Developed Method Application for Nitrite Ion (NO_2^-) Analysis of Tib -186 Macrophage Like Cell Lines by Rapid Isocratic HPLC System with High Sensitive Glassy Carbon Electrochemical Detector

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ABSTRACT: *A rapid isocratic method of high performance liquid chromatography system (HPLC) with a glassy carbon working electrode of electrochemical detector is set up for quantitative detection of trace amount of nitrite ion (NO_2^-) in aqueous protein containing cell lysate, cell media, plasma, serum, urine and other body fluids. The solid extraction reversed phase cartridges (Sep-pak) are used for deproteinizing and purification of the samples. Nitrite ion is the only stable end product of autoxidation of nitric oxide (NO) ; which is a highly reactive paramagnetic molecule produced via the enzymatic conversion of L-arginine to L-citroline. The enzyme involved in this process is the inducible nitric oxide synthase (iNOS), the main isoform of the enzymes in macrophage and macrophage like cell lines such as Raw-264, J774, and Ic-21. Nitrite ion (NO_2^-) in nanomolar concentration range is measured by the ECD detector with an amperometric cell, applied voltage of + 800 mV and Ag-AgCl as the reference electrode. Elusion buffer is 8 mM ammonium chloride containing 25% methanol, flow rate of 1 ml/min and column temperature set at 20° C. The reproducibility of sample preparation and analysis had a coefficient of variance (c.v.) less than 10 % in the cell lysates and cell media of the Tib-186 cell lines. Therefore, this will be a reliable analytical method for the nitrite ion analysis under various conditions of cytokines, LPS, irradiation, or other chemical applications for evaluation of the probable over expression of the inducible nitric oxide synthase (iNOS) gene in these type of cells.*

KEY WORDS: *Isocratic, Glassy carbon electrode, Sep-pak cartridge, Inducible nitric oxide synthase (iNOS).*

INTRODUCTION

Biochemistry of NO

Nitric oxide (NO) is an endogenous mediator of numerous physiological process that range from regulation of cardiovascular function and neurotransmission to

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antipathogenic and tumoricidal responses [1, 3].

Nitric oxide is a highly reactive paramagnetic molecule with a biological half life of 1-10 s which is converted to the oxidation products of nitrite (NO_2^-) and nitrate (NO_2^-) ions depending on ambient redox condition [4]. It can be diffused to a target cell located nearby and hits its plasma memberane. Oxygene and NO closely resemble each other in diffusibility and fluid-phase membrane of cells are not diffusion barrier for (NO) [5]. In the presence of superoxide anion ($O^{\circ}_2^-$) at oxidation phosphorylation chain reactions of mitochondrial membranes, NO rapidly converts to very reactive and toxic peroxynitrite anion ($ONOO^-$), which then decomposes into highly reactive intermediates.

Nitric oxide ultimately exerts its biological effects either directly or through the other reactive nitrogen intermediates. Its interaction with various enzymes containing heme group, Fe-S or Zn-S clusters, sulphydryl groups or the other chemical substances, will distruct them (like the citric acid cycle enzyme of aconitase or ferrochelatase which catalyses the insertion of Fe into protoporphyrin) [6,7].

Nitric Oxide Analysis Methods

Three major nitric oxide synthase enzyme isoforms characterized up to date (endothellial eNOS, macrophage mNOS and neronal nNOS isoforms) produce small amount of nitric oxide [7]. In vivo at sufficient concentrations, of NO and its derivatives (like anion peroxinitrate ($ONOO^-$) have cytotoxic and mutagenic effects, so considerable research efforts has been devoted to develope more reliable and precise methods for its analysis[8]. Methods like chemiluminescence headspace-gas analysis[9], elecrochemical measurment of NO for in situ [10] and colorimetric methods based on reaction of NO with a reagent like 3,5-dibromo- 4 - nitrosobenzen-sulphonate (DBNBS) [10], are useful for different type of samlpes analysis. High performance liquid chromatography (HPLC) plays important role specially for ion analysis. New series of microbore anion exchange (HR) columns with high resolusion are the best choice for nitrite ion (NO_2^-) analysis. Deproteinizing the samples like cell lysate, body fluids and cell media will keep the columns more efficient and decreases the retention times of the injected samples.

MATERIALS AND METHODS

HPLC System

High performance liquid chromatography (HPLC) system Waters™ in isocratic mode with a pump model 510, U6K universal liquid chromatography injector with capacity of a microliter to 2 milliliter and analytical column of anion exchange HR 4.6 mm × 75mm are applied. The detection system was a Waters™ 460 Glassy carbon elecrochemical detector with a response sensitivity of nano molar (linear range of 5 to 500 nmolar of NO_2^- content, Fig. 1), temperature sensitive and an interface module (SIM) based workstation software of MAXIMA-820 for easily save and retrieve of data processingin isocratic mode with a polar elusion buffer of ammonium chloride with 25 % ethanol and the flow rate of 1 mL/min at 20° C have prepared the best condition of resolution with low retention time for the fast analysis.

Sep-Pak treatment of the samples

Samples such as cell lysates, urine and serum after pre-treatment (centrifuge,…), need more purification before injection to HPLC system, so applying Sep-Pak cartridges of reversed phase types are more suitable for this purposes [12]. There are two simple strategies for isolation and cleanup of sample components of interst: choosing cartridge type and sample solvent to cause first the component(s) of interest to be (a)- unretained while matrix interferences are absorbed or (b)- retained while matrix interferences pass through unretained. The first strategy for adsorption of matrix interferences of bio-logic samples, aqueous environmental drugs and drug metabolites in serum, plasma, or urine, peptides in serum or plasma, water soluble vitamines, is the best way of purification of the samples and unretaining the nitrite ions (NO_2^-) or probable nitrate ions (NO_3^-) in the medium of the cell lines like Tib-186. This is achived via the application of the Sep-pak C18 cartriedges which is eluted with high polar eluent buffer solution through the non-polar C_{18} as a packing material.

Materials

All chemicals used were of the highest purity (HPLC grade) available. Buffers prepared with deionized double distilled water, Sep-pak cartridges (PN 11188) purchased from Waters™(Division of MILLIPORE), $NaNO_2$ used as standard material.

Tib-186 Cell line

This cell line (IC-21) is derived by transformation of normal C57BL/6 mouse pretoneal macrophages with SV4011 [13]. This cell line shares many properties with normal mouse macrophages and display macrophage specific antigens.

They have phagocytic and cytolytic propertiese, can lyse tumor target in vitro and appear to be more differentiated than cells of the P388D1 macrophage line [14]. Like the other macrophage like cell lines the main expressed nitric oxide synthase enzyme is the inducible isoform (iNOS) of the enzyme which is Ca^{2+} and calmoduline independent type and is involved in the conversion of L-Arginine to nitric oxide (NO) and L-citroline [15].

Cell Subculturing

The monolayer rinsed with 10 to 15 ml of calcium, magnesium free phosphate buffer saline PBS ,then added additional 10 to 15 ml of the same solution. The culture stays for 5 to 10 minutes at room temperature, the flask is struck to dislodge cells, 5 to 7 ml of the cell suspension is added to a flask containing less than 10 ml of growth medium. Additional medium is added once the cells have attached after one to two days (ATCC, Ic-21). Sampling for NO ion analysis are taken a) from the cell medium after 70 to 80% confluence of the plates or b) cells are lysised by cell lysate buffer of 50 mM Tris. Hcl (pH = 8), 150 mM NaCl, 0.02%sodium azide, 0.1%SDS, 100 µg/mL PMSF, 1% NP - 40, 0.5% sodium deoxycholate, then 10 minutes incubation on dry ice, scrape the cells, chilled microfuge (12000g, 2 min, 4ºC), the supernatant is treated by Sep-Pak cartriedges for deproteinizing the samples [16]. Extra purification of the samples with 0.22 µm milipore microfilters make them ready for HPLC system injection**.**

RESULTS AND DISCUSSION

Numerous methods are developed for measurment of Nitrite ion (NO_2^-) as the stable end product of autoxidation of nitric oxide ions in aqueous solution [17,22]. The short half-life and low in- vivo concentration of nitric oxide reduces the practicality of these methods for its analysis in biological samples [23]. The difficulties inherent to quantitation of NO can be eliminated by measuring its stable metabolites in particular, nitrite

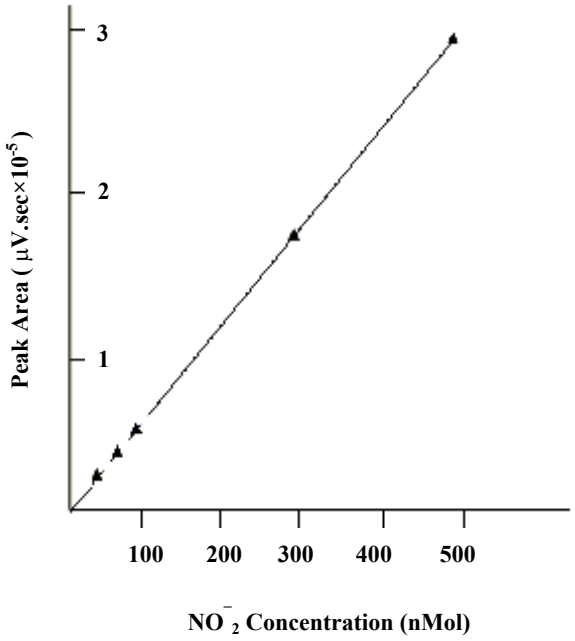


Fig. 1: NO_2^- concentration VS peak area. Various concentrations of NO_2^- (5 to 500 nmol) of NO^-2 ml were injected . Peak area (µV.Sec) was calculated by maxima-820 software of waters™ ECD-HPLC system .

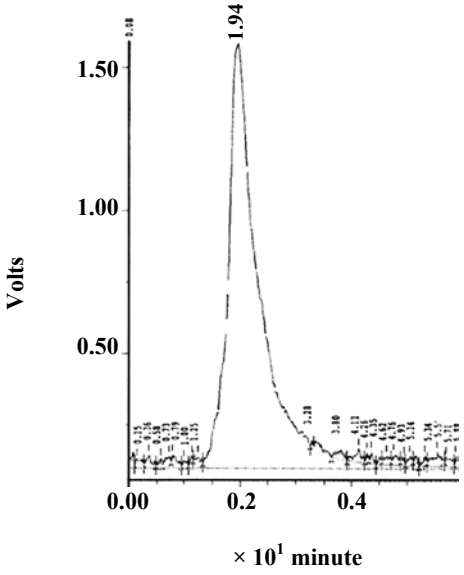


Fig.2: A sample chromatogram of nitrite ion (NO_2^-) analysis with IC-pak (HR) column, mobile phase (buffer of amonium chloride 8 mM with pH 5.8, 25% of methanol), +800 mV of applied voltage of electrochemical Detector, Flow rate 1mL / min, T =20 ºC .

(NO_2^-) and nitrate (NO_3^-) ion. The nitrate ion (NO_3^-) can be formed by reaction of NO with superoxide anion ($O^{\circ}{}_2^-$) or oxyhemoglubin [24]. Consequently plasma, serum and urine predominantly contain nitrate (NO^-_3),while significant amount of nitrite ion (NO_2^-) can be accumulated in non-heme containing fluids. The classic colorimetric Griess method [25] has limitation regarding both sensitivity (1~5μM), and inability to detect nitrate (NO_3^-) ions[26]. The detection limit of this assay can be enhanced by substitution of dapson for sulfanilamide followed by ultracentrifugation (linear to 0.2 μM of NO_2^-) [27] or by sequential addition of the reagents at 4ºC (25% increase in sensitivity) [28]. The second developed viable method which would be used for huge number of the samples without any extra treatment is the automation of Griess method in applying the flow injection analysis (FIA) system [29]. In early 1970'S this method developed for analysis of pharmaceuticals, food ingredients, and now is used for clinical purposes. Hemoglubine assay which is quantitative oxidation of oxyhemoglubin (HbO_2) to methemoglubin (metHb) in aqueous solutions due to nitric oxide, will have maximume absorption at 405 nm. The mixture of oxyHb and metHb has an isobestic point at 411nm. Using the ratio of absorption of 405 to 411 nm (A405/A411) compared with a calibration line, will give the $NO^{\cdot}$ concentration [30]. The oxyhemoglubine assay method has also the problem of the long procedure of oxyHb preparation and adjustment of the stecheometric reaction between oxyHb and nitric oxide ion. Using the high performance liquide chromatography, Leuenberger et al. (1980), detected authentically applied NO_2^- with a low detection limit [31]. Davenport and Johnson (1974) and Gerritse (1979) also determined NO_2^- by HPLC with an anion xchange column using a spectrophotometric flow-throw detector [32]. The detection limit of these methods were 0.01 mMol in the samples.

Quantitative determination of the trace nitrite ion (NO_2^-) as a stable ion of the enzymatic conversion of the L- arginine to L-citroline and nitric oxide NO from purified samples such as plasma, urine and cell lysates with optimized condition was achived in this method (Figs. 1,2). In non-treated macrophage and macrophage like cell lines by cytokines, lipopolysaccharides (LPS) or by the other chemicals the trace amount of nitric oxide ion (NO) produced by the inducible isoform of nitric oxide

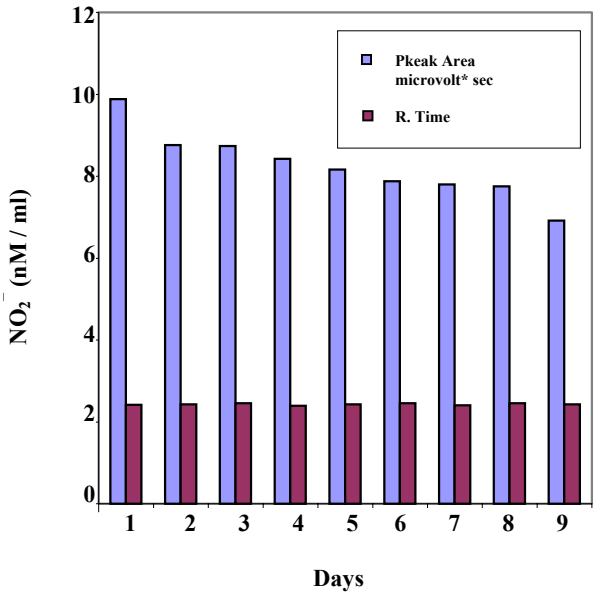
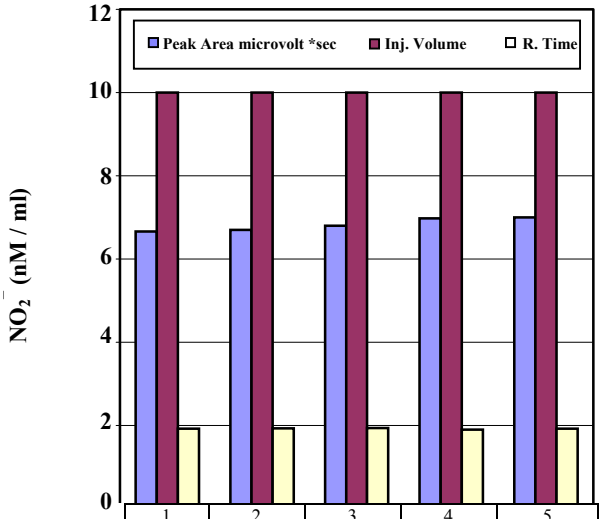


Fig. 3: Day to day analysis of nitrire ion (NO_2^-), showing the constant retention times but a decreased amount in nitrite (ion) concentration (about 1% per day), condition of the analysis is the same as Fig.1.



	1	2	3	4	5
Pkeat Area microvolt* sec	6.66	6.7	6.8	6.98	7
Lnj. Volume	10	10	10	10	10
R. Time	1.92	1.93	1.94	1.9	1.92

Fig.4: Demonstration of the Reproducibility of Analysis; with constant injected volumes (10 μL) and Concentration (10 nM / mL), shows the coefficient of variation less than 10 % in peak area and Retention times.

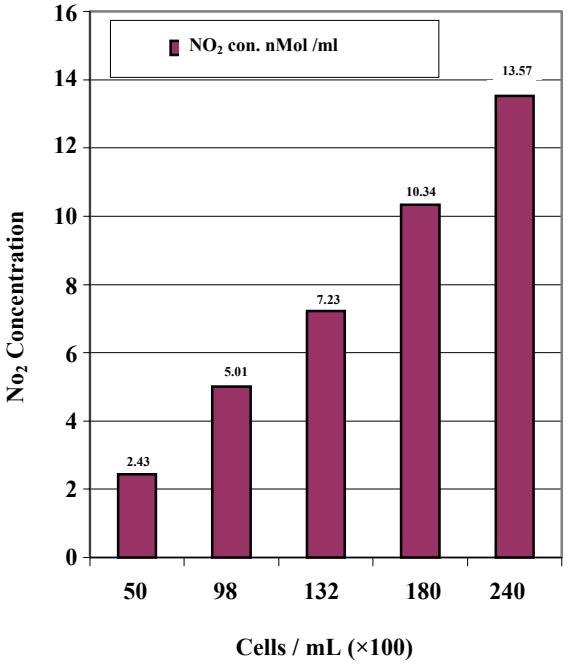


Fig. 5: NO_2^- assay in TIB-186 macrophage like cell line. Basal trace amount of nitric oxide anion in the stable nitrite anion (NO_2^-) form in the nanomolar concentration range is analysed with optimized conditions of the ECD- HPLC system.

synthase (iNOS or NOS2) need a reliable and high sensitive method for quantitative assay of this ion.

In our project we have applied these idea with some changes of the conditions; using Sep-pak cartridges, Ic-Pak anion HR high Resolution but short (75mm) column ,the ECD detector with a sensitive working electrode have made the analysis more precise and reproducible (Fig. 4).

The day to day analysis will cause about 1% decreasement per day in NO_2^- concentrations (Fig. 3). Applying Sep-pak cartidges for rapid deproteinizing of biological samples with isocratic mode of ECD-HPLC has prepared a best method of precise analysis system for nano-molar oncentration ranges of the nitrite ion (NO_2^-) in macrophage and macrophage like cell line of TIB-186 in treated condition (Fig.5).

CONCLUSIONS

The optimized applied voltage on working electrode (Glassy Carbon) and treatment of the samples by SEP-Pak cartridges, produce the best choice on nitrite (NO_2^-) ion analysis with high sensitivity (nanomolar range) and simple Isocratic HPLC systems. For precise and accurate assessment of the nitric oxide synthase enzymes activity which produce trace amount of the nitric oxide in biological systems and its changes under different conditions (IFN-γ, LPS and ... effects) this will be more reliable and also fast method of analysis.

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REFERENCES

[1] Moncada, S., Palmer, R.M.J. and Higgs, E.A., Nitric Oxide: Physiology, Pathophysiology and Pharmacology, *Pharmacol, Rev*., **43**, p. 109 (1991).

[2] Hibbs, J.B., Jr. Synthesis of Nitric Oxide from L-Arginine: A Recently Discovered Pathway Induced by Cytokines with Antitumour and a Imicrobial Activity, *Res Immunol*, **142**, p. 565 (1991).

[3] MacMicking, J., Xie, Q.W. and Nathan, C., Nitric Oxide and Macrophage Function, *Annu. Res. Immunol*, **15**, p. 323 (1997).

[4] Lancaster, J.R. Jr., Simulation of the Diffusion and Reaction of Endogenously Produced Nitric Oxide Proc, *Natl. Acad. Sci. USA*, **91**, p. 8137 (1994).

[5] Vanderkooi, J.M., Wright, W.W. and Erecinska, M., *Biochem. Biophys. Acta*, **1207**, p. 249 (1994).

[6] Kim, Y.M., Bergonia, H.A., Muller, C., Pitt, B.R., Watkins, W.D. and Lancaster, J.R., *J. Biol. Chem.* **270**, p. 5710 (1995).

[7] Knowles, R.G., Moncada, S., *Biochem. J.*, **298**, p. 249 (1994).

[8] Demiryurek, A.T., Cakici, I., Kanzik, I., *Pharmacol. Toxicol.*, **82**, p. 113 (1998).

[9] Demiryurek, A.T., Cakici, I., Kanzik, I., *Pharmacol. Toxicol.*, **82**, p. 113 (1998).

[10] Joshi, M.S., Lancaster, J.R., Liu, X. and Ferguson, T.B., *Nitric oxide, Biology and chemistry,* **5** (6), PP. 561-565 (2001).

[11] Michaeil, P. Murphy, Nitric Oxide and Cell Death, *Biochim, Biophys. Acta*, **1411**, p. 401 (1999).

[12] Waters SEP-PAK Cartridge, Care and Use Manual, PN 11188, Rev 4, september, (1990).

[13] Mauel, J., Defendi, V., Infection and Transformation of Mouse Peritoneal Macrophages by Simian Virus 40, *J. EXP. Med.*, **134**, p. 335 (1971).

[14] Mauel, J. and Defendi, V., Infection and Transformation of Mouse Peritoneal Mmacrophages by Simian Virus 40, *J. EXP. Med.*, **134**, p. 335 (1971) PubMed: 71255451.

[15] Marletta, et. al., Macrophage oxidation of L-Arginine to nitrite and nitrate, *Biochemistry*, **27**, p. 8706 (1988).

[16] Sambrook, J., Fritsch, E.F. and Mainatis, T., Molecular cloning, A laboratory manual, **13**, p. 18 (1998).

[17] Miles, AM., Wink, DA., Cook jc. and Gnsham, MB., Determination of nitric oxide using fluorsence spectroscopy, *Methods Enzymology*, **268**, p. 105 (1996).

[18] Brien, J.F., Mc Laughhin B.E., Chemiuluminescence head space-gas analysis for determination of nitric oxide formation in biological system, *Methods Enzymol*, **268**, p. 83 (1996).

[19] Nazhat, N.B., et. al., Nitric oxide in human plasma and synovial fluid using reaction of nitric oxide with 3,5-dibromo-4-nitrobenzene sulphonate, *BBA-General Subjects,* **1427** (2), pp. 276-286 (1999).

[20] Mesaros, S., Determination of nitric oxide saturated solution by amperometry on modified micro-electrode, *Methods Enzymol*, **301**, p. 160 (1999).

[21] Misko, T.P., Schilling, R.J., Salvemini, D.S. and Moore, W.M., A Fluorometric Assay for the Measurment of Nitrite in Biological Samples, *Anal. Biochem.*, **214**, p. 11 (1993).

[22] Balcioglu, A. and Watkins, CJ., Use of a Hemoglubin-trapping Approach in the Determination of Nitric Oxide in Vitro and in Vivo Systems, *Neurochem Res.*, **23** (5), p. 815, May. (1998).

[23] Xiaoping Liu., et. al., Diffusion Limited Reaction of Free Nitric Oxide with Erythrocytes J. B. C., *J. Biol. Chem.*, **273**, p. 18709, Jul. (1998).

[24] Awad, H.H. and Stanbury, D.M, Autoxidation of Nitric Oxide in Aqueous Solution, *Int. J. Chem. Kinet.* **25**, p. 375 (1993).

[25] Griess, j.p., Determination of Nitrite and Nitrate by Griess Reaction, *Ber. Deutsch Chem. Ges.*, **12**, p. 426, (1897).

[26] Green, L.C., wagner, D.A., Analysis of Nitrate, Nitrite and N^{15} Nitrate in Biological Fluids, *Anal. Biochem.*, **126**, p. 131 (1982).

[27] Marzin zig, M. et. al., Improved Methods to Measure and Products of NO in Biological Fluids, *Nitric oxide*, **1**, p. 177 (1997).

[28] Gueara,I., et. al., Determination of Nitrite/Nitrate in Hum an Biological Material by the Simple Griss Reaction, *Clin. Chim. Acta.*, **274**, p. 177 (1998).

[29] Schulz, K.,et. al., Reevaluation of the Griss Method for Determining. No/No^{-}_{2} in Aqueous and Protein – Containing Samples, *Nitric Oxide, Biology and Biochemistry* (1999).

[30] Kelm, M., Quantative and Kinetic Characterization of NO and EDRF Release from Cultured Endothelial Cells, *Biochem. Biophys. Res. Commun.*, **154**, p. 236 (1988).

[31] Leuenberger, R.G., et. al., Nitrite Ion Analysis, *J. Chromatography.*, **202**, p. 461, (1980).

[32] Davenport, R.J., et. al., Determination of Nitrate and Nitrite by Forced-Flow Liquid Chromatography with Electrochemical Detection, *Anal. Chem.*, **46**, (1971)