Journal of Chromatography B, 911 (2012) 55–58

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

Separation of intermediates of iron-catalyzed dopamine oxidation reactions using reversed-phase ion-pairing chromatography coupled in tandem with UV–visible and ESI-MS detections

Lin Zhang[a], Gargey Yagnik[b], Dianlu Jiang[b], Shuyun Shi[a], Peter Chang[b], Feimeng Zhou[a,b,*]

[a] College of Chemistry and Chemical Engineering, Central South University, Changsha, Hunan 410083, People's Republic of China
[b] Department of Chemistry and Biochemistry, California State University, Los Angeles, Los Angeles, CA 90032, United States

ARTICLE INFO

Article history:
Received 23 July 2012
Accepted 24 October 2012
Available online 29 October 2012

Keywords:
Reversed-phase ion-pairing chromatography
Electrospray ionization-mass spectrometry
Dopamine oxidation
6-Hydroxydopamine
Short-lived intermediates

ABSTRACT

Reversed-phase ion-pairing chromatography (RP-IPC) is coupled on-line with electrospray ionization-mass spectrometry (ESI-MS) through an interface comprising a four-way switch valve and an anion exchange column. Regeneration of the anion exchange column can be accomplished on-line by switching the four-way switch valve to interconnect the column to a regeneration solution. Positioning the anion exchange column between the RP-IPC and ESI-MS instruments allows the ion-pairing reagent (IPR) sodium octane sulfonate to be removed. The IPC–ESI-MS method enabled us to separate and detect four intermediates of the Fe(III)-catalyzed dopamine oxidation. In particular, 6-hydroxydopamine, which is short-lived and highly neurotoxic, was detected and quantified. Together with the separation of other intermediates, gaining insight into the mechanism and kinetics of the Fe(III)-catalyzed dopamine oxidation becomes possible.

Published by Elsevier B.V.

1. Introduction

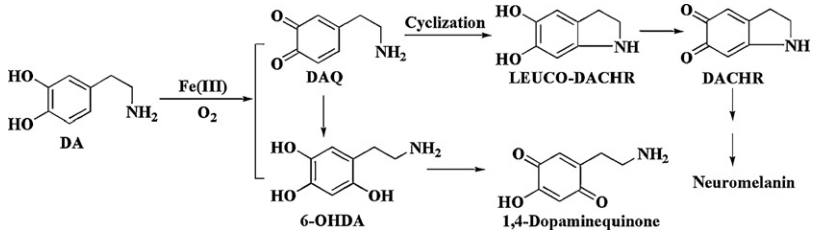
Dopamine (DA) is an important neurotransmitter that mediates a variety of functions of the central nervous system (e.g. motor control, cognition, emotion, memory processing and endocrine regulation) [1,2]. In brain DA is tightly controlled, as under certain conditions, similar to its oxidation intermediates, it can be neurotoxic [2,3]. Therefore, DA molecules that are not bound by DA receptors need to be cleared either through metabolic processes or be recaptured through endocytosis. Disruption of DA homeostasis is closely associated with the pathogenesis of Parkinson's disease (PD) [3]. Identification and quantification of the oxidation intermediates of DA have therefore attracted a broad range of interests.

Oxygen is abundant in brain, and can slowly oxidize DA [4]. In the presence of iron, the oxidation of DA is drastically accelerated [5]. Since iron overloading is known to be particularly acute among the elderly, studies of the iron-catalyzed DA oxidation reaction are considered to be physiologically relevant. A simplified mechanism of the iron-catalyzed DA oxidation reaction is presented in Scheme 1. Briefly, dopamine quinone (DAQ) undergoes a rapid ring closure (cyclization reaction) to produce leuco-dopaminechrome (leuco-DACHR). Leuco-DACHR is then gradually oxidized to dopaminechrome (DACHR), which is the monomer that constitutes the polymer neuromelanin. Neuromelanin is a black, insoluble substance that contributes to the black color of dopaminergic neurons in the *substantia nigra*. In competition with the above route is the formation of the short-lived, highly neurotoxic intermediate 6-hydroxydopamine (6-OHDA), which can be readily oxidized to its quinone form (1,4-dopaminequinone). Thus far, studies using off-line methods [6] only captured the oxidized form of 6-OHDA, 1,4-dopaminequinone. As delineated by Prota and coworkers [7], the mechanism behind the Fe(III)-catalyzed DA oxidation reaction is actually more complicated. The tautomer of DAQ, quinomethide, can react with water in the presence of a metal ion (e.g. Mn(II) or Fe(III)) to form norepinephrine as a stable product [7].

Reversed-phase ion-pairing chromatography (RP-IPC) with UV–visible (UV–vis) detection or ion exchange chromatography in conjunction with electrochemical detection has been developed for separation and detection of monoamine neurotransmitters [7,8]. However, unambiguous identification of unknown or short-lived products/intermediates of the Fe(III)-catalyzed dopamine oxidation by these methods is not trivial. Mass spectrometry (MS) is perhaps the most powerful technique due to its high sensitivity and capability of elucidating compound structures [9]. A problem inherent in coupling RP-IPC with MS is that non-volatile salts necessary for IPC could clog the LC–MS interface and suppress the analyte signals [10]. The on-line IPC trapping method was developed by

* Corresponding author at: Department of Chemistry and Biochemistry, California State University, Los Angeles, Los Angeles, CA 90032, United States.
Tel.: +1 323 343 2390; fax: +1 323 343 5587.
E-mail address: fzhou@calstatela.edu (F. Zhou).

1570-0232/$ – see front matter. Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.jchromb.2012.10.026



Scheme 1. A simplified mechanism of the Fe(III)-induced DA oxidation reaction. DAQ, dopamine quinone; DACHR, dopaminechrome; 6-OHDA, 6-hydroxydopamine.

Forngren and other groups for coupling RP-IPC with ESI-MS and successfully used for the separation of biopterin, sulphonated azo dyes, sulfonated compounds and other molecules [9,11,12]. To our knowledge, there has been no report on the use of RP-IPC coupled on-line with ESI-MS for detecting short-lived DA oxidation products. Herein we describe the tandem combination of a RP-IPC column and a strong anion exchange column for on-line removal of IPCs prior to analyte detection by UV–vis spectrometry and ESI-MS. As shown in Fig. 1, unlike the work conducted by Forngren et al. [9], a four-way rotary valve is used to either interconnect the RP-IPC and anion exchange columns (detection mode) or to divert a regeneration solution to the anion exchange column (regeneration mode). The resultant interface enabled us to detect four key intermediates of the Fe(III)-catalyzed DA, with the short-lived, highly neurotoxic 6-OHDA clearly identified and quantified by ESI-MS.

2. Materials and methods

2.1. Chemicals

Dopamine (DA), norepinephrine (NE), and sodium octane sulfonate were purchased from Sigma–Aldrich Corp. (St. Louis, MO). Two of the possible intermediates of the DA oxidation reaction, 3,4-dihydroxybenzaldehyde and 6-hydroxydopamine (6-OHDA) were acquired from MP Biomedical Inc. (Solon, OH). All other chemicals and solvents were obtained from Thermo-Fisher Scientific (Pittsburgh, PA) or Alfa Aesar (Ward Hill, MA).

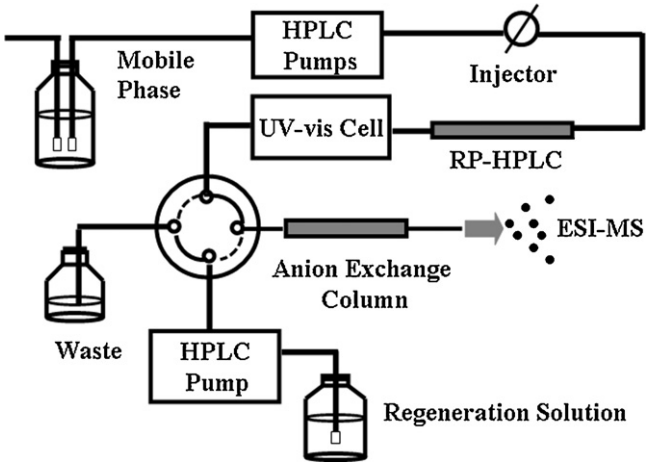


Fig. 1. Schematic of RP-IPC coupled to ESI-MS via an interface comprising a four-way switching valve and a strong anion exchange column. In one mode (denoted by the solid curves within the circle that symbolizes the four-way valve), species separated by RP-IPC are eluted to the anion exchange column before being detected by ESI-MS. In the other mode (dotted curves), regeneration solution is diverted to the anion exchange column to strip ion-pairing agent off the column.

2.2. Instruments

The mass spectrometer (MS) was an Xcaliber LCQ Fleet model (Thermo, San Jose, CA) equipped with an ESI source. The HPLC system included two pumps, an injector, a C_{18} column (5 μm particle size, 2.0 mm × 100 mm, Agilent Technologies, Santa Clara, CA) and a photodiode array detector (PDA). A strong anion exchange column (5 μm; 40 mm × 2.1 mm) from Higgins Analytical Inc. (Mountain View, CA) was used for the IPR removal. A Hitachi L-6000 pump was used to deliver the regeneration solution. As shown in Fig. 1, in a typical operation, once the PDA detected the first chromatographic peak, the four-way valve was switched from the regeneration mode to the detection mode. Alternatively, the valve can be switched back to the regeneration mode after an entire chromatographic run.

2.3. Sample preparation

For separation and identification of the Fe(III)-catalyzed DA oxidation products and intermediates, a mixture of DA (0.5 mM) and $FeCl_3$ (0.25 mM) was prepared in an HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (pH 7.2). Standards of different 6-OHDA concentrations were prepared with this buffer. The mobile phase for the RP-IPC column was 50 mM phosphate in a water/acetonitrile mixture (v/v = 95/5) containing 0.5 mM sodium octane sulfonate and 5 mM formate acid (pH 3.4), while the regeneration solution was 50 mM phosphate in a water/acetonitrile mixture (v/v = 95/5) containing 150 mM ammonium formate (pH 3.0).

2.4. Procedures

The separated species were detected with the PDA by setting the wavelength at 280 and 300 nm. For optimal separation, a flow rate of 0.2 mL/min was used. Typically, 20 μL sample was injected for each separation. To monitor how intermediates from the Fe(III)-catalyzed DA oxidation vary over time, 20-μL aliquots from the incubated solution were taken out at different time intervals and diluted with the mobile phase solution. The incubation was carried out over a period of 72 h for monitoring the concentration variations of different DA oxidation products and intermediates. Quantification of 6-OHDA was carried out by integrating the chromatographic peak area and compared the value to a calibration curve constructed with a series of 6-OHDA standards.

3. Results and discussion

The unequivocal identification of the Fe(III)-catalyzed DA oxidation intermediates is desirable for understanding the DA oxidation pathway [7]. But owing to the facts that some of the intermediates are short-lived and some of their standards are not commercially available, their separation and identification have been difficult with the conventional HPLC methods [8]. In our work, RP-IPC coupled with ESI-MS was employed for the separation and detection of intermediates/products from the Fe(III)-catalyzed DA oxidation

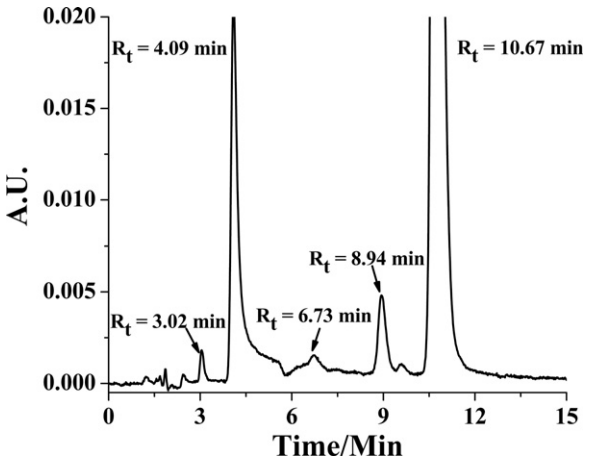


Fig. 2. RP-IPC separation of intermediates/products generated from oxidation of 0.5 mM DA by 0.25 mM Fe(III) in HEPES buffer (pH 7.2) for 1 h at 37 °C. The detection was carried out with UV–vis detection at 280 nm. Mobile phase: acetonitrile/H_2O (v/v = 95/5, pH 3.4) containing 0.5 mM sodium octane sulfonate and 5 mM formate acid. The flow rate used was 0.2 mL/min.

reaction (cf. Fig. 1). Collected with a UV–vis detector, the chromatogram of an aliquot taken out from a mixture of DA/Fe(III) incubated for 1 h is shown in Fig. 2. The peak with a retention time (R_t) of 10.67 min was assigned to unreacted DA, since injection of an unoxidized DA sample yielded a chromatographic peak at the same R_t. In addition to the peak attributable to unreacted DA, four other peaks with varying intensities are discernible. Fraction collection of these peaks followed by direct infusion into the MS did not allow the species with R_t = 3.02 and 6.73 min to be identified, suggesting they are shorted-lived or of low abundance. Thus the UV–vis spectrometric detection is insufficient for the identification of all of the analytes in Fig. 2.

With on-line IPR removal by the anion exchange column, mass spectra corresponding to all of the chromatographic peaks in Fig. 2 can be obtained. These mass spectra are depicted in Fig. 3. Fig. 3A is the mass spectrum of the chromatographic peak at R_t 3.02 min. The molecular ion at *m/z* 150.25 is equivalent to the molecular mass of monoprotonated dopaminechrome or DACHR (149.18+1H$^+$). Fig. 3B is the mass spectrum of the species eluted at 4.09 min. The molecular ion at *m/z* 170.08 is equivalent to the molecular mass of protonated norepinephrine (NE; 169.18+1H$^+$). This spectrum is essentially identical to that obtained by directly infusing an NE standard into the ESI-MS. As a product of DA oxidation, NE is formed through the aforementioned quinomethide pathway [7,13]. Fig. 3C is the mass spectrum of the species eluted at 6.73 min, which clearly shows a peak at *m/z* 170.17 and can be assigned to the protonated form 6-OHDA. The concentration was determined to be 3 μM by comparing the peak area to a calibration curve constructed with a series of 6-OHDA standards. It is interesting to note that both NE and 6-OHDA have the same molecular weight. However, a daughter ion at *m/z* 152.25 is displayed in the mass spectrum of NE (Fig. 3B), which corresponds to the detachment of the hydroxyl group on its side chain. Notice that this peak is of greater intensity than the molecular ion at 170.08. In contrast, 6-OHDA does not fragment easily and consequently the peak at 152.25 is rather small. Also the chromatographic peak height of 6-OHDA is substantially smaller than that of NE in Fig. 2, which is indicative of the short lifetime of 6-OHDA. The fact that the NE peak height is higher than those of other intermediates/products suggests that the reaction via the quinomethide intermediate is more dominant under our experimental condition. To further confirm that the peak with R_t 6.73 min is indeed given rise by 6-OHDA, we spiked NE into the reaction mixture and did not see any enhancement of the peak at 6.73 min. As 6-OHDA is known to be easily converted to its quinone form (1,4-dopaminequinone), we also prepared a 6-OHDA solution and injected it into the column. In addition to a peak at R_t 6.73 min, a new peak with R_t of 8.94 min appeared. Another interesting observation is that, when the 6-OHDA solution was incubated for a longer period, the peak at 8.94 min increased at the expense of the peak at 6.73 min. These results strongly suggest that the short-lived 6-OHDA can be readily converted to its quinine form. A representative mass spectrum of the species with R_t = 8.94 min is shown in Fig. 3D. The mass peak at 168.17 Da can be

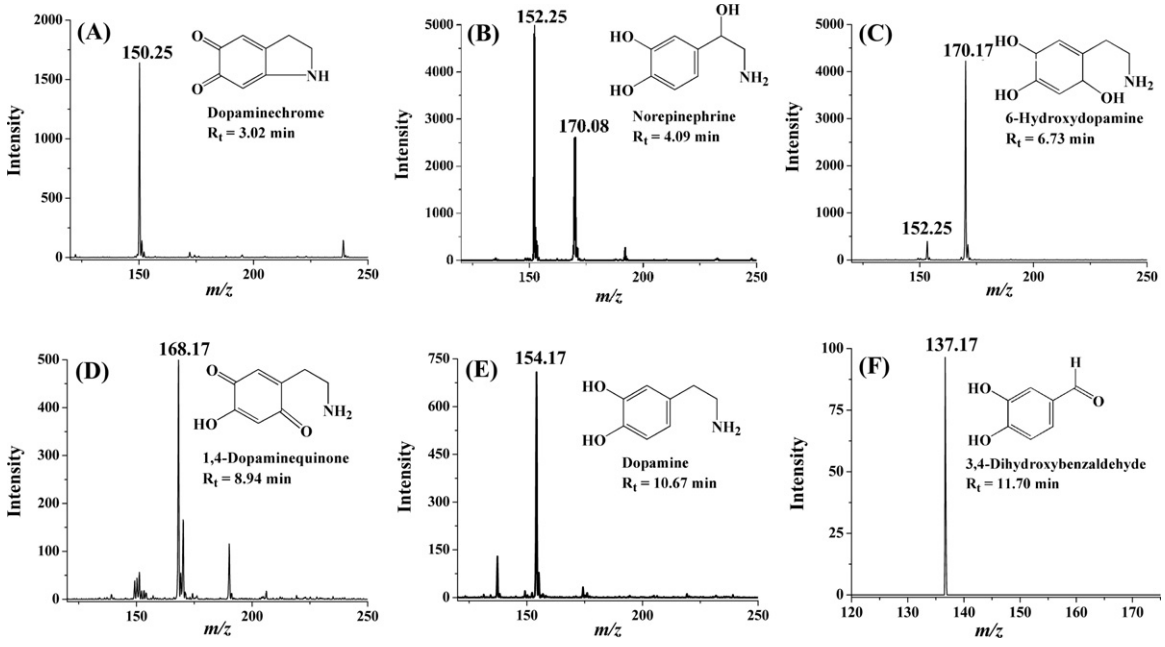


Fig. 3. Mass spectra corresponding to species eluted at different retention times after post-separation IPR removal: (A) R_t = 3.02 min; assigned to dopaminechrome, (B) R_t = 4.09 min; assigned to norepinephrine, (C) R_t = 6.73 min; assigned to 6-hydroxydopamine, (D) R_t = 8.94 min; assigned to 1,4-dopaminequinone, and (E) R_t = 10.67 min; assigned to unreacted dopamine. The mass spectrum of 3,4-dihydroxybenzaldehyde, obtained by direct infusion to the MS in the negative ion mode, is shown in panel (F).

assigned to the protonated 1,4-dopaminequinone (167.18+1H^+). By oxidizing 6-OHDA in the presence of sodium periodate, a spectrum essentially the same as Fig. 3D was obtained, further confirming that the peak at R_t 8.94 min is 1,4-dopaminequinone. Finally, Fig. 3E is the mass spectrum of corresponding to the peak at R_t 10.67 min. The molecular ion at *m/z* 154.17 is equivalent to the singly charged DA (153.2+1H^+), confirming our earlier assignment of this peak with the UV–vis detection. It is worth mentioning that we did not observe the formation of 3,4-dihydroxybenzaldehyde, which could be produced from the reaction between the quinomethide intermediate and H_2O_2 [7]. When 3,4-dihydroxybenzaldehyde was injected into the column, a chromatographic peak appeared at 11.70 min and the MS spectrum exhibited a peak with *m/z* = 137.17 in the negative ion mode. This *m/z* value is equivalent to that of deprotonated 3,4-dihydroxybenzaldehyde (138.18−1H^+). Thus we verified that 3,4-dihydroxybenzaldehyde is not a product of the Fe(III)-catalyzed reaction under our experimental condition. This observation can be rationalized on the basis that H_2O_2 is absent in the solution. Among the identifications of the intermediates/products shown in Figs. 2 and 3, detection of 6-OHDA is the most significant given its neurotoxicity and the fact that it has not been separated and identified by other HPLC methods [6,8,14–16]. We believe that in previous studies either 6-OHDA was completely oxidized or the sensitivity of the methods used was not sufficiently high to detect the trace amount of 6-OHDA.

4. Conclusion

RP-IRC is coupled on-line with ESI-MS through an interface that facilitates effective removal of the ion-pairing reagent (sodium octane sulfonate). With a four-way rotary valve, analytes from the RP-IRC column can be directed to a strong anion exchange column prior to being introduced to the mass spectrometer. By removing IPR with an anion exchange column, signals of the separated species are no longer suppressed by the non-volatile salt. The method was successfully applied to the separation and identification of four key intermediates of the Fe(III)-catalyzed dopamine oxidation reaction. In contrast to the UV–vis detection, ESI-MS enabled us to identify norepinephrine, dopaminechrome, 6-hydroxydopamine, and 1,4-dopaminequinone. The concentration of the highly neurotoxic 6-hydroxydopamine was detected to be 3 μM after 0.5 mM DA had been oxidized in the presence of 0.25 mM Fe(III) for 1 h at 37 °C. The method also allowed us to study the conversion of 6-hydroxydopamine to 1,4-dopaminequinone as the Fe(III)-catalyzed DA oxidation proceeds, thus demonstrating the feasibility for kinetic studies of the Fe(III)-catalyzed DA oxidation reaction. With the effective removal of IPR, the method should be applicable to the separation and ESI-MS detection of other mixtures wherein polar components are short-lived, of low abundance, or lacking commercially available standards.

Acknowledgements

Partial support of this work by Central South University, a grant from the National Institutes of Health (SC1NS070155-01), a National Science Foundation (NSF) grant (no. 1112105), and the NSF-CREST Program at California State University, Los Angeles (NSF HRD-0931421) is gratefully acknowledged.

References

[1] A. Carlsson, Pharmacol. Rev. 11 (1959) 490.
[2] R.A. Nicoll, R.C. Malenka, J.A. Kauer, Physiol. Rev. 70 (1990) 513.
[3] F. Zoccarato, P. Toscano, A. Alexandre, J. Biol. Chem. 280 (2005) 15587.
[4] S.D. Iversen, L.L. Iversen, Trends Neurosci. 30 (2007) 188.
[5] Y. Izumi, H. Sawada, N. Sakka, N. Yamamoto, T. Kume, H. Katsuki, S. Shimohama, A. Akaike, J. Neurosci. Res. 79 (2005) 849.
[6] A. Pezzella, M. d'Ischia, A. Napolitano, G. Misuraca, G. Prota, J. Med. Chem. 40 (1997) 2211.
[7] A. Napolitano, A. Pezzella, G. Prota, Chem. Res. Toxicol. 12 (1999) 1090.
[8] S.D. Ochs, T.C. Westfall, H. Macarthur, J. Neurosci. Methods 142 (2005) 201.
[9] B.H. Forngren, J. Samskog, S.A. Gustavsson, N. Tyrefors, K.E. Markides, B. Langstrom, J. Chromatogr. A 854 (1999) 155.
[10] K.W.M. Siu, R. Guevremont, J.C.Y. Le Blanc, G.J. Gardner, S.S. Berman, J. Chromatogr. 554 (1991) 27.
[11] A. Plum, A. Rehorek, J. Chromatogr. A 1084 (2005) 119.
[12] G. Socher, R. Nussbaum, K. Rissler, E. Lankmayr, Chromatographia 54 (2001) 65.
[13] A. Slivka, G. Cohen, J. Biol. Chem. 29 (1985) 15466.
[14] Z.D. Zhou, T.M. Lim, Neurochem. Res. 34 (2009) 316.
[15] A. Napolitano, A. Pezzella, G. Prota, Tetrahedron Lett. 40 (1999) 2833.
[16] A.J. Nappi, E. Vass, G. Prota, S. Memoli, Pigment Cell Res. 8 (1996) 283.