Talanta 79 (2009) 1071–1075

Contents lists available at ScienceDirect

Talanta

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

Automated on-line microdialysis sampling coupled with high-performance liquid chromatography for simultaneous determination of malondialdehyde and ofloxacin in whole blood

Guan-Wen Cheng[a], Hsin-Lung Wu[a], Yeou-Lih Huang[b,c,d,*]

[a] Graduate Institute of Pharmaceutical Sciences, Kaohsiung Medical University, Kaohsiung 807, Taiwan
[b] Department of Medical Laboratory Science and Biotechnology, Kaohsiung Medical University, Kaohsiung, Taiwan
[c] Graduate Institute of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan
[d] Center of Excellence for Environmental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan

ARTICLE INFO

Article history:
Available online 4 February 2009

Keywords:
Malondialdehyde
Ofloxacin
On-line
Microdialysis
Liquid chromatography

ABSTRACT

We have developed a system that couples an on-line microdialysis (MD) system with flow injection high-performance liquid chromatography (HPLC)-fluorescence detection for simultaneous measurement of the concentrations of malondialdehyde (MDA) and ofloxacin (OFL) in whole blood samples. The sample matrix was first cleaned with an MD system using an MD probe. A continuously flowing dialysate stream was derivatized on-line and auto-injected into a separation column. MDA and OFL were separated through a reverse-phase C18 column (250 mm × 4.6 mm) at a flow rate of 0.8 mL min^{-1} and then detected using a fluorescence detector (excitation: 532 nm; emission: 553 nm); the system's components were connected on-line using a valve control. Validation experiments demonstrated good linearity, precision, accuracy, and recovery. The precisions for the determinations of MDA and OFL, measured in terms of relative standard deviations, were 6.5% and 4.6%, respectively, for intra-day assays and 7.5% and 8.7%, respectively, for inter-day assays. The average recoveries of MDA and OFL spiked in plasma were each close to 100%. The use of this on-line MD-HPLC system permitted continuous monitoring of MDA and OFL in OFL-treated whole blood subjected to UV-A irradiation. Based on our results, the UV-A irradiation markedly increased the level of MDA in the OFL-treated whole blood.

1. Introduction

The use of antimicrobial quinolones, such as ofloxacin (OFL), in the treatment of urinary tract infections, respiratory tract infections, osteomyelitis, and tendon disorders has led to concerns regarding harmful side effects [1–3]. These antibacterial agents can cause several side effects to the gastrointestinal system [4] and central nervous system [5] and can exhibit phototoxicity [6,7]. Matsumoto et al. [8] revealed that the eight position of the quinolone nucleus is very unstable under irradiation with UV-A, leading to reduced antibacterial activity and increased cytotoxicity toward cultured mammalian cells. This damage is induced through phototoxic reactions: in which the drug absorbs photonic energy and subsequently releases photoactivated agents that can damage cell components and lead to the formation of free radicals that can cause acute inflammation or skin allergies [9,10]. Animal studies have provided evidence that phototoxic reactions occur dose-dependently after UV-A exposure [11]. Moreover, although some quinolones degrade by UV-A radiation [12–14], studies with murine ears have revealed that not all quinolones cause obvious inflammation or edema [15]. This photodegradation process lowers the antibacterial activity of the quinolones, but generates some unknown products [10]. Wagai and Tawara [16] provided evidence that these photoproducts do not necessarily induce phototoxicity directly; they also determined the types of reactive oxygen species (ROS) [17].

Cellular oxidative damage is a well-established general mechanism for cell and tissue injury [18,19]. It is caused primarily by free radicals and ROS. Free radicals have the ability to bind to most normal cellular components. Hydroxyl radicals, singlet oxygen, and superoxide anions have been found in quinolone solutions after UV radiation [20,21]. Saniabadi et al. [22] revealed that the generation of ROS produces leukocyte stimulants and causes leukocyte accumulation at the irradiated site. This adverse effect from photodegradation damages parts of the host tissue, such as DNA, and can lead to retinal degeneration [23]. Cuquerella et al. [24] provided evidence that OFL mediates oxidative DNA damage, as estimated from the determination of 8-OH-dGuo (a biomarker of oxidative damage). All of this evidence suggests that quinolones can produce

* Corresponding author at: Department of Medical Laboratory Science and Biotechnology, Kaohsiung Medical University, 100, Shih-Chuan 1st Road, Kaohsiung 807, Taiwan. Tel.: +886 7 312 1101x2251; fax: +886 7 311 3449.
E-mail address: yelihu@kmu.edu.tw (Y.-L. Huang).

0039-9140/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2009.01.044

oxidative effects on cells or their components through the generation of ROS after UV-A exposure. An increasing amount of research is being published in the field of antibiotic-induced oxidative stress in which malondialdehyde (MDA) is commonly employed as a biomarker [25].

Few studies have been devoted to establishing a simultaneous monitoring method for oxidative stress markers and antibiotics; however, in our previous work [26], a procedure for off-line determination of quinolone and MDA was proposed. In the present study, we developed an on-line microdialysis (MD) sampling system coupled with a flow injection HPLC system for simultaneous on-line determination of OFL and MDA in whole blood. Microdialysis sampling is a diffusion-based separation process that requires analytes to diffuse through a semi-permeable membrane before being transferred to the outlet through a flowing perfusion fluid [27]. The MD system permits the efficient sampling of small target molecules in plasma by excluding large interfering molecules, such as proteins. In addition to being a sampling technique, microdialysis has also been shown to be an effective sample clean-up technique, is readily automated, and can be coupled on-line to various kinds of analytical systems (e.g., liquid chromatographs) for biomedical and environmental investigations [28].

Because the phototoxic reactions of OFL that result from UV-A irradiation can be accompanied with oxidative stress, a method that simultaneously monitors the OFL level and oxidative stress biomarkers would provide further information for investigating their relationship. Therefore, the present study was aimed at the development of an on-line MD-HPLC method for continuous, real time, dynamic and simultaneous determination of MDA and OFL. The major advantage of this proposed method is that both MDA and OFL can be determined using a single chromatographic system. This method reduces the amount of pre-column work, is not time-consuming, and provides MDA and OFL profiles during UV-A radiation.

2. Experimental

2.1. Chemicals and reagents

All chemicals and reagents were of analytical or HPLC grade. 1,1,3,3-Tetraethoxypropane (TEP) and OFL were obtained from Sigma–Aldrich (St. Louis, MO, USA); thiobarbituric acid (TBA), sodium hydroxide, potassium hydroxide, potassium phosphate, ethanol, and methanol from Merck (Darmstadt, Germany); phosphoric acid from J.T. Baker (Phillipsburg, NJ, USA). Ultrapure water was prepared using a Barnstead nanopure water system (Dubuque, IA, USA); the filtered water was used to prepare mobile phases and other related solutions.

2.2. Instrumentation

A schematic representation of the on-line MD-HPLC system used for the simultaneous determination of MDA and OFL in biological samples is presented in Fig. 1. The microdialysates were perfused through an MD probe and were continuously pumped into a micro-Tee (Valco Instruments, Houston, TX, USA) connector. Fluorinated ethylene polypropylene (FEP) tubing (CMA, Stockholm, Sweden) was used to connect the microinjection syringe pump to the micro-Tee to introduce TBA for the derivatization reaction. A delivery tube containing a derivatized sample was connected to the sample loop, and then injected into the chromatographic system.

2.3. MD system

The MD system was purchased from Carnegie Medicine Associates (CMA, Stockholm, Sweden). The MD sampling system consisted of a microinjection syringe pump (CMA/100) and a 10 mm long MD probe (CMA/20) equipped with a 0.5-mm-diameter, metal-free, polyarylethersulfone (PAES) membrane with a molecular mass cut-off of 20 kDa. The probe was perfused (1 μL min^{-1}) with this syringe pump. FEP tubing was used to connect the microinjection syringe pump to the inlet of the MD probe and from the outlet of the MD probe to the six-port on-line injector (CMA/160). FEP tubing was connected to the microinjection syringes, the probe, and the other FEP tubing by tubing adaptors (CMA, Stockholm, Sweden), which ensured tight and zero-internal-volume connections.

2.4. Derivatization procedure

A microinjection syringe pump was employed to propel the microdialysate into the micro-Tee connector, and the eluent was pumped continuously. FEP tubing was used to connect the microinjection pump to the micro-Tee to introduce TBA for derivatization of samples, which was performed by mixing the eluent (TBA/H_3PO_4) in the flow tubing that was heated at 85 °C. The delivery tube containing the derivatized sample was connected to a sample loop within the on-line injector and then the sample was injected into the chromatographic system. The on-line injector was specially designed for the direct injection of dialysates into chromatographic columns during microdialysis experiments.

2.5. LC conditions

The HPLC system consisted of a Jasco PU-2085 solvent delivery pump, a silica-based reverse-phase analytical column (Supelco LCC18, 5 μm particle size, 150 mm × 4.0 mm i.d., Bellefonte, PA, USA), a guard column packed with the same material, and a Jasco FP-920 fluorescence detector operated at an excitation wavelength of 532 nm and an emission wavelength of 553 nm. The analytical column was maintained at ambient temperature. Samples were introduced through an injector valve equipped with a 20-μL sample loop. Chromatographic control, data collection, and processing were performed using a personal computer running SISC software (Chem-Lab, Taipei, Taiwan). The mobile phase consisted of 50:50 (v/v) methanol/0.05 M phosphate buffer; the pH was adjusted to 4.8 using KOH and filtered through an advanced cellulose ester 0.2-μm filter (Tokyo, Japan) before use; the flow rate was 0.8 mL min^{-1}.

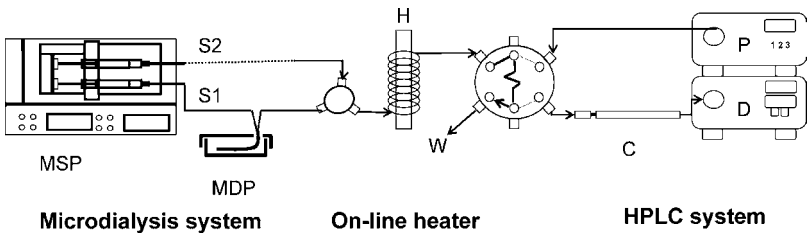

Fig. 1. Schematic representation of the on-line MD-HPLC system developed for the simultaneous determination of MDA and OFL in blood. MSP, microinjection syringe pump; MDP, microdialysis probe; S1, Ringer's solution; S2, TBA solution; H, heater; W, waste; C, column; P, HPLC pump; D, fluorescence detector.

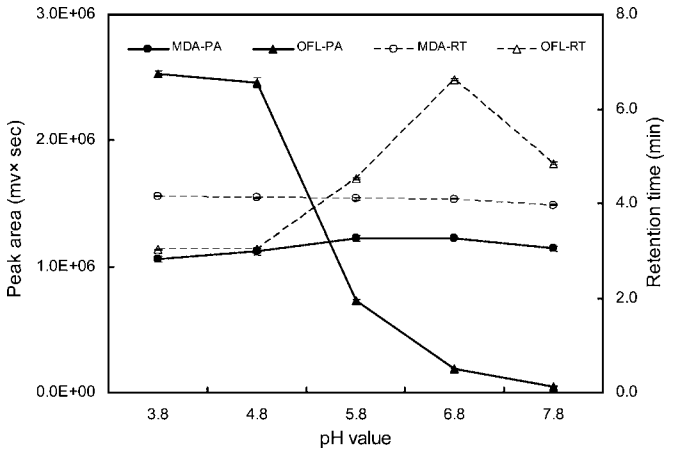

Fig. 2. Effect of the pH of the mobile phase on the signals of MDA-PA (-●-) and OFL-PA (-▲-) and the retention time of MDA-RT (-○-) and OFL-RT (-△-) detected by the HPLC column. (PA: peak area; RT: retention time).

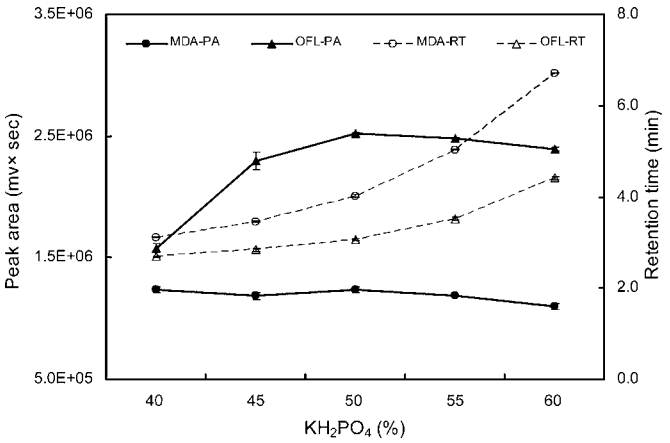

Fig. 3. Effect of the percentage of KH_2PO_4 in the mobile phase on the signals of MDA-PA (-●-) and OFL-PA (-▲-) and the retention time of MDA-RT (-○-) and OFL-RT (-△-). (PA: peak area; RT: retention time).

2.6. Analytical characteristics

The linearity, intra- and inter-day assay precisions, and recovery were determined by analyzing samples containing known concentrations of MDA and OFL. The linear responses of MDA and OFL were assessed in the ranges 0.03–2.43 μM and 0.02–1.00 mM, respectively, for both the standard solutions and the standards added in water samples. The intra- and inter-day assay precisions were evaluated using samples of three different concentrations; these were calculated in terms of their relative standard deviation [R.S.D. (%) = (S.D./mean) × 100; S.D. = standard deviation] after quantifying seven replicates on the same day (intra-day assay) or 5 consecutive days (inter-day assay). The recovery tests were performed using samples spiked with MDA and OFL. The limit of detection (LOD) was defined as the lowest concentration that produced a signal-to-noise (S/N) ratio of 3:1 with the limit of quantitation (LOQ) being defined as a signal-to-noise ratio of 10:1.

3. Results and discussion

To optimize the proposed HPLC method for simultaneous determination of MDA and OFL, we studied the effects of several parameters, including the concentrations of the derivatizing agent (TBA), potassium phosphate, and methanol and the pH and flow rate of the mobile phase. The effects of these parameters were evaluated and optimized based on the peak areas and retention times of the resulting signals for MDA and OFL.

3.1. Effect of pH and flow rate of the mobile phase on the signals of MDA and OFL

pH is the one of main factors influencing the signal shapes and retention times. In this study, we examined the effect of pH by varying it from 3.8 to 7.8. The pH of the mobile phase was adjusted using 1 M HCl or 1 M KOH. Fig. 2 indicates that OFL provided higher signals at lower pH, but its peak was positioned too close to that of MDA at pH 3.8. Therefore, we selected a pH of 4.8 for subsequent experiments. Next, we tested the effects of various flow rates, ranging from 0.6 to 1.4 mL min^{-1}, on the elution process. The elution of MDA and OFL at 0.8 mL min^{-1} provided the optimal retention times for both analytes; at higher flow rates, the resolution decreased. Thus, we selected a flow rate of 0.8 mL min^{-1} for the elution of MDA and OFL in the following experiments.

3.2. Optimization of the percentages of phosphate buffer and methanol in the mobile phase

When varying the phosphate buffer to methanol ratio from 40:60 to 60:40, we found that the resolution of MDA and OFL was poor (overlapping peaks) at lower percentages of phosphate buffer. Additionally, we obtained weak signals and prolonged retention times at higher percentages of phosphate buffer (Fig. 3). Therefore, we selected a phosphate buffer to methanol ratio of 50:50 for subsequent experiments. Table 1 summarizes the optimal parameters for the on-line MD-HPLC system.

Table 1
Main method parameters for the simultaneous determination of MDA and OFL using the developed on-line MD-HPLC system.

Microdialysis sampling	
Probe	10 mm × 0.5 mm PAES membrane (CMA/20)
Dialysate solution	
Syringe 1	Ringer's solution
Derivatizing reagent	
Syringe 2	39.9 mM TBA, 0.15 M H_3PO_4
Heater	85 °C, 30 min
HPLC	
Mobile phase	Methanol/0.05 M phosphate buffer, 50:50 (v/v), pH 4.8
Flow rate	0.8 mL min^{-1}
Column	Reverse-phase Phenomenex, RP-C18 column (250 mm × 4.60 mm, 5 μm)
Fluorescence detection	λ_{ex}: 532 nm; λ_{em}: 553 nm

Table 2
Analytical performance of the on-line MD-HPLC system.

	MDA	OFL
Calibration curve		
Linear range[a]	0.03–2.43	0.02–1.00
Correlation coefficient (γ)	0.9992	0.9994
LOD[a]	0.004	0.0004
LOQ[a]	0.014	0.0016
Precision (R.S.D., %)		
Intra-day assay[b]	4.5–6.5	3.6–4.6
Inter-day assay[c]	4.7–7.5	7.0–8.7
Recovery (n = 3, %)	96.3 ± 1.0	105.0 ± 0.8

[a] Units of μM and mM for MDA and OFL, respectively.
[b] Intra-day assay variance from seven replicate measurements of MDA and OFL at three intervals within a single day.
[c] Inter-day assay variance from triplicate measurements of MDA and OFL on 5 consecutive days.

3.3. Calibration, precision, and recovery

We determined the linearities of the MDA and OFL analyses using both a standard calibration method and a standard addition technique. Calibration graphs were established with the concentration of the analytes on the *x*-axes and the peak areas of the respective compounds on the *y*-axes. We obtained the following linear regression equations: for MDA, $y = 2000000x + 26115.3$ (± 37627.7) (correlation coefficient: $r = 0.9995$); for OFL, $y = 1000000x + 124626$ (± 26528) ($r = 0.9998$). We evaluated the precision of the method by using samples of five different concentrations of MDA and OFL and determining the LODs (S/N ratio = 3) and limits of quantification (LOQs; S/N ratio = 10). We also obtained intra- and inter-day assay precision data for the analyses of MDA and OFL. The correlation between the samples was acceptable within the accuracy of the method. We performed recovery analyses with this analytical system for blood dialysate samples spiked with 1.22 μM MDA and 0.5 mM OFL. Table 2 summarizes the relevant analytical data.

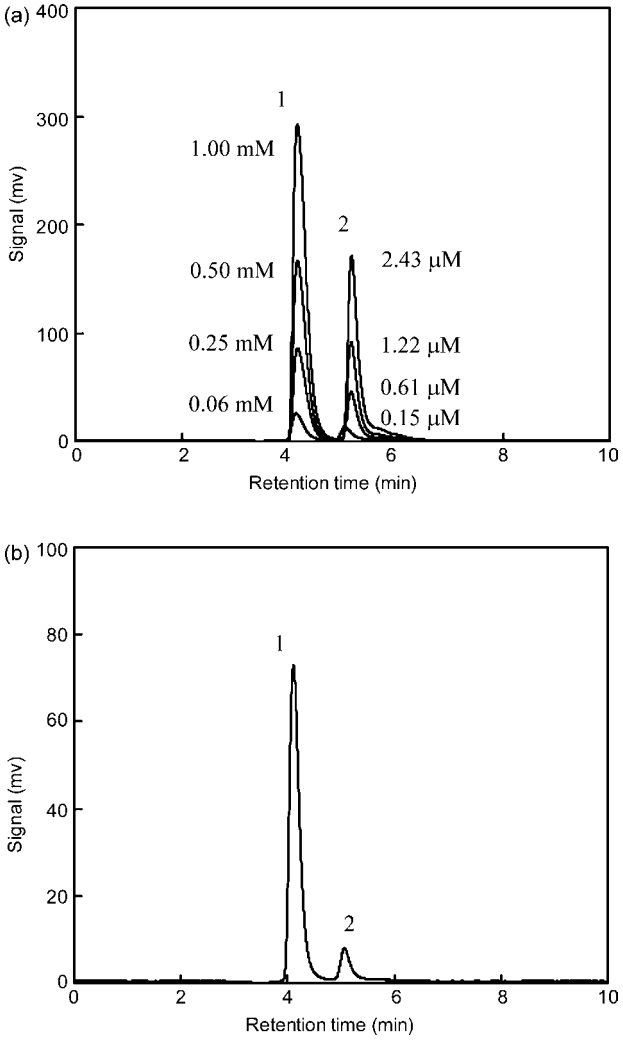

Fig. 4. Chromatograms obtained for the simultaneous determination of MDA and OFL using the developed on-line MD-HPLC system. (a) The standard mixtures contained 0.15, 0.61, 1.22, or 2.43 μM MDA and 0.06, 0.25, 0.50, or 1.00 mM OFL, respectively. (b) Blood spiked with 0.25 mM OFL and exposed to UV-A irradiation. Peak labels: (1) OFL; (2) MDA. The conditions were the same as those described in Table 1.

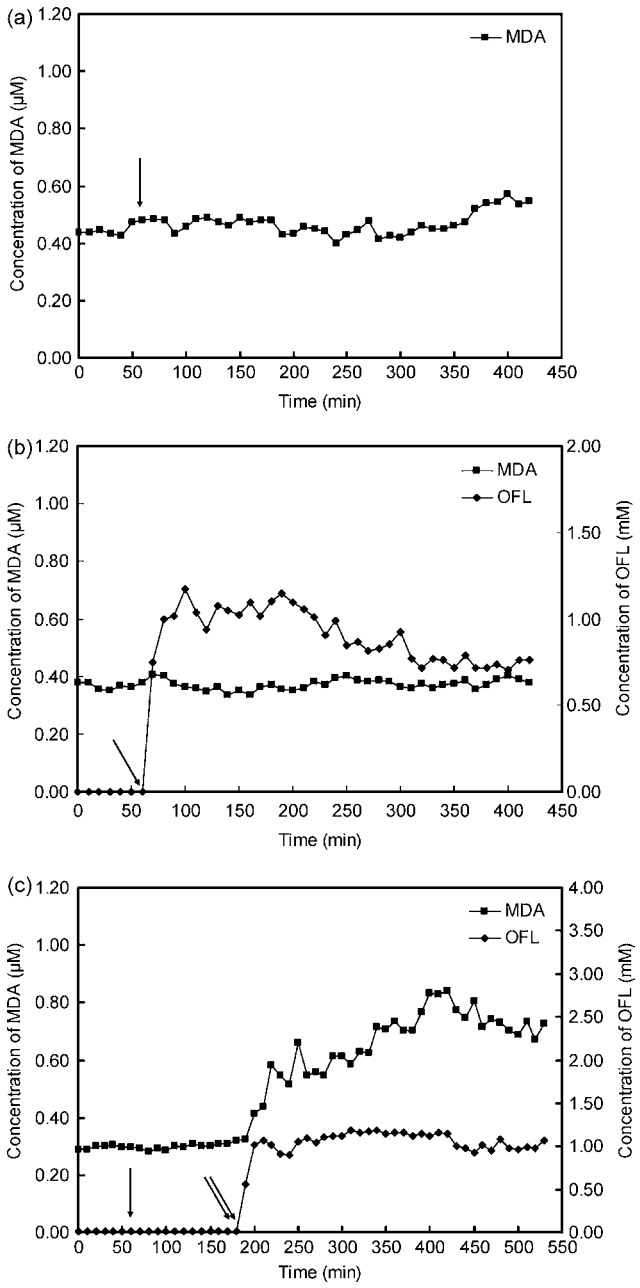

Fig. 5. Continuous on-line determination of the concentrations of MDA and OFL using the developed MD-HPLC detection system ($n = 5$). (a) Effect of UV-A irradiation on the blood concentration of MDA in the absence of added OFL; ↓: irradiated with UV-A (at 60 min). (b) Effect of OFL on the blood concentration of MDA in the absence of UV-A irradiation; ↓: treated with OFL (at 60 min). (c) Effect of both OFL exposure and UV-A irradiation on blood MDA concentration; ↓: onset of UV-A irradiation (60 min); ↓ ↓ onset of treatment with OFL (180 min). The conditions were the same as those described in Table 1.

3.4. Simultaneous determination and chromatographic separation of MDA and OFL in spiked blood dialysate

Under the optimized conditions, we evaluated the quantitative applicability of the method through simultaneous determination of the concentrations of MDA and OFL in spiked blood dialysate samples over the ranges 0.15–2.43 μM and 0.06–1.00 mM, respectively. Fig. 4 displays a set of typical chromatograms. The mobile phase conditions that provided the optimal selectivity, efficiency, and sensitivity were investigated. To the best of our knowledge, the proposed system is the first method reported that uses on-line MD

sampling coupled with HPLC devices for the detection of MDA and OFL without prior sample preparation. The overall analysis time for each sample in the present study was rather rapid compared with previous studies [29–32]. Previously reported single-separation methods, for MDA or fluoroquinolones, require more than twice the time for complete analysis. However, in the present study, both MDA and OFL can be detected simultaneously when using this on-line system. In addition to the pre-column derivatizations of the proposed method, the proposed method distinctively features an on-line MD coupled with an HPLC system. This method reduces the time interval resolution and provides real time, dynamic alteration of analytes in biological samples.

3.5. Application

To determine the practical applicability of using the developed system, we collected five blood samples, where each sample was divided for three different treatment conditions. Fig. 5(a) depicts the effect of UV-A exposure on the concentration of MDA in the samples in the absence of added OFL. We monitored the baseline for 60 min prior to UV-A exposure, and then the irradiation process was performed continuously for another 6 h. The concentration plot remained nearly constant from the beginning to the end of the UV-A exposure process, i.e., the MDA concentration was not obviously affected under irradiation. Fig. 5(b) displays the effect of added OFL on the blood MDA concentration in the absence of UV-A exposure. We monitored the system continuously for 6 h. After OFL was added to the blood, an apparent initial increase in concentration was observed; however, we did not observe any significant change in the MDA concentration under these conditions. Fig. 5(c) displays the effect of OFL on the blood concentration of MDA under UV-A exposure. It is clear that the MDA concentration underwent an obvious elevation after OFL had been added and UV-A irradiation was applied. Although some reports have suggested that OFL undergoes photodegradation upon the absorption of light energy [12–14], we did not observe such phenomena in this study. This work showed evidence that OFL is photosensitive under UV irradiation. Therefore, MDA can be used as an ROS marker for oxidative injury induced by UV irradiation of OFL.

4. Conclusion

We have developed a reliable, automatic, on-line method – involving MD sampling, derivatization with heating, and HPLC measurement – for continuous and simultaneous monitoring of MDA and OFL concentrations in real samples. This method does not require any time-consuming sample preparation. The responses for MDA and OFL were linear over practical concentration ranges; additionally, this method is highly reproducible. To the best of our knowledge, our proposed system is the only report of a method that uses on-line MD sampling coupled with a heated derivatizing device and an HPLC system for the detection of MDA and OFL in biological samples without the need for prior sample preparation. In addition to the practical application of analyzing biological samples, this procedure can also be used to monitor the effects of UV-A radiation-induced oxidative stress of OFL-treated samples. The system has been successfully used for a dynamic study of the effects of OFL on the levels of MDA.

Acknowledgments

We thank the National Science Council of Taiwan (NSC95-2113-M-037-020-MY3) and the Center of Excellence for Environmental Medicine of Kaohsiung Medical University (KMU-EM-97-3.3) for financial support.

References

[1] G. Carlucci, J. Chromatogr. A 812 (1998) 343.
[2] F. Belal, A.A. Al-Majed, A.M. Al-Obaid, Talanta 50 (1999) 765.
[3] R. Stahlmann1, H. Lode, Drugs Aging 20 (2003) 289.
[4] W. Christ, T. Lehnert, B. Ulbrich, Rev. Infect. Dis. 10 (Suppl. 1) (1988) S141.
[5] H. Halkin, Rev. Infect. Dis. 10 (Suppl. 1) (1988) S258.
[6] D.A. Birkett, M. Garretts, C.J. Stevenson, Br. J. Dermatol. 81 (1969) 342.
[7] Y. Kawabe, N. Mizuno, S. Sakakibara, Photodermatology 6 (1989) 58.
[8] M. Matsumoto, K. Kojima, H. Nagano, S. Matsubara, T. Yokota, Antimicrob. Agents Chemother. 36 (1992) 1715.
[9] D.E. Moore, V.J. Hemmens, H. Yip, Photochem. Photobiol. 39 (1984) 57.
[10] K. Marutani, M. Matsumoto, Y. Otabe, M. Nagamuta, K. Tanaka, A. Miyoshi, T. Hasegawa, H. Nagano, S. Matsubara, R. Kamide, T. Yokota, F. Matsumoto, Y. Ueda, Antimicrob. Agents Chemother. 37 (1993) 2217.
[11] T. Horio, H. Miyauchi, Y. Asada, Y. Aoki, M. Harada, J. Dermatol. Sci. 7 (1994) 130.
[12] G.B.E. Phillips, J. Johnson, Ferguson, J. Antimicrob. Chemother. 26 (1990) 783.
[13] J. Sunderland, C.M. Tobin, A.J. Hedges, A.P. MacGowan, L.O. White, J. Antimicrob. Chemother. 47 (2001) 271.
[14] P. Sukul, M. Spiteller, Rev. Environ. Contam. Toxicol. 191 (2007) 131.
[15] K. Yabe, K. Goto, T. Jindo, M. Sekiguchi, K. Furuhama, Toxicol. Lett. 157 (2005) 203.
[16] N. Wagai, K. Tawara, Arch. Toxicol. 65 (1991) 495.
[17] N. Wagai, K. Tawara, Arch. Toxicol. 66 (1992) 392.
[18] M. Ott, V. Gogvadze, S. Orrenius, B. Zhivotovsky, Apoptosis 12 (2007) 913.
[19] L. Chen, H.M. Lee, G.H. Greeley Jr., E.W. Englander, Free Radic. Biol. Med. 42 (2007) 385.
[20] T. Araki, H. Kitaoka, Chem. Pharm. Bull. 46 (1998) 1021.
[21] N. Umezawa, K. Arakane, A. Ryu, S. Mashiko, M. Hirobe, T. Nagano, Arch. Biochem. Biophys. 342 (1997) 275.
[22] A.R. Saniabadi, K. Wada, K. Umemura, S. Sakuma, M. Nakashima, J. Photochem. Photobiol. B: Biol. 33 (1996) 137.
[23] V. Wiebe, P. Hamilton, J. Am. Vet. Med. Assoc. 221 (2002) 1568.
[24] M.C. Cuquerella, F. Boscá, M.A. Miranda, A. Belvedere, A. Catalfo, G. de Guidi, Chem. Res. Toxicol. 16 (2003) 562.
[25] D.R. Janero, Free Radic. Biol. Med. 9 (1990) 515.
[26] G.W. Cheng, H.L. Wu, Y.L. Huang, Anal. Chim. Acta 616 (2008) 230.
[27] D.J. Weiss, C.E. Lunte, S.M. Lunte, Trends Anal. Chem. 19 (2000) 606.
[28] G. Jin, Q. Cheng, J. Feng, F. Li, J. Chromatogr. Sci. 76 (2008) 276.
[29] Y. Sun, Z. Zhang, Z. Xi, Anal. Chim. Acta 623 (2008) 96.
[30] S. Bielecka-Grzela, A. Klimowicz, Pol. J. Pharmacol. 55 (2003) 613.
[31] C.S. Yang, P.J. Tsai, W.Y. Chen, J.S. Kuo, J. Chromatogr. B 752 (2001) 33.
[32] J.Y. Sheu, H.P. Ku, W.C. Tseng, M.T. Chen, L.Y. Tsai, Y.L. Huang, Anal. Sci. 19 (2003) 621.