with previous findings [17]. Immunohistochemical studies with antibodies specific for caveolin, NOS and the ARG transporter (cationic amino acid transporter-1) demonstrated that these proteins co-localize in plasma membrane caveolae in porcine pulmonary artery endothelial cells [18].

The present method is specific for nitrite only. If nitrate concentration is of interest, it can be converted first to nitrite before applying this method. This approach is identical to that taken by other assays, such as the Griess reaction and fluorescent assays involving DAN or the diaminofluorescein derivatizing reagent. It has been suggested that nitrite rather than nitrate more appropriately reflects NOS activity [9,10], and the extent to which nitrite and nitrate represent quantitative NO production under diverse experimental conditions is unknown [8].

5. Conclusion

A sensitive and selective LC–MS/MS method was developed and applied to quantify ^{15}NO production when $^{15}N_4$-ARG was used in human endothelial cells. This stable-isotope labeled LC–MS/MS method offers an advantage over other traditional methods to elucidate NOS derived NO production resulting from exogenous ARG sources with higher sensitivity and selectivity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2011.08.017.

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