

# Measurement of 3-Nitrotyrosine by High Performance Liquid Chromatography

## YÜKSEK BASINÇLI SIVI KROMATOĞRAFİSİ İLE 3-NİTROTİROZİN ÖLÇÜMÜ

Halil YAMAN\*, Ali ÜNLÜ\*, Ufuk KARABIÇAK\*\*, Behzat ÇİMEN\*\*, Barbaros BALABANLI\*\*, Mehmet Kemal ERBİL\*, Nurten TORKÖZKAN\*\*

\* Dept. of Biochemistry, Gülhane Military Medical Academy,

\*\* Dept. of Biochemistry, Medical School of Gazi University, Ankara, TURKEY

### Summary

Several methods including immunohistochemical, spectrophotometric and GC-MS have been performed to determine 3-nitrotyrosine level in soluble fractions. In this study, 3-nitrotyrosine level was measured by sensitive and simple isocratic-high performance liquid chromatographic method (HPLC) using UV detector after addition of peroxynitrite (1-4mM) to plasma from healthy donors. The detection limit of the method was 0.1 pM. 3-nitrotyrosine could not be detected in the plasma of healthy person. When we treated normal plasma with peroxynitrite, 3-nitrotyrosine peak could be detected dependent on the concentration of peroxynitrite.

Since 3-nitrotyrosine is a stable product of peroxynitrite (an oxidant formed by a reaction of nitric oxide and superoxide radicals), the measurement of its plasma concentration may be useful as a marker of nitric oxide-dependent oxidative damage.

**Key Words:** Peroxynitrite, 3-nitrotyrosine, High performance liquid chromatography

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Nitric oxide (NO), a diffusible gas with a short half-life, is produced by endothelial cells, macrophages and neurons (1,2) and involved in a wide spectrum of activities such as, smooth muscle relaxation, inhibition of platelet activation, neurotransmission (3). However, excess NO<sup>•</sup> can exert cytotoxic and cytostatic effects. These may involve both direct toxicity, e.g. by reaction of NO<sup>•</sup> with other free radicals (4).

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**Correspondence:** Halil YAMAN  
Dept. of Biochemistry  
Gulhane Military Medical Academy  
Ankara, TURKEY

### Özet

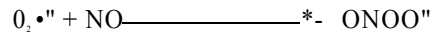
İmmunohistokimyasal, spektrofotometrik ve GC-MS gibi değişik metodlar sulu fraksiyonlardaki 3-nitrotirozin seviyesinin tayininde kullanılmıştır. Bu çalışmada sağlıklı donörlerden elde edilen plazmaya peroksinitrit ilavesinden sonra UV detektör kullanılarak hassas ve basit isokratik HPLC yöntemiyle 3-nitrotirozin seviyesi ölçülmüştür. Bu yöntemin hassasiyet sınırı 0.1 pM'dir. Sağlıklı plazmada 3-nitrotirozin seviyesi tespit edilememiştir. Ancak normal şahıslardan elde edilen plazma, peroksinitrit ile muamele edildikten sonra, 3-nitrotirozin piki, peroksinitrin konsantrasyonuna bağlı olarak tespit edilebilmiştir.

3-nitrotirozin, peroksinitritin (nitrik oksit ve superoksit radikalinin reaksiyonuyla oluşan bir oksidan) stabil bir ürünü olduğundan, plazma konsantrasyonunun ölçümü nitrik oksit aracılı oksidatif hasarın bir belirleyicisi olarak kullanılabilir.

**Anahtar Kelimeler:** Peroksinitrit, 3-nitrotirozin, Yüksek basınçlı sıvı kromatografisi

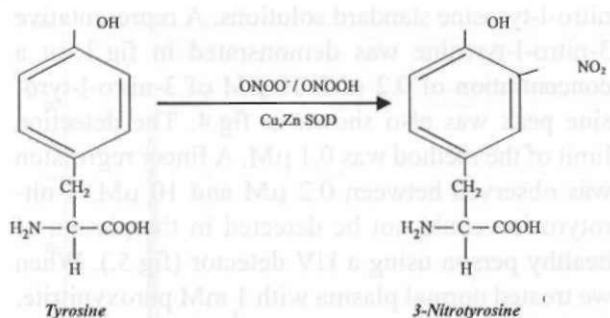
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Excess NO<sup>•</sup> reacts with O<sub>2</sub><sup>•-</sup> to give peroxynitrite (5).

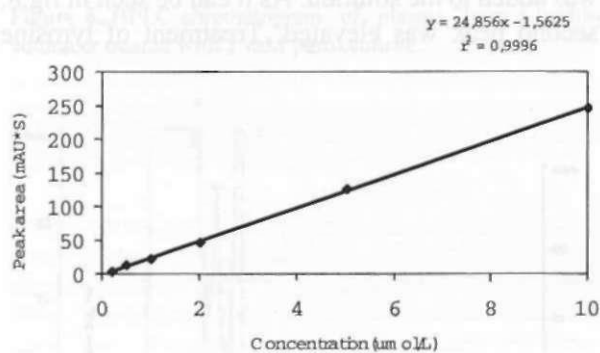


Peroxynitrite can be directly cytotoxic and it can also decompose to give a range of products, including hydroxyl radicals (OH<sup>•</sup>), nitrogen dioxide (NO<sub>2</sub><sup>•</sup>) and nitronium ion (NO<sub>2</sub><sup>+</sup>). NO<sub>2</sub><sup>•</sup> is a species with well described ability to cause oxidative damage and OH<sup>•</sup> is one of the most reactive free radicals (6).

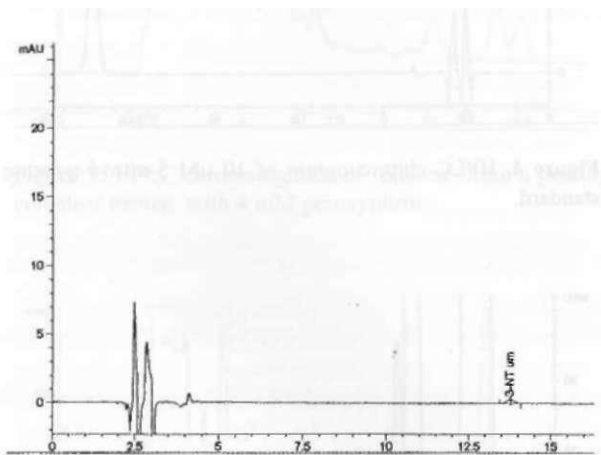
Peroxynitrite and peroxynitrous acid (ONOOH) are able to oxidize biomolecules such as, deoxyribose, lipids, methionine and thiols (5). However, the reaction of peroxynitrite with tyrosine yields 3-nitrotyrosine, which appears to be a



**Figure 1.** Peroxynitrite nitration of tyrosine catalyzed by Cu, Zn-SOD.



**Figure 2.** Standard curve of 3-nitrotyrosine.



**Figure 3.** MPLC chromatogram of 0.2 µM 3-nitro-L-tyrosine standard.

stable and specific footprint. Nitration of the 3-position (ortho) of tyrosine by peroxynitrite occurs spontaneously, but is also catalyzed by transition metals such as iron (III) and copper (II) as well as the copper-containing protein superoxide dismutase (SOD) (Fig.1) (7).

The determination of 3-nitrotyrosine may reflect the degree of peroxynitrite-dependent tissue damage, since it is difficult to demonstrate the other oxidative products of peroxynitrite (3).

Determination of 3-nitrotyrosine was described by various methods such as, spectrophotometric, immunohistochemical, GC-MS and HPLC. However, the most sensitive method has been suggested to be HPLC.

In this study, we aimed to measure 3-nitrotyrosine level in plasma using HPLC with UV detector after addition of peroxynitrite (1-4 mM) to plasma from healthy donors.

## Materials and Methods

### Reagents

D-tyrosine and 3-nitro-L-tyrosine were obtained from Sigma Chemical Co. (St. Louis, MO, USA).  $\text{H}_2\text{O}_2$ , sodium acetate (NaOAc), citrate, NaOH, manganese dioxide ( $\text{MnO}_2$ ),  $\text{H}_3\text{PO}_4$ ,  $\text{KH}_2\text{PO}_4$  and  $\text{K}_2\text{HPO}_4$  were purchased from Merck Chemical Co. (Germany). All organic solvents were HPLC grade.

### Peroxynitrite Synthesis

An aqueous solution of 0.6 M sodium nitrite was rapidly mixed with an equal volume of 0.6 M  $\text{H}_2\text{O}_2$ , containing 0.7 M HCl and immediately quenched with the same amount of 12 M NaOH. All reactions were performed on ice. Excess  $\text{H}_2\text{O}_2$  was removed by addition of  $\text{MnO}_2$  powder to the peroxynitrite solution. The mixture was shaken for five minutes and then  $\text{MnO}_2$  was removed by passage over a cellulose disposable filter. Solutions were used freshly or kept frozen at  $-20^\circ\text{C}$  for a week. The final concentration of peroxynitrite was determined spectrophotometrically in 12 M NaOH ( $\epsilon_{302} = 1670 \text{ M}^{-1} \text{ cm}^{-1}$ ). Dilutions of this peroxynitrite stock solution were made in 12 M NaOH to give final dilution of 0.1 M NaOH before each use (8).

### Incubation with Peroxynitrite

Blood samples were drawn out from normolipidaemic volunteers (aged 20-22 years) into heparinized syringes and plasma was separated after centrifugation for ten minutes at  $750 \times g$  a.v. In the study several plasma samples from different donors were used. Aliquots (5ml) of freshly isolated plas-

ma were incubated in test tubes at 37°C. After five minutes of preincubation, 40 µl peroxy-nitrite was added from the solution (1-4 mM) and was rapidly vortexed. Plasma pH was measured after each incubation and found concentration of 1 mM (from  $7.9 \pm 0.1$  to  $8.2 \pm 0.2$ ). When higher concentrations used, plasma was diluted with 50 mM  $\text{KH}_2\text{PO}_4$ / $\text{K}_2\text{HPO}_4$  buffer, pH 7.4. Reaction mixtures were incubated 15 min at 37°C and plasma samples were assayed immediately after incubation with peroxy-nitrite for 3-nitrotyrosines (5).

### Measurement of tyrosine nitration

A stock concentration (10mM) of D-tyrosine was prepared in 10 ml by adding 8 ml of water to 250 µl of 10 % (w/v) KOH followed by 250 µl of 5 % phosphoric acid with 15 ml of water. 0.1 ml of tyrosine solution together with 0.1 ml of a solution of the compound to be tested was added to a glass test tube containing 0.795 ml of buffer (50 mM  $\text{K}_2\text{HPO}_4$ / $\text{KH}_2\text{PO}_4$ , pH 7.4) and incubated in a water bath at 37°C for 15 min. After incubation, peroxy-nitrite (typically 40 µl) was added to a final concentration of 1-4 mM, the tubes vortexed for 15 sec and incubated for a further 15 min. The pH was measured after the addition of peroxy-nitrite and found between 7.46-7.52 (9). We analyzed 3-nitrotyrosine contents in plasma proteins after acid-hydrolysis with 6 M HCl at 105°C for 24 h (2).

All samples were analyzed on an Hewlett Packard 1050 high performance liquid chromatography (HPLC). The analytical column with a 5 mm pore-size Allsphere ODS-2 C18 reverse-phase column was used (4.6 mm x 250 mm; Alltech, Deerfield, IL, USA.). Guard column was a 5 mm pore-size Spherisorb ODS-2 C18 cartridge (Alltech, Deerfield, IL, USA). The mobile phase was 50 µM NaOAc / 50 mM citrate / 8% (v/v) methanol, pH 3.1. HPLC analysis was performed under isocratic conditions at a flow rate of 1 ml min<sup>-1</sup> and UV detector set at 274 nm. The 3-nitrotyrosine detection was confirmed by spiking with standards. Peak areas of 3-nitrotyrosine were measured and concentrations were calculated from a standard curve.

### Results

As seen in fig.2 the standard curve of 3-nitrotyrosine was prepared using 0,2-10 µmol/L of 3-

nitro-l-tyrosine standard solutions. A representative 3-nitro-l-tyrosine was demonstrated in fig.3. at a concentration of 0.2 µM. 10 µM of 3-nitro-l-tyrosine peak was also shown in fig.4. The detection limit of the method was 0.1 µM. A linear regression was observed between 0.2 µM and 10 µM. 3-nitrotyrosine could not be detected in the plasma of healthy person using a UV detector (fig.5.). When we treated normal plasma with 1 mM peroxy-nitrite, 3-nitrotyrosine peak was detected (fig.6). The highest peak was obtained when peroxy-nitrite dose was increased 4 mM (fig.7.). In order to identify the 3-nitrotyrosine peak, exogenous 3-nitro-l-tyrosine was added to the solution. As it can be seen in fig.8, second peak was elevated. Treatment of tyrosine

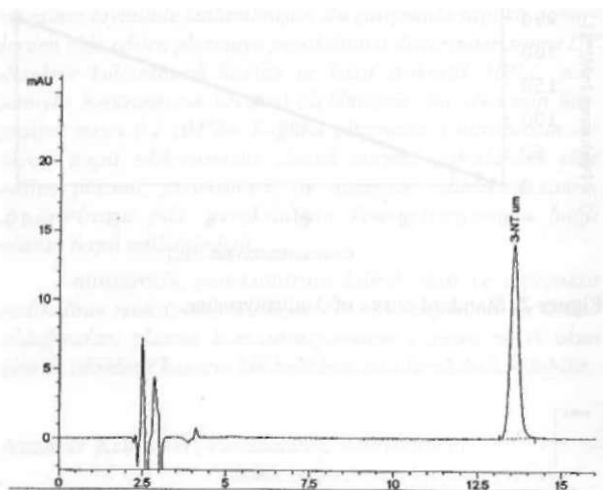


Figure 4. HPLC chromatogram of 10 µM 3-nitro-l-tyrosine standard.

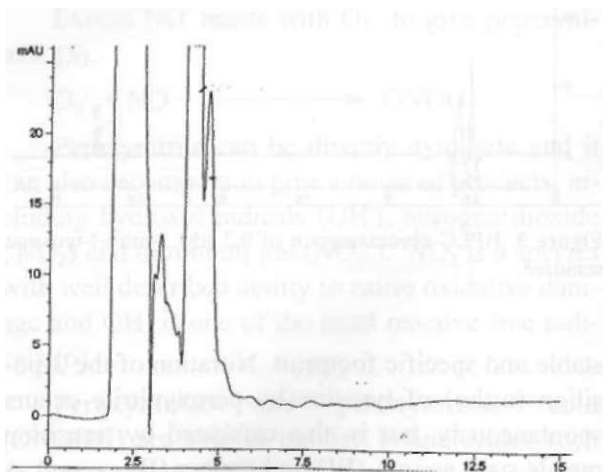
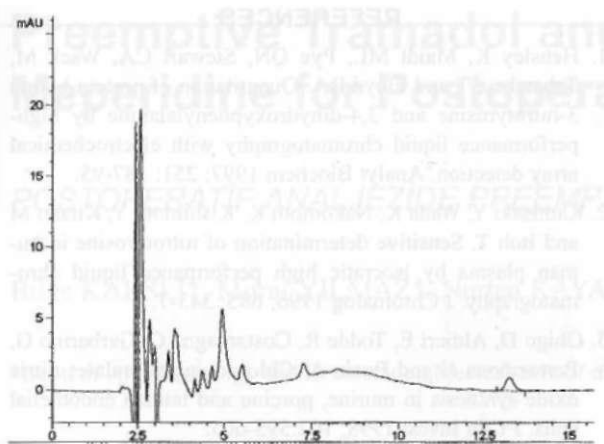
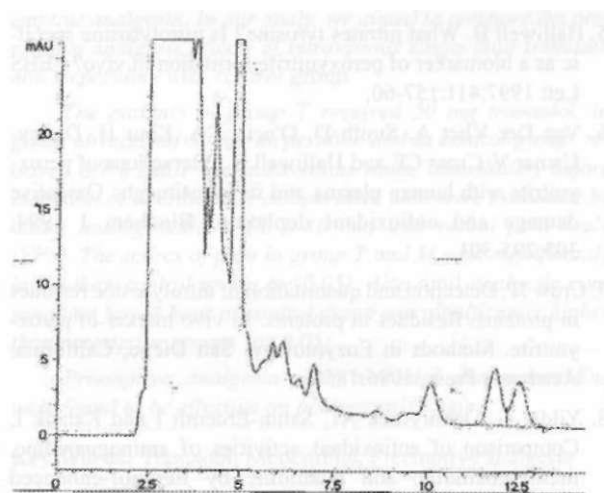


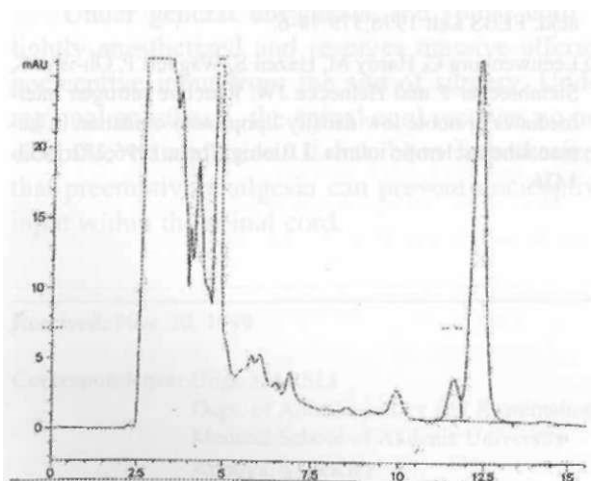
Figure 5. A 3-nitrotyrosine peak could not be detected in a healthy volunteer.



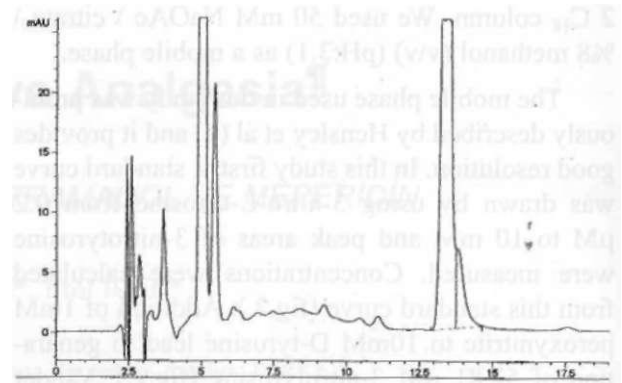
**Figure 6.** HPLC chromatogram of plasma from a healthy volunteer treated with 1 mM peroxynitrite.



**Figure 7.** HPLC chromatogram of plasma from a healthy volunteer treated with 4 mM peroxynitrite.



**Figure 8.** Chromatogram of human plasma containing exogenous 3-nitro-L-tyrosine (4 p.M) standard.



**Figure 9.** Addition of 1 mM peroxynitrite to 10 mM D-tyrosine lead to generation of 52.81 p.M 3-nitrotyrosine.

solution with peroxynitrite was also resulted with peroxynitrite formation in in vitro condition (fig.9). In this study, 96-100 % recovery was obtained for each 3-nitrotyrosine studied.

### Discussion

We have demonstrated the formation of 3-nitrotyrosine after the reaction of peroxynitrite with plasma from healthy volunteers. Nitration on the ortho position of tyrosine is a major reaction. Therefore, the determination of 3-nitrotyrosine may reflect the degree of peroxynitrite-dependent tissue damage. Since it is difficult to demonstrate the other oxidative products of peroxynitrite (2).

Immunohistochemical investigations have demonstrated the existence of 3-nitrotyrosine residue in proteins of damaged tissue, using polyclonal and monoclonal antibodies. However, no sensitive method has been developed to detect 3-nitrotyrosine levels in soluble fractions. Although, in some pathological conditions, elevated 3-nitrotyrosine levels were demonstrated by reversed-phase HPLC with a direct detection of its own UV absorbance (274 nm), the detection limit was 0.2  $\mu$ M in this technique. Plasma 3-nitrotyrosine levels in healthy controls were undetectable. In addition, for the analysis of 3-nitrotyrosine and its metabolites in urine, the gas chromatographic system with a thermal energy analyser was reported (2,10).

In this paper we also described a sensitive and simple isocratic HPLC method for determination of 3-nitrotyrosine in human plasma with UV detection of 274nm using a 5 mm-pore size Allsphere ODS-

2 C<sub>18</sub> column. We used 50 mM NaOAc \ citrate \ %8 methanol (v/v) (pH:3.1) as a mobile phase.

The mobile phase used in this study was previously described by Hensley et al (1) and it provides good resolution. In this study firstly, standard curve was drawn by using 3-nitro-L-tyrosine from 0.2 uM to 10 mM and peak areas of 3-nitrotyrosine were measured. Concentrations were calculated from this standard curve (fig.2.). Addition of 1mM peroxynitrite to 10mM D-tyrosine lead to generation of 52,81 uM 3-nitrotyrosine (fig.9.). Vander Vliet et al (6) performed the similar procedure and obtained  $53 \pm 11$  uM 3-nitrotyrosine.

The 3-nitrotyrosine level of plasma from healthy volunteers could not be detected with the UV detector. Our results were correlated with the others used UV detector (1,2). Our detection limit was 0,1 uM with this detector.

We analyzed 3-nitrotyrosine in plasma after acid-hydrolysis with 6 N HCl at 105°C for 24 h. Although some assays were performed immediately after incubation with peroxynitrite by HPLC, without acid hydrolysis we could not obtained a nice chromatogram. Kamisaki et al (2) suggested the same hydrolysis procedure. In our study, as shown in fig.7, the 3-nitrotyrosine levels were increased by peroxynitrite in a dose dependent manner. In order to identify 3-nitrotyrosine peak from other peaks in human plasma, a certain amount of 3-nitrotyrosine standard was added to human plasma (fig.8.).

In this study, we described a sensitive and simple isocratic HPLC method for determination of 3-nitrotyrosine in biological fluids with UV detector. The rapid HPLC method for determination of 3-nitrotyrosine in plasma may be one of the important diagnostic procedures for peroxynitrite-dependent tissue damage.

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