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Optimization of post-column photolysis and electrochemical detection for the liquid chromatographic determination of 3-nitro-L-tyrosine

Huwei Liu[a,b,c], Chester T. Duda[b], Tiehua Huang[b], Wayne O. Aruda[d], Peter T. Kissinger[b,c,*]

[a]*Department of Chemistry, Peking University, Beijing 100871, China*
[b]*Bioanalytical Systems, Inc., 2701 Kent Avenue, West Lafayette, IN 47906, USA*
[c]*Department of Chemistry, Purdue University, West Lafayette, IN 47907, USA*
[d]*Agrenetics, 81 Salem Street, Wilmington, MA 01887-1319, USA*

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Abstract

A new post-column photolysis technology has been developed based on the use of a low pressure, low temperature UV lamp and TiO_2 coated knitted reaction coil. As a test case the developed technique was used for the determination of 3-nitro-L-tyrosine by liquid chromatography with electrochemical detection. Different photolysis lamps and reactor tubing lengths were evaluated in terms of their effect on the separation efficiency and/or photolysis efficiency. A detection limit of 0.5 n*M* (10 fmol) for 3-nitro-L-tyrosine was achieved under optimized conditions, with a linear correlation coefficient of $R^2=0.9898$ over a concentration range of 2–100 μ*M*. Pre-injection photolysis of 3-nitro-L-tyrosine indicated that dihydroxyphenylalanine is the main photolysis product. In general, use of the photoreactor prior to liquid chromatography is an excellent method for exploring photodegradation products of an analyte in conjunction with the full range of available liquid chromatography detectors.

Keywords: Photolysis; Detection, LC; Electrochemical detection; Nitrotyrosine

1. Introduction

Electrochemical detection (ED) has been an excellent alternative to UV and fluorescence (FL) detection in liquid chromatography [1]. For compounds without suitable electrophores in their structures, post-column photolysis has been used for modification (derivatization) of analytes [2–7]. Even for analytes containing a suitable electrophore, improvement of detection has been achieved by using post-column photolysis prior to ED detection (*hv*–ED) [8]. Combining the high separation efficiency of liquid chromatography (LC) with the low detection limits of ED and the high selectivity of photochemistry, LC–*hv*–ED has received more and more attention in the last decade and has proved to be a very useful technique for detection of nitro compounds [9–11], as well as peptides and proteins [8,12]. Since the photolysis reaction can be simply realized in a short knitted Teflon reactor tube around a UV lamp [13], another obvious advantage of photolysis over other derivatization methods is that

*Corresponding author.

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most often the addition of reagents is avoided. Quite recently a low cost photolysis reactor has been developed based on the use of a TiO_2 catalyst. The purpose of this paper was to evaluate this new technology for detection of an analyte of biological interest.

In recent years, the metabolic role of nitric oxide (NO), peroxynitrite ($ONOO^-$) and related compounds has received increasing attention in neuroscience [14]. 3-Nitro-L-tyrosine (NO_2-Tyr), results from the reaction of $ONOO^-$ with tyrosine (Tyr) in vivo [15], is considered to be a marker for $ONOO^-$ mediated tissue damage [16]. Maruyama et al. used LC with multielectrochemical detection to identify NO_2-Tyr in human brain [17] while Kamosaki et al. determined NO_2-Tyr in human plasma by LC with fluorescence detection after pre-column derivatization of NO_2-Tyr [18]. We have developed an LC method with amperometric redox detection for the determination of NO_2-Tyr while comparing different detection methods including UV, oxidative ED, reductive ED and redox ED [19]. In the present report, we applied LC–*hv*–ED to the determination of NO_2-Tyr and optimized the photolysis conditions. A detection limit of 0.5 n*M* (10 fmol) for NO_2-Tyr was achieved with a linear correlation coefficient of $R^2 = 0.9898$ over a concentration range of 2 n*M* to 100 μ*M*. The main photolysis product of NO_2-Tyr was identified to be diphdroxyphenylalanine (DOPA) by pre-injector photolysis and LC–ED. Monitoring NO_2-Tyr in the microdialysate from a biological sample was briefly explored.

2. Experimental

2.1. Chemicals

D,L-Tyrosine, 99%, nitrotyrosine, 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, KY, USA). NO-Generating reagent 3-morpholino-sydnonimine (SIN-1), which can produce nitric oxide (NO) spontaneously in aqueous solution, was purchased from Cayman (MI, USA).

All LC mobile phase solutions were prepared using deionized water, Barnstead NANOpure ultrapure water system (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), then diluted with mobile phase as outlined below.

2.2. Instrumentation and conditions

A BAS 200 LC system with an internal UV and dual ED detectors, and an ODS (3 μm) 100×2 mm column [Bioanalytical Systems (BAS), USA] were employed. The mobile phase was 90 m*M* sodium acetate–35 m*M* citric acid buffer (pH 4.4) containing 3 m*M* SOS, 1.0 m*M* EDTA and 3% (v/v) methanol. Flow-rate was 0.4 ml/min. The mobile phase was sparged with helium using the BAS 200 integral manifold. An Agrenetics PhotoBlaster® System-1 (BAS) with varying length LuxTube® knitted reactors (0.25 mm I.D.×1/16 in. O.D. Teflon FEP tubing; internal surface coated with TiO_2; 1 in.=2.54 cm) was used for post-column photolysis. The ED cell employed dual glassy carbon working electrodes (6 mm upstream and 3 mm downstream) and a Ag/AgCl reference electrode. The applied potential at the two electrodes was varied as indicated. Injection volume was 20 μl.

2.3. Microdialysis of rat blood

As described previously [19], a syringe drive and a microfraction collector were used for in vitro microdialysis, at a constant temperature of 37°C. BAS DL microdialysis probes with 2 cm acrylonitrile membranes were utilized throughout the study. The microdialysis flow-rate was maintained at 1.5 μl/min.

The microdialysis recoveries of NO_2-Tyr using different probes at different flow-rates were evaluated with standard NO_2-Tyr in Ringer's solution. Blood was taken from a rat at sacrifice, and EDTA was added (final concentration of 100 μ*M*) as an anti-clotting agent prior to microdialysis. In order to investigate the relationship between the content of NO_2-Tyr and the reaction time of $ONOO^-$ with Tyr, standard solutions of NO_2-Tyr, Tyr, and SIN-1 were spiked into blood to the final concentrations of 1.5

n*M*, 1.0 μ*M*, and 10 μ*M* respectively. Since superoxide dismutase (SOD) is present in blood, NO produced by SIN-1 will react with O_2^-, producing $ONOO^-$. Finally, $ONOO^-$ reacts with Tyr, resulting in NO_2-Tyr, which can be separated and detected by LC–*hv*–ED. The reaction was sampled at 30 min intervals.

3. Results and discussion

3.1. Comparison of different photolysis reactors and fittings

Photolysis reactors (LuxTube and tubing not coated with TiO_2) were evaluated in terms of their contribution to band broadening and their effects on photolysis efficiency. We first compared LuxTube reactors of different lengths in order to evaluate band broadening due to the tubing. Photolysis tubing was connected between the column and the detector, but the UV lamp (PhotoBlaster System-1, low-pressure mercury lamp) was off, and redox detection of NO_2-Tyr was employed as previously described [19]. Table 1 illustrates that with the increase in tubing length, the retention time and peak width of NO_2-Tyr were increased, while the peak height and separation efficiency were decreased, indicating the expected extra-column effect. It should be noted that the extra-column band broadening can be caused not only by the tubing itself but also by the connection fittings between the tubing and column, and detector. Photolysis efficiency of NO_2-Tyr did not increase for tubing longer than 1.5 m (Table 1). For this reason, a 1.5 m FEP LuxTube reactor was used in the following experiments.

Two different 1.5 m LuxTube reactors coated with titanium dioxide (TiO_2) were compared with two tubes (of similar I.D.) without TiO_2 coating (i.e. blanks). The TiO_2 coated reactors (LuxTube) resulted in significantly larger peak for NO_2-Tyr than the blank tubing. For instance, the peak height for 0.5 μ*M* NO_2-Tyr detected at +800 mV after photolysis was less than 1 nA with blank tubing while it was 73 nA with TiO_2-coated tubing. We monitored detector background under lamp ''ON'' and ''OFF'' conditions. When the blank tubing was used, there was only a 1 nA difference within the backgrounds. When coated tubing was used, the difference became 20 nA.

We were initially concerned about the possible ''poisoning'' of the catalyst. The results listed in Table 2 show that after three months of continuous use, the reactor tubing remained effective under the conditions of this experiment, indicating the TiO_2 coating was stable.

3.2. Comparison of different lamps for photolysis of NO_2-Tyr

Three different photolysis lamps were evaluated in this study including a PhotoBlaster System-1 (low pressure mercury lamp), a prototype flash U-type lamp, and a xenon flash lamp. The results indicated that all three lamps were effective in carrying out the photolysis of NO_2-Tyr. Fig. 1 illustrates a typical chromatogram obtained with the PhotoBlaster System-1. It can be seen that very little response was

Table 1
Comparison of photolysis reactor tubing of different lengths [a]

Tube length (m)	t_R (%)	$W_{1/2}$ (%)	N (%)	h_1 (%)	h_2 (%)
0 [b]	100	100	100	100	0
0.5	102	108	89	93	85
1.0	103	110	88	91	99
1.5	104	112	86	89	100
4	106	115	85	86	101
9	112	122	84	82	102

[a] All data were obtained by ED redox detection when photolysis lamp was off, except for h_2 which was obtained by oxidative detection at +850 mV with photolysis lamp on. t_R=Retention time; $W_{1/2}$=peak width at half peak height; N=theoretical plate number; h=peak height.
[b] The outlet of the column was connected to the detector with a short piece of 0.005 in. I.D. polyether ether ketone (PEEK) tubing. Twenty μl of 0.5 μ*M* NO_2-Tyr were injected. See text for other conditions.

Table 2
Comparison of the reactor tubing before and after 3 months of use

Tubing	Peak height (nA) of repeat injections of NO_2-Tyr (0.5 μM) [a]							
	1	2	3	4	5	Mean	S.D.	R.S.D. (%)
Used	70	74	75	72	73	72.8	1.92	2.64
Freshly prepared	72	75	69	75	72	72.6	2.51	3.46

[a] Detected at +800 mV using chromatographic conditions as outlined in Fig. 1A.

detected, even at +850 mV, in the lamp OFF mode. In the lamp ON mode, a very large peak (DOPA, see Section 3.3.2) appeared at the same retention time as NO_2-Tyr.

3.2.1. Performance of the U-type flash lamp

Tyr and NO_2-Tyr (0.5 μM) were injected to evaluate the performance of a U-type flash lamp with a 9.0 m reactor. The flash rate was an important factor affecting the photolysis efficiency. The peak height increased with an increase in the lamp flash rate (10–40 s^{-1}, energy: 0.5 J per flash). This was reasonable, because the higher the flash rate, the higher total light flux. Rates higher than 40 s^{-1} (40–100 s^{-1}) resulted in a constant peak height. It should be noted that the response to injected Tyr was not enhanced by the present photolysis conditions, using either PhotoBlaster System-1 or U-type flash lamp, indicating that NO_2 group was involved in the photolysis reaction.

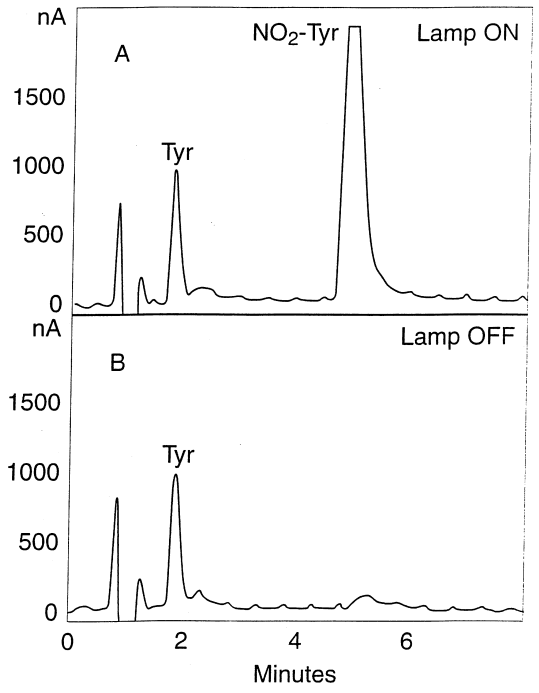

Fig. 1. Typical LC–*hv*–ED chromatograms of Tyr and NO_2−Tyr, each at 50.0 μM. A PhotoBlaster System-1 was used with a 1.5 m×0.25 mm photolysis tubing coated with TiO_2 (LuxTube); ED was at +850 mV vs. Ag/AgCl. Chromatographic conditions as outlined in Section 2.2. (A) Photolysis lamp ''ON''. (B) photolysis lamp ''OFF''.

The U-type flash lamp is an effective light source for the photolysis of nitro compounds. The flash rate is an additional parameter which may be used to control photochemical reactions. However, the detection sensitivity under the present LC–*hv*–ED conditions with the prototype flash lamp was not as high as with PhotoBlaster non-flash lamp. The peak height for a 0.5 μM NO_2-Tyr sample using non-flash lamp was more than 20 nA, but was less than 13 nA using flash lamp at the rate of 50 s^{-1}. The detector background was higher with the flash lamp than with non-flash lamp.

3.2.2. Performance of Agrenetics xenon lamp

A xenon lamp with a 4.0 m LuxTube was effective for the photolysis of NO_2-Tyr, but did not display any advantage over the PhotoBlaster System-1 and U-type flash UV lamp.

In summary, the PhotoBlaster System-1 shows better performance in terms of photolysis efficiency for NO_2-Tyr. Its continuous light output results in lower background noise level and a more stable baseline, so lower detection limits can be obtained with this system. The dynamic range is from 2 nM to 100 μM, with a correlation coefficient of $R^2=0.99$, and a detection limit of 0.5 nM (10 fmol or 2.3 pg injected).

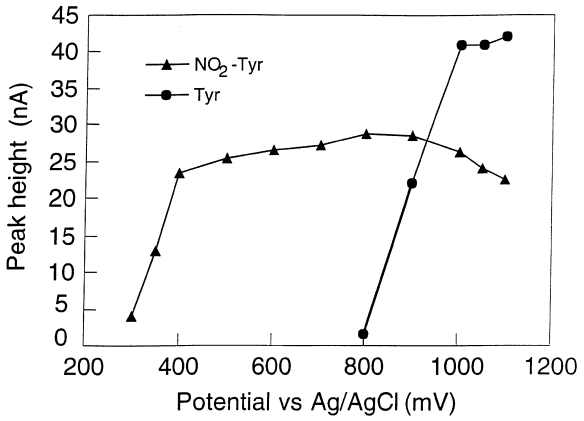

Fig. 2. Peak height of NO_2-Tyr and Tyr (0.5 μ*M* each) as a function of oxidative potential. A PhotoBlaster System-1 was used in the lamp ''ON'' condition. Detection was at the upstream electrode maintained at the indicated potentials. Chromatography conditions as outlined in Section 2.2.

Fig. 2 presents the response vs. potential curves for NO_2-Tyr and Tyr using the LC–*hv*–ED system. A potential of +850 mV was chosen for the determination of NO_2-Tyr and +1050 mV for the determination of Tyr.

3.3. Pre-injector photolysis

Pre-injection photolysis of NO_2-Tyr and Tyr was carried out in order to identify the products of photolysis. It has been reported that DOPA is one of the major photolysis products of an irradiated Tyr solution [20]. Our pre-injector photolysis experiments supported this report and further demonstrated DOPA is also the major photolysis product of an irradiated NO_2-Tyr solution.

3.3.1. Photolysis of the mobile phase

As mentioned above, the background at the ED system increased following post-column photolysis. Irradiation of mobile phase alone, prior to injection, produced a number of chromatographically distinct peaks. The concentration of the major electrochemically active photolysis product increased with the length of the irradiation period. Also, the magnitude of the response increased as the applied potential. Both parameters must be considered when optimizing the signal-to-noise ratio of an LC–*hv*–ED system. Precolumn irradiation may prove useful in this respect.

3.3.2. Photolysis of NO_2-Tyr

NO_2-Tyr is readily photolyzed and the main product is DOPA (Fig. 3) [21]. DOPA is readily oxidized at low potentials (<+700 mV vs. Ag/AgCl). Direct detection of NO_2-Tyr requires a high oxidation potential (>+950 mV vs. Ag/AgCl), in the order of that for Tyr itself (Fig. 2) [19]. This is why conversion of NO_2-Tyr by irradiation, prior to detection, increases the sensitivity and lowers the

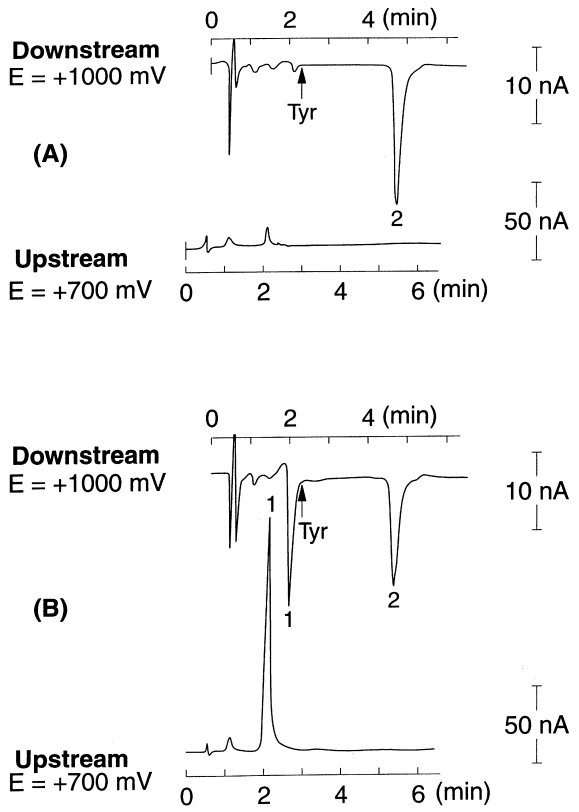

Fig. 3. Pre-injector photolysis of a NO_2-Tyr. A 0.5 μ*M* solution of NO_2-Tyr was irradiated in a LuxTube for varying lengths of time and then injected onto an LC–ED system. Chromatography conditions as outlined in Section 2.2. The ED cell employed dual glassy carbon working electrodes in a series configuration with the upstream electrode maintained at +700 mV and the downstream electrode maintained at +1000 mV vs. Ag/AgCl. (A) Injection of NO_2-Tyr solution prior to irradiation, and (B) injection after 30 s irradiation. Peak 1 is DOPA and Peak 2 is NO_2-Tyr. The arrow indicates the Tyr peak position if it had been injected.

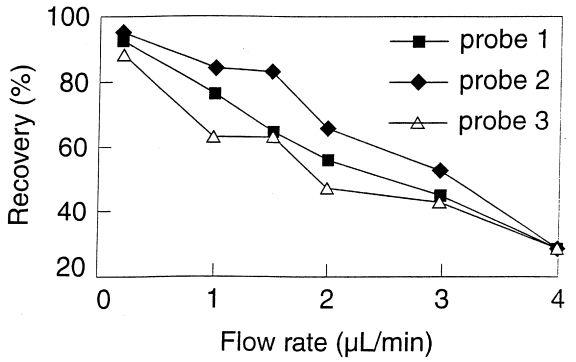

Fig. 4. In vitro recovery vs. flow-rate. A 2.5 μ*M* solution of NO_2-Tyr in Ringer's was used. Samples were collected from three separate probes, at the flow-rates indicated. Ringer's solution was used for the dialyzing medium. Other conditions as outlined in Section 2.3. The concentrations of NO_2-Tyr in the dialysates were determined as outlined in Section 2.2.

detection limit [19]. There was no indication that Tyr was a photolysis product of NO_2-Tyr irradiation (Fig. 3, no peak corresponding to the retention time of Tyr was observed in the pre-injector irradiated samples).

3.4. Application to microdialysis samples

The in vitro recovery of NO_2-Tyr as a function of dialysis flow-rate is presented in Fig. 4. Subsequent experiments utilized a flow-rate of 1.5 μl/min.

A NO generating system added to rat blood resulted in the synthesis of NO_2-Tyr. Microdialysis sampling was found to be a convenient way to follow the production of NO_2-Tyr (Fig. 5). The 0-time-point represents a concentration of 1.5 n*M* NO_2-Tyr in the incubation mixture. Direct injection of the dialysates onto the LC–*hv*–ED system produced clean chromatograms [19].

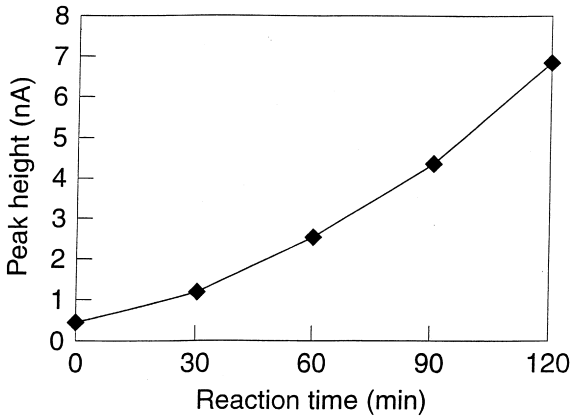

Fig. 5. Time course of in vitro NO_2-Tyr synthesis. A blood incubation mixture, as outlined in Section 2.3, was sampled by microdialysis. Dialysates were collected at 1.5 μl/min every 30 min and 20 μl injected on the LC–*hv*–ED system (for conditions see Fig. 1A).

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