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A LAZAR LABORATORY HANDBOOK

MEASUREMENT OF NITRIC OXIDE IN BIOLOGICAL SAMPLES

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NITRIC OXIDE MEASUREMENT IN BIOLOGICAL SAMPLES AN OVERVIEW

Nitric oxide (NO), which is produced by nitric oxide synthases plays a major role in a variety of mammalian biological processes including blood pressure homeostasis, immune regulation, and nervous system signal transmission. The concentration of NO determines whether NO acts as a beneficial signal molecule or when overproduced acts to cause various stress symptoms due to the production of other nitric oxide species.

NO is very unstable and has a physiological half life of only 1 to 40 seconds and is only produced in picomolar to nanomolar quantities. It is very difficult to measure the production of NO directly due to its short half life and also due to the extremely low quantities of NO that are produced. In order to get around the problem of direct measurement of NO, analytical techniques have been developed which determine the stable end products of NO oxidation, namely NO_3^- and NO_2^- ions. NO_2^- is commonly measured using a Griess colorimetric assay. This technique usually requires the reduction of NO_3^- to NO_2^- using either chemical or enzymatic methods.

This booklet will take a brief look at the various methods, be direct or indirect, for the analysis of NO in biological fluids.

DIRECT ANALYSIS OF NO IN BIOLOGICAL FLUIDS

The direct measurement of NO in biological systems is very difficult due to the short half life of NO, the small quantities of NO produced, and the potential interferences from other components in the biological system. The direct analysis of NO can be divided up into two basic methods, namely chemiluminescence and electrochemical. These will be examined below.

1. Chemiluminescence or fluorometric methods for direct determination of NO concentration

Chemiluminescence can be defined as luminescence (light) produced by the direct transformation of chemical energy into light energy. Many researchers consider chemiluminscence to be the most accurate method for determination of small amounts of NO on a real time basis. The problems with this technique include the need of very expensive analytical instruments and the potential interference from other components found together with NO in the biological medium.

The fluorometric approach to direct NO analysis utilizes some sort of chemical reaction involving NO or its oxidation products (NO_2^- or NO_3^-) which converts a nonfluorescent precurser into a fluorescent product. The fluorescent product can then be measured using an spectrophotometric instrument which emits a light that excites the fluorescent product to emit light at a specific wavelength. The quantity of light emitted can be accurately measured and directly related to the concentration of the fluorescent product. The concentration of the fluorescent product is directly related to the concentration of NO or its oxidation products. Due to the potential interference from other chemical compounds found in the biological medium, purification of the fluorescent product often needs to be done prior to fluorescence measurement. As an example of a purification method, the collected fluorescent compound is purified using reverse phase HPLC. Fluorescence of the purified fluorescent product is then measured. The drawbacks of this method, besides requiring a relatively expensive HPLC system, is that it requires some fairly complex chemical reaction and purification steps, each one affecting the overall accuracy of the NO determination. Any reaction which does not go to completion or a faulty purification step may significantly affect the NO assay.

2. Electrochemical methods using amperometry

In the electrochemical method for the direct determination of NO in biological fluids, NO is either oxidized or reduced by imposing a constant or varying potential (voltage) on a small electrode and measuring the resultant current which is produced. The current is hopefully directly related to the concentration of NO in the sample. Micro electrodes for the electrochemical analysis of NO are made out of platinum and more recently from carbon formulations. The imposition of the

desired potential and the measurement of the resultant current requires a sophisticated (expensive) electrochemical analyzer which can measure electrical currents in the low picoampere range. Beyond the design of the electrochemical instruments, the measurement of such small electrical currents requires that all cables leading from the electrochemical instrument to the measurement electrodes be properly shielded and that any electrical noise in the vicinity of the test equipment be minimized. Sometimes the use of a Faraday cage is required in order to effectively eliminate electrical interference from outside sources. Occasionally, improper grounding can lead to ground loops in the test setup which may totally negate the accuracy of the current readings.

Standardization of the amperometric system is done by preparing NO standards by bubbling NO gas through an aqueous solution which had been carefully purified to remove any traces of oxygen. The purification process involves the use of O2 scavengers for removal of O2 from both the NO gas and the N2 gas used to purify the aqueous
solution. NO standards are prepared by diluting the original NO standard solution. Since NO gas is toxic to humans, the preparation of the standards has to be done using a functioning fume hood. Any trace quantities of oxygen which remain in the original NO standard or the diluted standards will lead to the oxidation of NO and the resultant lowering of accuracy of the standards and the electrochemical assay procedure.

Since the biological half life of NO is so short, the measurement of NO using either the chemiluminescense or electrochemical techniques requires a very rapid response time in order to insure that the NO is measured before it is oxidized.

INDIRECT ANALYSIS OF NO IN BIOLOGICAL FLUIDS

As mentioned earlier, the indirect methods of assaying O production measure the products of NO oxidation, namely NO2- and NO3-. These indirect methods have one feature in common which often makes them superior to the direct measurement of NO. NO2- and NO3- are relatively very stable compared to their NO precurser. Therefore speed of measurement is not nearly as important as the measurement of unstable NO.

1. Colorimetric determination of NO2- using the Greiss reagent

The Greiss method for assaying NO2- is the most common method for measuring NO oxidation products. In this method NO2- reacts with sulfanilic acid to give diazonium salt which then reacts with N-(naphthyl)ethylenediamine to give an azo dye whose absorbance at 550 nm is measured to give a quantitative determination of the NO2- ion present in the sample. Since the oxidation of NO produces both NO2- and NO3-, there is still the problem of determining NO3- ion. The most common methods for the analysis of NO oxidation products involve the reduction of NO3- ion to NO2- ion and the subsequent measurement of the total amount of NO2- present. The total NO2- present is then directly related to the original amount of NO present in the sample. NO3- can be reduced to NO2- in several different ways either using a bacterial enzyme, metallic reducing agents such as zinc or cadmium, or a chemical reducing agent such as hydrazine.

To insure that the Greiss method gives good analytical results two potential problems need to be considered. The first problem is to make sure that the reaction of NO2- with the Greiss reagents always gives consistant results for the amount of chromophore (azo dye) which is produced versus the amount of NO2- that is present in the sample. Obviously if the yield of chromophore varies with regard to the NO2- present then accuracy can also vary or be reduced. In some of the commercial kits available for NO2- analysis the two reagents in the production of the azo dye can either be mixed together in a single cocktail for reaction with NO2- or they can react with NO2- in a sequential manner. Time of reaction, degree of mixing, as well as temperature can effect the yield of the azo dye.

Another potential problem involves the reduction of NO3- to NO2-. Here the yield is again an important factor. If NO3- is not completely converted to NO2- then the resultant overall concentration of NO2- will be low giving erroneously low results for the original concentration of NO. The reducing agents or enzymes used to reduce NO3- to NO2- need to bring the reduction reaction to completion without going further and also possibly reducing the NO2- to other species which cannot be assayed with the Greiss reagent. The type, quantity, and surface area of the reducing agent used are all factors which can affect the final yield of NO2-. Other factors include time, mixing efficiency, temperature, and pH of the solution.

A final factor which can lower the accuracy of results using the Greiss reagent is the sample color prior to the Greiss reaction and sample turbidity. Both these sample characteristics could effect the subsequent colorimetric determination of the azo dye using a colorimeter or spectrophotometer and therefore affect the overall assay of the original NO present in the sample.

2. HPLC techniques for the measurement of NO3- and NO2-

Both reverse phase and ion exchange HPLC or IC (ion chromatography) methods have been used to analyze for NO3- and NO2-. HPLC detectors which have been used include UV-VIS absorbance, fluorescence, electrochemical, and conductivity. Electrochemical detectors are usually the most sensitive but suffer from significant interferences from other substances found in the samples. Procedures used to remove potential interferences may lead to other problems such as incomplete separations and low recoveries. Furthermore, the HPLC approach is relatively expensive for both the HPLC system itself and the specialized HPLC columns required for the analysis.

3. Direct measurement of NO3- and NO2- using micro ion selective electrodes

Ion selective electrodes have been used for over two decades to measure various ions in solution. An ion electrode for NO3- has been available for some time which can detect NO3- down to the low microMolar concentrations. The nitrate ion selective electrode can be used over a wide pH range (2 to 11) and has very few interferences coming from biological samples. More recently an NO2- electrode has been developed which can measure NO2- ions in solution with a detection limit also down to the low microMolar values. The nitrite ion selective has a somewhat more limited pH range (4.4 to 8) which is applicable to most biological samples. Interferences from biological solutions are also not usually a problem for this electrode.

The primary problem for the use of ion selective electrodes in biological fluids has been a matter of size. Up until recently ion selective electrodes have been too large to measure biological solutions below 20 to 30 milliliters of solution. Recently Lazar Research Laboratories, Inc. has developed a series of micro ion electrodes with tip diameters down to 1 to 3 mm which can be used in very small biological solutions contained in 96 well plates, microcentrifuge tubes, and micro sample vials. With these micro ion electrodes samples as small as 10 microliters can be measured.

Electrodes for both NO3- and NO2- are now available in two micro forms for use in the assay of NO in biological systems. The first configuration is a micro tip probe which can be dipped directly into the biological medium. Another configuration is a micro flow electrode where a micro biological sample can be injected into the electrode using a micro syringe. Both configurations can measure both NO3- and NO2- down to the low microMolar concentrations in samples

between 10 to 50 microliters.

An advantage of the micro ion electrodes in assaying for NO is that the results are not affected by yields of either the NO3- to NO2- reaction and also not affected by yield of the Griess reaction or sample color or turbidity when the absorbance of the chromophore is measured. Overall the ion selective method is a much simpler approach which potentially increases the accuracy of measurement by avoiding any intermediate chemical reaction steps. Furthermore, this method is much less labor intensive than the other methods described above.

A final advantage of the dip micro ion electrodes for both NO2- and NO3- is that they can continuously measure changes in the concentration of either ion. This method is superior to removing aliquotes from the sample for remote measurement.

The ion selective approach to measuring NO concentration involves the use of the ion electrodes for NO2- and NO3- in conjunction with an ion analyzer which is connected to a PC using an RS232 port. The price of the whole system is a fraction of the spectrophotometric and liquid chromatography systems described above.

D. Micro ion analyzer for measuring NO in biological samples

Nitric oxide (NO) plays a major role in a variety of mammalian biological processes including blood pressure homeostasis, immune regulation, and nervous system signal transmission. NO is very unstable and has a physiological half life of only 1 to 40 seconds. Even though there are electrochemical (amperometric) methods for detecting NO, its rapid degradation to other nitrogen oxide compounds makes it difficult to determine quantitatively with a high degree of accuracy. The amperometric method for assay of NO is expensive, time consuming, and operator dependent. NO oxidizes into two stable end products, namely nitrite (NO2-) and nitrate (NO3-) ions. The concentration of these two end products can be used to quantify NO production without the measurement problems caused by the transient nature of NO.

There are various methods for quantitatively determining the concentration of both NO2- and NO3- ions. The most common methods involved the use of the Greiss reagent which reacts with NO2- ion to produce a stable azo end product which is purple in color and can be quantified using colorimeteric or spectrophotometric analytical techniques. There are are three basic problems with this colorimetric technique. First of all, the Greiss reagent only reacts with the NO2- compound thereby requiring an additional difficult step to completely reduce NO3- ion to NO2-. Secondly, like with all colorimetric methods, the Greiss technique can be affected by original sample color or turbidity. Thirdly, this method is very time consuming and tedious because it involved several intermediate chemical reactions.

Recently a potentially more accurate, less time consuming, and less tedious method has been developed for assaying both NO2- and NO3- which relies on the use of ion selective electrodes for both NO2- and NO3- ions. These electrodes have been miniaturized to be able to measure samples down to 30 to 40 microliters with detection limits down to the low microMolar range for both species. Since both NO2- and NO3- ions can be measured individually no reaction to reduce NO3- to NO2- is required. This micro electrode system comes complete with a sophisticated ion analyzer which attaches via RS232 port to a PC or laptop to give accurate concentration measurements for both ions.

FEATURES

- Measure both NO2- and NO3- in microliter samples
- Detection limits for both NO2- and NO3- down to low microMolar values
- Increase accuracy by avoiding colorimetric methods that can be affected by sample color or turbidity
- Avoid the tedious and time consuming step of converting NO3- to NO2-
- Save time and bother
- Significant cost savings over amperometric electrochemical methods
- Easily connects to PC or laptop

SPECIFICATIONS

Nitrite micro ion electrode
electrode size: 6.0 mm diameter x 70 mm length
Lower detection limit: 5 microMolar
Temperature range: 0 to 50 °C
pH range: 4 to 8
Response time: 25 seconds

Nitrate micro ion electrode
electrode size: 6.0 mm diameter x 70 mm length
Lower detection limit: 7 microMolar
Temperature range: 0 to 50 ºC
pH range: 2 to 11
Response time: 25 seconds

ORDERING INFORMATION

Model ISM-146NOXM NO3- and NO2- measurement system. Includes NO2- and NO3-micro ion electrodes, micro reference electrode, electronic ionanalyzer system, software program, RS232 connector cable to PC or laptop