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Journal of Chromatography B, 713 (1998) 289–295

JOURNAL OF CHROMATOGRAPHY B

Comparison of detection methods for liquid chromatographic determination of 3-nitro-L-tyrosine

Huwei Liu[a,c], Tiehua Huang[b], Candice B. Kissinger[b], Peter T. Kissinger[a,b,*]

[a]*Department of Chemistry, Purdue University, West Lafayette, IN 47907, USA*
[b]*Bioanalytical Systems, Inc., 2701 Kent Ave., West Lafayette, IN 47906, USA*
[c]*Department of Chemistry, Peking University, Beijing 100871, China*

Received 24 June 1997; received in revised form 17 March 1998; accepted 23 March 1998

Abstract

A liquid chromatographic method has been developed for the determination of 3-nitro-L-tyrosine. Different detection methods, including UV, oxidative and redox electrochemistry, and postcolumn photolysis followed by electrochemical detection, have been optimized and compared in terms of analysis time, detection limit and dynamic range. It was demonstrated that liquid chromatography with postcolumn photolysis followed by electrochemical detection is the most effective method, with an analysis time of 5 min, detection limit of 0.01 pmol, and a linear dynamic range from 2 n*M* to 100 μ*M*.

Keywords: 3-Nitro-L-tyrosine

1. Introduction

Nitric oxide (NO), produced in the endothelial cell and neurons by nitric oxide synthase, plays a major physiological role in the nervous system [1], and may regulate cellular respiration by competition with oxygen at the level of mitochondrial cytochrome oxidase [2]. As a result, the physiological role of NO has received attention in recent years [3–6]. It has been suggested that NO combines with superoxide (O_2^-) in vivo producing peroxynitrite ($ONOO^-$) [4,7], a relatively long-lived (1.9 s at pH 7.4) oxidant that is suggested to be the major product of activated macrophage-derived NO [8]. Recent studies [9–12] show that $ONOO^-$ oxidizes sulfhydryl residues in tissue proteins and inhibits the function of enzymes, causing direct tissue damage and limitation of vascular relaxation. Consequently, a sensitive method for the determination of $ONOO^-$ is highly desired. However, thus far it has been impossible to directly and quantitatively detect this unstable molecule in tissue [13].

On the other hand, $ONOO^-$ can react in vivo with L-tyrosine (L-Tyr) residues, causing alterations of protein phosphorylation or perturbation of protein tertiary structure [14]. The product, 3-nitro-L-tyrosine (NO_2-Tyr, see Fig. 1), might be a marker for $ONOO^-$ mediated tissue damage in humans [15]. Therefore, the determination of NO_2-Tyr in biological samples is potentially very beneficial. There are several analytical methods reported in the literature, including an immunohistochemical technique [15], gas chromatography (GC) [16], and liquid chromatography (LC) with UV [11] or multielec-

*Corresponding author.

0378-4347/98/$19.00
PII: S0378-4347(98)00167-4

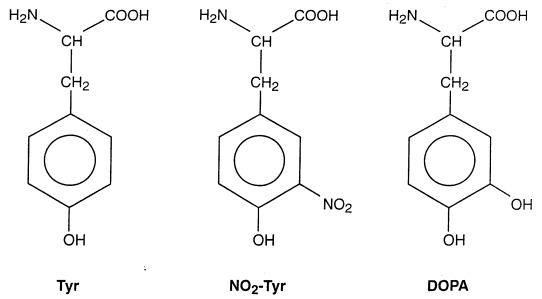

Fig. 1. Structures of Tyr, NO_2-Tyr and DOPA.

trode amperometry [17–21]. Recently, Maruyama et al. used LC with a multichannel electrochemical detector to identify NO_2-Tyr in the human brain [13], while Kamisaki et al. determined NO_2-Tyr in human plasma by LC with fluorescence (FL) detection [22]. The determination of NO_2-Tyr by LC with reductive amperometric electrochemical (EC) detection, or by LC with a postcolumn photoreactor followed by EC detection (*hv*-EC) has not been reported. GC methods require derivatization of NO_2-Tyr, while quantitation is difficult for the immunohistochemical technique. Precolumn derivatization is also necessary for LC–FL analysis. Conventional LC with multielectrode (oxidative) detection is used more often than other detection techniques, but the detection limit is not very low. LC–EC with dual channel detection [23] and LC with postcolumn photoreactor followed by EC detection (LC–*hv*-EC) [24–27] are very selective and sensitive; however, these techniques have not been utilized for the determination of NO_2-Tyr. In this study, we used LC to separate Tyr and NO_2-Tyr, and compared different detection methods including UV, amperometric (oxidative and redox) EC and *hv*-EC, in terms of analysis time, sensitivity and dynamic range. As an example, the content of NO_2-Tyr in microdialysates from rat blood spiked with NO_2-Tyr was determined by using the developed method. This methodology has not been 'validated' in a regulatory sense.

2. Experimental

2.1. Chemicals

DL-Tyrosine, 99%, nitro-tyrosine, 99%, and sodium octyl sulfate (SOS), 95%, were obtained from Aldrich (Milwaukee, WI, USA). Sodium acetate, 99.46%, citric acid monohydrate, 99.85%, EDTA and potassium phosphate monobasic (analytical grade) were from Mallinckrodt Baker (Paris, Kentucky, USA).

All LC mobile phase solutions were prepared with deionized water purified by a NANOpure system (Barnstead, Dubuque, IA, USA) and filtered through a 0.2-μm nylon membrane. The stock solutions of standard NO_2-Tyr and Tyr were prepared in water–methanol (3:1, v/v), then diluted with the mobile phase.

2.2. Instrumentation and conditions

A BAS 200A LC system with component UV and dual EC detectors, and an ODS, 100×2 mm, 3 μm, PEEK column (Bioanalytical Systems, USA) were employed. The mobile phase contained 90 m*M* sodium acetate–35 m*M* citric acid buffer (pH 4.4), 3 m*M* SOS, 1.0 m*M* EDTA and 3% (v/v) methanol, sparged with helium, at a flow-rate of 0.4 ml/min. The UV detector was set at 278 nm, and the EC detector used two glassy carbon working electrodes in series. Electrodes of both 3- and 6-mm diameter were explored using a gasket thickness of 16 μm. The applied potentials for LC–EC oxidative detection was +1100 mV and for redox detection was −750 mV and +600 mV vs. an Ag/AgCl reference electrode, at upstream (W1) and downstream (W2) dual series electrodes, respectively. For LC–*hv*-EC oxidation detection the potential was maintained at +850 mV vs. Ag/AgCl. For postcolumn photolysis, an Agrenetics PhotoBlaster System-1 (Bioanalytical Systems) with a 1.5 m LuxTube® photolysis tube was used.

A BAS 100B/W electrochemical analyzer was used for cyclic voltammetric measurements of the redox properties of Tyr and NO_2-Tyr. Both compounds were dissolved in the mobile phase (pH 4.6), each at a concentration of 1.0 m*M*.

2.3. Microdialysis of rat blood

A BAS Baby Bee syringe drive and a BAS HoneyComb fraction collector were utilized during in vitro microdialysis, and the temperature was

maintained at 37°C with a Fisher Scientific Model 9100 Isotemp refrigerated circulator and Model 120MR Thermix stirrer. BAS DL microdialysis probes (polyacrylonitrile) were utilized. New probes were washed with deionized water for 60 min before microdialysis.

Different probes and flow-rates for microdialysis were evaluated using NO_2-Tyr in Ringer's solution. The DL 2-cm probe and 1.5 μl/min were chosen for the microdialysis of blood. Blood was taken from a rat at sacrifice and made 100 μ*M* with respect to EDTA; a portion was then spiked to 1.5 n*M* with NO_2-Tyr.

3. Results and discussion

3.1. UV detection at 278 nm

UV detection has been used with LC for the determination of Tyr and NO_2-Tyr [11,15], which have a maximum UV absorption at about 280 nm [28]. In this study, UV detection at 278 nm was utilized for comparison with EC detection. Linearity with a correlation coefficient $R^2=0.9986$ was obtained over the range 50 n*M* to 100 μ*M* of NO_2-Tyr. The detection limit for NO_2-Tyr ($S/N=3$) was estimated to be 33 n*M* with an injection amount of 150 pg (0.67 pmol), which is lower than that reported previously. The advantage of UV detection includes the simultaneous determination of Tyr and NO_2-Tyr with equivalent sensitivity. Neither the selectivity nor detection limit are as favorable as for EC redox detection (see Section 3.3). Therefore, UV detection is not recommended for the determination of NO_2-Tyr in biological samples.

3.2. Oxidative EC detection

Both background noise and detector response to NO_2-Tyr are increased with an increase in applied potential at the electrode. Since NO_2-Tyr is not readily oxidized at a potential below +1000 mV, at pH 4.6, a higher potential is necessary. Oxidative detection at +1100 mV exhibited good linearity over a range of 10^4, but due to high noise, the detection limit (20 n*M*) for NO_2-Tyr was not much lower than for UV detection. The selectivity of such a direct high potential oxidative detection is also not favored.

3.3. Reductive and redox EC detection

3.3.1. Cyclic voltammetry results and the redox property of NO_2-Tyr

Cyclic voltammetry (CV) of Tyr and NO_2-Tyr was conducted at pH 3.0 (50 m*M* KH_2PO_4, adjusted with H_3PO_4), pH 4.6 (LC mobile phase) and pH 7.0 (50 m*M* KH_2PO4). When the potential was scanned first in a positive direction (pH 4.6), an anodic wave appeared $E_p=+1082$ mV and a cathodic peak at $E_p=-744$ mV (reduction of NO_2 moiety) for NO_2-Tyr (Fig. 2B). Only an anodic wave appeared for Tyr at about +1000 mV. These results indicated that either oxidative (1082 mV) or reductive (−744 mV) detection could be used for NO_2-Tyr. Oxidative detection, however, is not promising as discussed in Section 3.2 due to the high potential required. Reductive detection at a negative potential gives

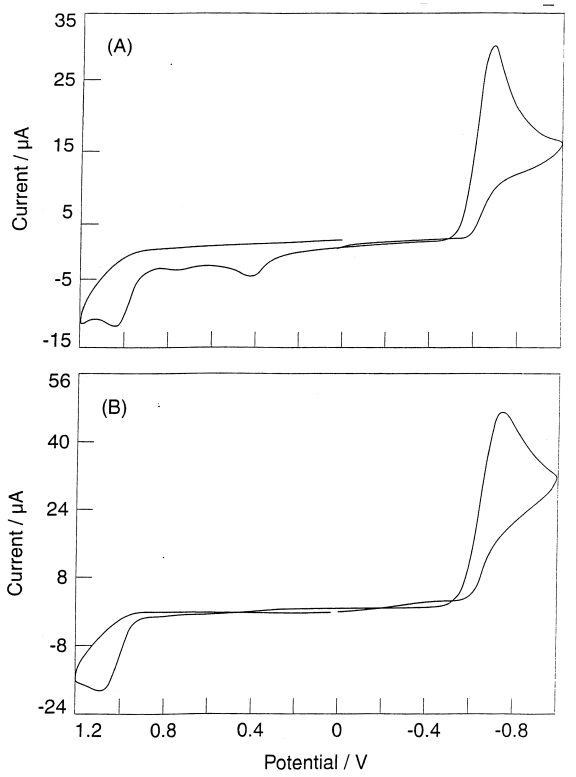

Fig. 2. Cyclic voltammetry of NO_2-Tyr at 1 m*M* in the mobile phase, pH 4.6; scan rate: 100 mV/s; (A) with an initial negative scan (B) with an initial positive scan.

excellent selectivity in biological systems except for the difficulty of excluding oxygen from the mobile phase and sample.

When the potential was scanned first in a negative direction (pH 4.6), as indicated in Fig. 2A, one reductive and three oxidative waves were obtained indicating that NO_2-Tyr can be reduced first and then the reduction product can be oxidized at lower positive potentials than needed for phenol oxidation. The primary mechanism for the redox reactions of NO_2-Tyr are summarized in Fig. 3. This redox detection can be realized on a system equipped with a series dual electrode EC detector.

3.3.2. Effect of varying detection electrode potential (W2)

Based on the CV results, a negative potential was applied at the upstream electrode (W1) of a dual series configuration for the reduction of NO_2-Tyr to its corresponding hydroxylamine, and a positive potential at the downstream electrode (W2) for the oxidation of the hydroxyl amine to the corresponding nitroso compound (redox detection). The two electrodes were in a series configuration and the signal could be monitored at either W1 (reductive detection) or W2 (redox detection). Since the reduction product of NO_2-Tyr is much easier to oxidize than NO_2-Tyr itself, in addition to higher selectivity, lower detection limits are obtained using the redox (reduction then oxidation) reaction than for oxidation alone.

Fig. 4 shows the peak height as a function of the potential applied at W2, indicating that the detector response increases at more positive potentials (similar to Tyr). On the other hand, the baseline noise also increases at higher potential. When both noise and response are considered, +600 mV is optimum for detection of NO_2-Tyr after reduction at W1.

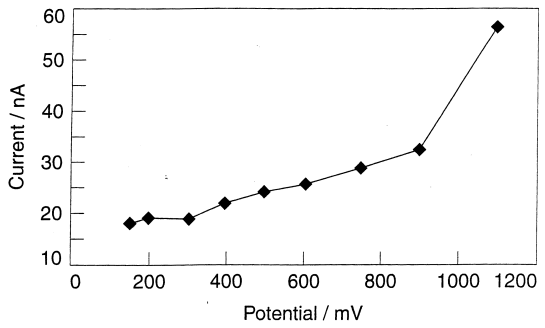

Fig. 4. Effect of varying potential on detection of upstream conversion product. The upstream electrode (W1, 3 mm) was maintained at −750 mV vs. Ag/AgCl while the downstream electrode (W2, 3 mm) potential was varied as indicated. Only the downstream electrode was monitored. Chromatographic conditions as outlined in Section 2.2. Injections were 20 μl of a 3.1 μ*M* solution of NO_2-Tyr.

3.3.3. Effect of varying reactor electrode potential (W1)

Experiments showed that the reduction potential at W1 not only affects the reductive detection, but also affects the oxidative detection at W2 as well, as illustrated in Fig. 5. The W2 (and W1) response increased as the W1 potential was made more negative. It can be concluded that, in order to achieve high sensitivity for both reductive and oxidative detection, a maximal negative potential is necessary at W1 for the reduction of NO_2-Tyr. Comparative tests indicated that an upstream electrode with a 6-mm diameter results in a greater reduction conversion of NO_2-Tyr than that with 3-mm diameter, leading to higher sensitivity. As a result, a dual series electrode configuration with a 6-mm diameter W1 and a 3-mm diameter W2 was used in both reductive and redox detection, and the optimum applied potential was −750 mV at W1 and +600 mV at W2.

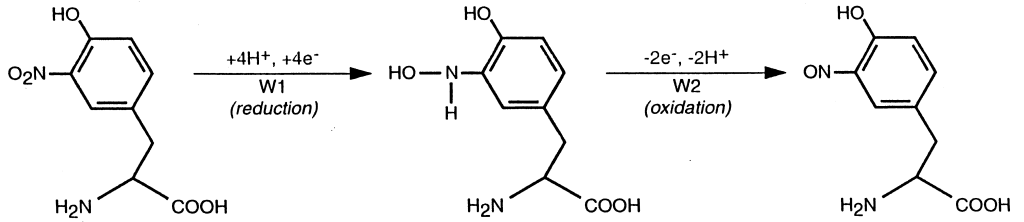

Fig. 3. Scheme of redox reaction mechanisms of NO_2-Tyr.

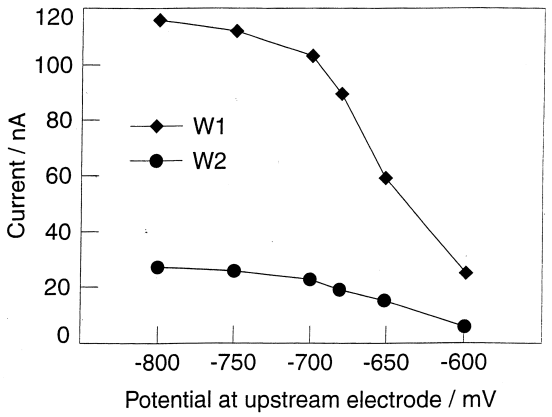

Fig. 5. Effect of varying upstream reactor electrode. The downstream electrode (W2, 3 mm) was maintained at +600 mV vs. Ag/AgCl while the upstream reactor electrode (W1, 3 mm) was varied as indicated. Both electrodes were monitored. Other conditions as in Fig. 4.

It was also observed from Fig. 5 that reductive detection at W1 would be much more sensitive than redox detection at W2. This was consistent with the CV results presented in Fig. 2, where the reduction peak of NO_2-Tyr (four electron transfer) is higher than its oxidative peak (two electron transfer). However, the detection limit for reductive detection was less favorable due to the higher detector background. Reduction detection requires deoxygenation for both mobile phase and sample in order to reduce background noise, making the procedure somewhat more cumbersome.

3.3.4. Effect of sample deoxygenation

The oxygen dissolved in samples not only caused a large reductive peak at W1, which can obscure the NO_2-Tyr peak at low concentration, but also resulted in a big negative peak on the redox chromatogram at W2. After sample deoxygenation for 5 min with helium, almost no oxygen peak can be detected at either W1 or W2. In redox detection, since NO_2-Tyr eluted after the negative oxygen peak, the requirement of deoxygenating the sample for redox mode is not as critical as for reductive detection. In the experiments for reductive detection, samples were bubbled with helium as described by Solomon [29].

3.3.5. Calibration curves and detection limits

Experiments using redox detection, under the optimized conditions, demonstrated a linear relationship between detector response and the concentration of NO_2-Tyr over a 10^4 range. The detection limit was calculated to be 2.1 n*M* ($S/N=3$), since a 5 n*M* solution (20-μl injection) produced a peak of 0.18 nA while the background noise was 0.025 nA. The mass detection limit was 0.042 pmol and 11 pg. Table 1 lists the reproducibility data obtained under the optimized conditions for a standard NO_2-Tyr solution, with acceptable R.S.D.s for both retention time and peak height.

3.4. Postcolumn photolysis followed by oxidative EC detection

LC–*hv*-EC, using a postcolumn photoreactor without addition of reagent, is a powerful method for the sensitive detection of a variety of organic compounds and biomolecules [30–32]. NO_2-Tyr can be photolyzed to the catechol 3,4-dihydroxyphenylalanine (DOPA) by UV light [33], and DOPA is readily oxidized at low potentials. Therefore, LC–*hv*-EC is potentially a more selective and sensitive method for quantitating NO_2-Tyr than direct electrochemical detection. Details regarding the TiO_2 catalyzed photochemical reactor are presented elsewhere

Table 1
Experimental reproducibility for determination of NO_2-Tyr by LC–EC[a]

Day	Peak height (nA)					Mean	R.S.D.
	1	2	3	4	5		(%)
1	0.100	0.110	0.120	0.098	0.100	0.106	9.32
2	0.120	0.120	0.104	0.110	0.106	0.112	7.18
3	0.100	0.112	0.114	0.116	0.100	0.108	7.80
Day-to-day						0.109	2.95

[a] Using redox detection, see text for conditions; concentration of NO_2-Tyr was 0.005 μ*M*.

Table 2
Comparison of methods for 3-nitro-L-tyrosine

Detection method	V_{inj}[a] (μl)	t_R[b] (min)	Concentration[c] (n*M*)	Mass[c] (pmol)	Mass[c] (pg)	Linear range
Reported in literature						
GC-TEA [16]	10	N/A	221	2.2	500	N/A
Array of 16 electrodes (EC) [17]	20	30	0.83	0.062	14	
LC–UV at 274 nm [11]	70	13	200	14	3164	
LC–FL (100-μl injection) [22]	20	17	6 (in plasma)	0.1	22.6	0.4–2.0 n*M*
Obtained in this study						
UV at 278 nm	20	4.8	33	0.670	150	$>10^4$
EC reductive (−750 mV)	20	4.8	2.1	0.042	11.3	$>10^4$
EC redox (−750/+600 mV)	20	4.8	2.1	0.042	11.3	$>10^4$
EC oxidative (+1100 mV)	20	4.8	20	0.400	90.0	$>10^4$
LC–*hv*-EC (EC at +850 mV)	20	4.8	0.5	0.010	2.3	$>10^4$

[a] V_{inj}=Injected volume.
[b] t_R=Retention time.
[c] Detection limit (S/N=3).

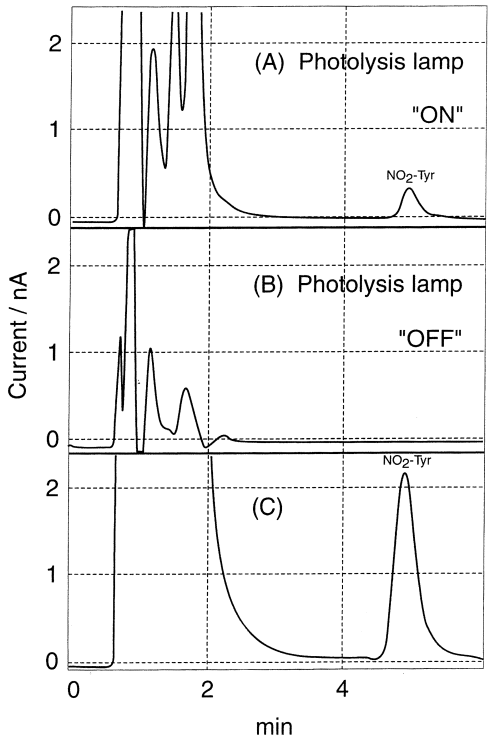

Fig. 6. Typical chromatograms of microdialysate from rat blood spiked with 1.5 n*M* NO_2-Tyr; (A) photolysis lamp 'ON', (B) lamp 'OFF', (C) lamp 'ON' for 5.0 n*M* NO_2-Tyr standard solution. LC–*hv*-EC system conditions as outlined in Section 2.2. Injection volume was 20 μl.

[33,34]. Table 2 presents a comparison of the different detection methods discussed above.

3.5. Application

As an example of a potential application, the LC method with postcolumn photochemical reactor followed by EC oxidative detection was used to determine NO_2-Tyr in microdialysates from rat blood. NO_2-Tyr was not detected in microdialysis samples from fresh rat blood; however, when the blood was spiked with NO_2-Tyr to 1.5 n*M* and a corresponding dialysis sample injected onto the LC–*hv*-EC system, a peak appeared at the retention time of NO_2-Tyr (Fig. 6). The identification of the NO_2-Tyr peak is based on the retention time (same as standard NO_2-Tyr), electrochemical behavior (DOPA, the main photolysis product of NO_2-Tyr, can be easily oxidized at +650 mV) and the photochemistry [34]. No peak appears at the NO_2-Tyr retention time when the photolysis lamp is turned off. The average microdialysis recovery for NO_2-Tyr was 73%. This indicates that direct microdialysis of NO_2-Tyr in blood is feasible, and that the several LC–EC or LC–*hv*-EC methods we have explored can be used to determine NO_2-Tyr in real world samples.

4. Conclusions

NO_2-Tyr was well separated from potential interference and selectively detected using LC–EC with redox detection or LC–*hv*-EC. The results show a shorter analysis time and lower detection limit than previously reported. The reproducibility and linearity are acceptable.

Acknowledgements

The authors would like to thank Wayne O. Aruda of Agrenetics and Dr. C. Duda for their helpful discussions regarding postcolumn photolysis, and Dr. J. Howell for his help with the CV experiments. Dr. C. Gunaratna and M. Cregor are acknowledged for their help in blood sample preparation. H. Liu was financially supported by the exchange program between Peking University, Beijing, China and Purdue University, Indiana, USA.

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