BIOMEDICAL CHROMATOGRAPHY
Biomed. Chromatogr. **20**: 787–793 (2006)
Published online 24 November 2005 in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/bmc.598

ORIGINAL RESEARCH

Assay of naproxen by high-performance liquid chromatography and identification of its photoproducts by LC-ESI MS

Yi-Hsin Hsu,[1] Yi-Bo Liou,[2] Jen-Ai Lee,[2] Chau-Yang Chen[3] and An-Bang Wu[2]*

[1]Division of Gastroenterology, Shin Kong Wu Ho-Su Memorial Hospital, Taipei 11101, Taiwan, Republic of China
[2]College of Pharmacy, Taipei Medical University, Taipei 11031, Taiwan, Republic of China
[3]Department of Pharmacy, Tajen University, Yan-pu, Pingtung 90741, Taiwan, Republic of China

Received 26 August 2005; accepted 28 September 2005

ABSTRACT: A rapid, accurate and reliable reversed-phase high-performance liquid chromatographic (HPLC) method for the determination of naproxen and its photodegradation products in methanol was developed and validated. An Inertsil 5-ODS-3V column (5 μm, C_{18}, 250 × 4.6 mm i.d.) was used with a mobile phase of acetonitrile–methanol–1% HOAc in H_2O (40:20:40, v/v/v). UV detection was set at 230 nm. The developed method satisfies system suitability criteria, peak integrity and resolution for the parent drug and its photoproducts. The intraday and interday standard deviations of five replicate determinations for five consecutive days at the working concentrations of 5.0, 10, 25, 50, and 100 μM were 0.23–0.98 with coefficients of variance (CVs) of between 0.96 and 4.56% for the former, and 0.14–1.15 with CVs of between 1.13 and 3.82% for the latter. The percentage recoveries were determined to be 98.34, 99.19, 100.18, 102.97 and 99.81%, respectively, at the five concentrations between 5.0 and 100 μM. The limit of quantitation of naproxen was determined to be 0.29 μg/mL, while the detection limit was 64 ng/mL. Four major photoproducts were observed from the HPLC chromatogram using a Panchum PR-2000 reactor which equipped with 8 W × 16 low-pressure quartz mercury lamps as the light source for irradiation of a naproxen sample in methanol. The structures of the photoproducts were confirmed by LC-ESI MS. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: naproxen; HPLC; validation; photoproducts; LC-ESI MS

INTRODUCTION

Naproxen, 2-(6-methoxy-2-naphthyl)propanoic acid, was first synthesized by Syntex Research (Harrison *et al*., 1970). Naproxen (a Cox-1 inhibitor) is a typical non-steroidal anti-inflammatory drug (NSAID) which has analgesic and antipyretic activities (Peswani and Lalla, 1990). Recently naproxen has become the one of the most popular NSAIDs prescribed in Taiwan (Kao *et al*., 2003). However, from October 27 to December 20, 2004, *FDAnews Drug Daily Bulletin* (from the USA) released a series of bad news concerning the withdrawal of Vioxx (rofecoxib, a Cox-2 inhibitor) from the market because of an increased risk of heart attacks and strokes in patients taking the drug. Similar worries extended to Celebrex (also a Cox-2 inhibitor) and naproxen.

*Correspondence to: A.-B. Wu, Graduate Institute of Pharmaceutical Sciences, College of Pharmacy, Taipei Medical University, No. 250 Wu-Hsing Street, Taipei 11031, Taiwan, Republic of China.
E-mail: anbangwu@tmu.edu.tw

Abbreviations used: IN, indomethacin; NAP, (*S*)-naproxen; NSAID, non-steroidal anti-inflammatory.

Contract/grant sponsor: Shin Kong Wu Ho-Su Memorial Hospital; Contract/grant number: SKH-TMU-93-09.

During the past two decades, there have been quite a few chromatographic methods reported for the quantitative determination of naproxen and its metabolites in biological samples (Wan and Matin, 1979; van Loenhout *et al*., 1982; Streete, 1989; Anderson and Hansen, 1992; Sidelmann *et al*., 2001; Tashtoush and Al-Taani, 2003). Some high-performance liquid chromatographic (HPLC) methods have also been developed for the quantitation of naproxen and related compounds or impurities (Moir *et al*., 1990; Ekpe *et al*., 2001; Monser and Darghouth, 2003). In the present study, we aim to examine the recent and eminent problems raised by naproxen by the following considerations. Firstly, when relatively larger quantities of drugs including NSAIDs are prescribed on a daily basis, the pharmaceutical manufacturers must clearly demonstrate that the drug or the dosage form they produce is sufficiently stable that it can be stored for reasonable lengths of time without changing to an inactive or toxic form. When drugs are stored on shelves, are they inevitably exposed to fluorescent lights, or if drugs are used externally, are they always in active forms even when subjected to sunlight irradiation? Furthermore, the applicability of the existing HPLC methods to samples containing photoproducts has yet to be fully clarified.

Secondly, in an attempt to quantify a series of NSAIDs including carprofen (Wu *et al*., 2001), zomepirac (Chen *et al*., 2003a), and tolmetin (Chen *et al*., 2003b), HPLC was proven to be a powerful tool. Here, we attempted to develop an HPLC assay method, which enables the simultaneous detection of photoproducts of naproxen. In addition, the structural identification of the photoproducts by LC-ESIMS is also attempted in order to solve the question 'have we overlook any constituent after photo-irradiation?'

EXPERIMENTAL

Chemicals and reagents. (*S*)-Naproxen (NAP) and indomethacin (IN) were purchased from Sigma Chemical (St Louis, MO, USA). LC-grade methanol, ethanol absolute and acetonitrile were from Merck (Darmstadt, Germany). Reagent-grade glacial acetic acid was the product of Riedel-deHaën (Seelze, Germany).

Preparation of naproxen standard solutions. An amount of 1.15 mg of naproxen was accurately weighed and placed in a 10 mL brown-colored volumetric flask. Methanol was added to volume to make the concentration of the stock solution exactly 500 μM. Samples of 100, 200, 500, 1000 and 2000 μL of the stock solution were respectively transferred to 10 mL brown-colored volumetric flasks. To each flask, 500 μL of indomethacin in methanol of 25 μM concentration was added as the internal standard and diluted with methanol to volume. The concentrations of the five standard solutions were 5.0, 10, 25, 50 and 100 μM, respectively. The samples were filtered by 0.45 μm Millipore membranes, and the filtrates were then subjected to HPLC analysis.

HPLC apparatus and assay conditions. For analytical purposes, a Hitachi L-6200 HPLC intelligent pump system (Tokyo, Japan) equipped with a Hitachi L-4200 UV–vis detector set at 230 nm, a DataApex Chromatography Station for Windows (CSW) version 1.7 integrator (Prague, The Czech Republic), and a GL Sciences Inertsil 5-ODS-3V column (5 μm, C_{18}) with a 250 × 4.6 mm i.d. (Tokyo, Japan) were used with a mobile phase of CH_3CN–CH_3OH–1% HOAc in deionized H_2O (40:20:40, v/v/v). The flow rate was 0.7 mL/min, and a Microliter™ 705 manual sample injector (Hamilton, Reno, NV, USA) was used with an injection volume of 20 μL. For preparative purpose, an Inertsil ODS-3 of 250 × 10 mm i.d. column (Tokyo, Japan) was used with the same mobile phase of CH_3CN–CH_3OH–1% HOAc in deionized H_2O (40:20:40, v/v/v). The deionized water was prepared using a Milli-Q filter system (Millipore, Milford, MA, USA).

LC-MS instrument and conditions. An HP series 1100LC/MSD (Palo Alto, CA, USA) instrument was used. The column was an Inertsil 3μ ODS (3) column (150 × 2.1 mm i.d.) and the mobile phase was CH_3OH–0.1% HOAc in deionized H_2O (70:30, v/v) at a flow rate of 0.2 mL/min. The UV detector was set at 230 nm and the injection volume 20 μL. The MS conditions were optimized as follows: API electron spray interface, positive mode polarity, drying gas flow 8 L/min, nebulizer gas pressure 48 psi, drying gas temperature 35°C, fragmentation voltage 80 V, capillary voltage 3500 V, and scan range *m*/*z* 0–500, 1.15 s/scan.

Photodegradation of naproxen. A Panchum PR-2000 reactor (Taipei, Taiwan), equipped with an 8 W × 16 low-pressure quartz mercury lamps (Sankyo Denki G8T5E) as the light source, was used. Irradiation was performed with the samples in stoppered quartz tubes mounted vertically on a merry-go-round rack at a speed of 6 rpm. The light intensity of the monochromatic radiation was measured at 306 nm to be 3.25 mW/cm^2 using a UVX Digital Radiometer Serial No. E. 16768 (UVP, Inc., Upland, CA, USA).

Naproxen (23.0 mg) was accurately weighed and placed in a 100 mL brown-colored volumetric flask. Methanol was added to volume to produce a sample concentration of exactly 1.0 mM (0.230 g/L). Four milliliters of the solution were transferred to a sample vial and capped. The sample was irradiated with the low-pressure Hg lamps for 3 days.

Validation of the HPLC method. The system suitability parameters, including the capacity factor (k'), selectivity (α), resolution (R_s), plate number (N) and asymmetric factor (A_s), of the HPLC system were established to adequate levels (Hsu and Chen 1994). The linearity of naproxen was assessed over the range 5.0–100 μM in methanol containing 25 μM of indomethacin as an internal standard. A calibration curve was constructed by plotting the NAP–IN response area ratio vs concentration. The precision of the method was assessed by intraday and inter-day variabilities at the usual working concentrations of 5.0, 10, 25, 50 and 100 μM with five replicate determinations for five consecutive days. The accuracy of the method was evaluated by the recovery test. Mimic excipients (starch/talc = 95/5, w/w) were compounded, and then 20 mg of the excipients was transferred to five individual 10 mL volumetric flasks. The 5.0–100 μM naproxen solutions containing 25 μM of indomethacin were prepared by adding adequate stock solutions of naproxen and indomethacin, which were then filled to the mark with methanol. After ultrasonication for 10 min and filtration through a 0.45 μm thickness of Millipore membrane, the filtrate was subjected to HPLC analysis.

RESULTS AND DISCUSSION

System suitability

The UV spectrum of naproxen showed four absorption maxima at 231 (1.884), 262 (0.153), 271 (0.152) and 332 (0.046) nm (absorbance). Thus for the HPLC assay of naproxen, the UV detector was set at 230 nm. The retention time of naproxen was found to be 13.83 min [Fig. 1(A)]. Indomethacin with a retention time of 28.89 min was chosen as an internal standard [Fig. 1(B)]. In order to determine the adequacy and suitability of the HPLC analytical system for method development and validation, the optimum conditions and suitability parameters, including the capacity factor

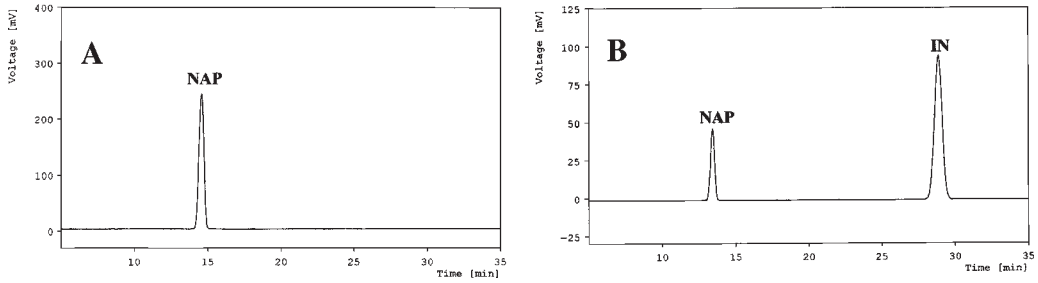


Figure 1. HPLC chromatograms of NAP in methanol: (A) standard solution; (B) NAP with IN as the internal standard.

Table 1. System suitability parameters for naproxen

Parameter	NAP	IN	Preferable levels
k'	6.5	16.5	
α	2.54		>1.02
R_s	100 (NAP-IN)		>1.50
	19 (NAP-**1**[a])		
	22.6 (NAP-**2**[a])		
	85 (NAP-**3**[a])		
	82.9 (NAP-**4**[a])		
A_s	1.1	1.01	0.9–1.3
N	360,000	217,777	

[a] Compounds **1**–**4** are the photoproducts of naproxen.

(k'), selectivity (α), resolution (R_s), plate number (N) and asymmetric factor (A_s), were established. The results are listed in Table 1. Peak specificity of naproxen was evaluated by comparing the ratio of the amount determined at two different wavelengths of 230 and 254 nm. The results of statistical comparison using one-way ANOVA are shown in Table 3.

Linearity

The linearity of the calibration curve was checked over a range of 5.0, 10, 25, 50 and 100 μM in methanol containing 25 μM of indomethacin as an internal standard. The calibration curve was constructed by plotting the NAP–IN response area ratio vs concentration. The calibration curve for naproxen was rectilinear in the concentration range studied (n = 5). The related coefficient, R^2 of the linear regression analysis was greater than 0.9987. The results of linear regression gave the equation $y = 0.3482\,x - 0.0874$. The analysis of variance for testing the significance of the regression is shown in Table 2. The F ratios for regression and lack-of-fit test confirm both the significance and the adequacy of the linear model.

Precision and accuracy

The intraday and inter-day standard deviations (SDs) of five replicate determinations for five consecutive days at the working concentrations of 5.0, 10, 25, 50 and 100 μM were between 0.23 and 0.98 with CVs of between 0.96 and 4.56% for the former, and 0.14–1.15 with CVs of between 1.13 and 3.82% for the latter (Table 4).

The accuracy of the method was evaluated by the recovery test. The results of the recovery test at the five concentrations of 5.0, 10, 25, 50 and 100 μM, were determined to be 98.34, 99.19, 100.18, 102.97 and 99.81%, respectively, which are shown in Table 5. There was no significant difference in a comparison with the results having 100% recovery ($p > 0.05$), which indicates good accuracy for the assay method.

Detection and quantitation limit

We began by analyzing a naproxen sample with HPLC which contained an amount equivalent to 5.0 μM of the drug. The signal response of naproxen in a signal-to-noise ratio of 10:1 was used to estimate the limit of quantitation (LOQ). The LOQ of naproxen was

Table 2. Analysis of variance of the naproxen calibration curve

Source of variation	d.f.[a]	SS[b]	MS[c]	F-ratio
Regression	1	3742.547	3742.547	67554.87[d]
Residual	23	1.274203	0.0554	
Lack-of-fit	3	0.1122935	0.03743	0.6453[e]
Pure error	20	1.1619094	0.0580	
Total	24	3743.822		

[a] d.f., degrees of freedom; [b] SS, sum of squares; [c] MS, mean square; [d] F-ratio > F, regression is significant; [e] F-ratio < F, there is no reason to doubt the linearity.

Table 3. Comparison between the peak area ratios of naproxen determined at 230 and 254 nm by ANOVA analysis

Component	Source of variation	d.f.[a]	SS[b]	MS[c]	*F*-ratio[d]
NAP	Between groups	3	2.357832	0.785944	0.671482
	Within groups	8	9.363692	1.170461	
	Total	11	11.72152		
IN	Between groups	3	0.001988	0.000663	0.604612
	Within groups	8	0.008769	0.001096	
	Total	11	0.010757		

[a] d.f., degrees of freedom; [b] SS, sum of squares; [c] MS, mean square; [d] *F*-ratio < *F*(3,8,0.95), difference between groups are not significant.

Table 4. Intraday and interday analytical precision values for naproxen ($n = 5$)

	Intraday			Interday		
Concentration (μM)	Mean (SD)	CV (%)	Relative error (%)	Mean (SD)	CV (%)	Relative error (%)
5	5.24 (0.23)	4.56	4.92	5.09 (0.19)	3.82	1.85
10	10.39 (0.44)	4.23	3.90	9.96 (0.14)	1.42	−0.32
25	25.34 (0.89)	3.52	1.38	25.57 (0.65)	2.55	2.29
50	51.19 (0.62)	1.21	2.38	50.86 (0.60)	1.18	1.72
100	101.30 (0.98)	0.96	1.30	101.24 (1.15)	1.13	1.24

Table 5. Spiked recovery (%) of naproxen ($n = 3$)

Calculated concentration (μM)	5	10	25	50	100
Concentration found	4.92	9.92	25.04	51.48	99.81
SD	0.26	0.32	0.25	1.58	1.92
CV (%)	5.19	3.21	0.99	3.07	1.92
Recovery (%)	98.34	99.19	100.18	102.97	99.81

determined to be 0.29 μg/mL as the average value of three consecutive injections, while the limit of detection (LOD) was determined to be 64 ng/mL with a signal-to-noise ratio of 3:1. In conclusion, the established assay method exhibits good selectivity and specificity and is suitable for stability measurements.

Photodegradation of naproxen

The HPLC chromatogram of naproxen photo-irradiated under the Hg lamps for 3 days is shown in Fig. 2(A). Naproxen was photodegraded to four major photoproducts which were observed with their respective retention times in increasing order as shown in Table 6. It is obvious that naproxen is very unstable when it exposes to light.

Structural identification of photoproducts by LC-ESI MS

The structural elucidation of the naproxen photoproducts by UV, IR, ^{1}H-, ^{13}C-, 2D-NMR and EI-MS spectroscopic methods had been reported recently (Ho *et al*., 2005). In the present study, LC-ESI MS technique was applied to re-examine the structures of the photoproducts including the two minor components as observed from the LC chromatogram [Fig. 2(A)]. The photoproducts derived from naproxen were subjected to a close examination on their chemical structures based solely on *m*/*z* characteristics. It is interesting to note that with a mild and optimized fragmentation voltage of 80 V, the quasimolecular ions of photoproducts **1**, **4a** and **4b** were fragmented with the disappearance of their original vital functional groups [Figs 2(B) and 3]. By a careful comparison of HPLC chromatogram (Ho *et al*., 2005) and MS signals appearing in LC-ESI MS, the chemical structures of the photoproducts were finally resolved: **1**, 1-(6-methoxy-naphthalen-2-yl)ethanol; **NAP**, 2-(6-methoxy-naphthalen-2-yl)propanoic acid; **2**, 1-(6-methoxy-naphthalen-2-yl)ethanone; **4a** and **4b**, methyl 2-(6-methoxy-naphthalen-2-yl)propanoate; **3**, 2-ethyl-6-methoxynaphthalene, as listed in Table 6. The results obtained from LC-ESI MS agreed perfectly with those from EI-MS and various spectroscopy methods, as reported previously (Ho *et al*., 2005).

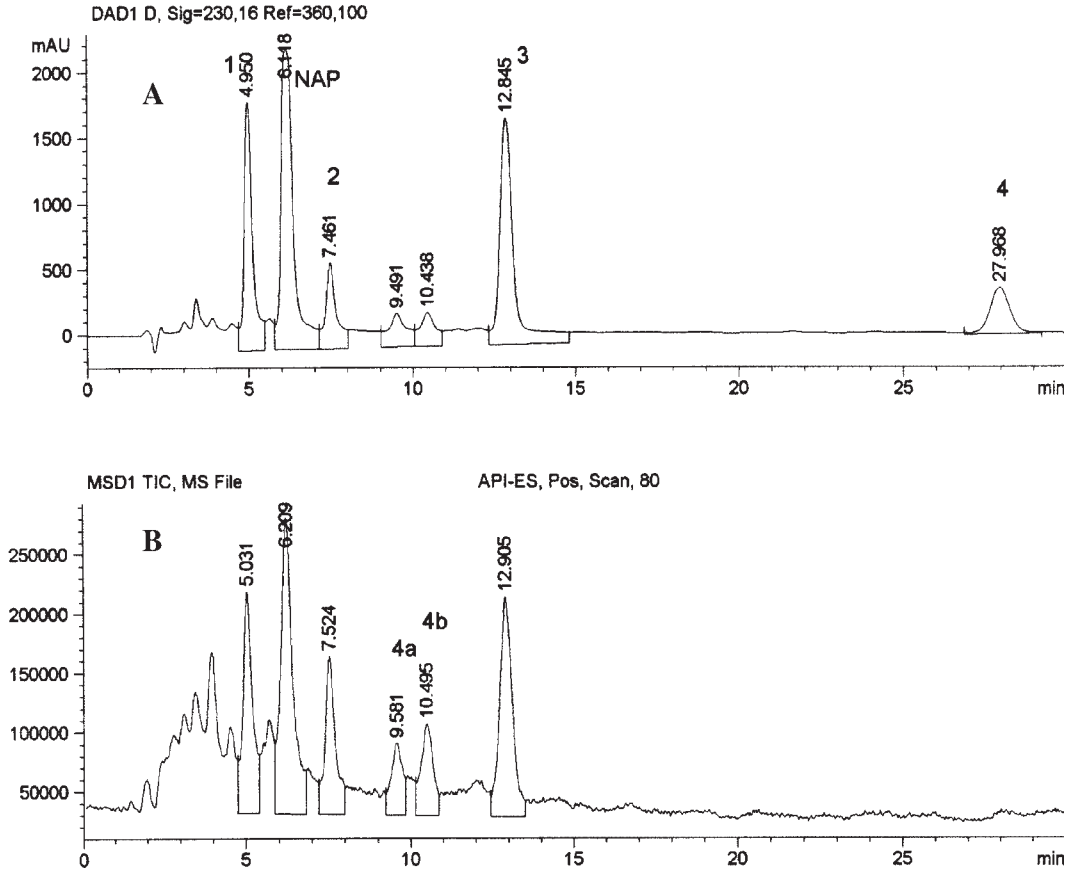


Figure 2. LC-ESIMS of an 1.00 mM NAP sample in methanol photo-irradiated by the low-pressure Hg lamps for 3 days. (A) LC signals with the four photoproducts numbered and arranged in increasing order of retention times; (B) MS signals after passing through ESI positive ion mode interface.

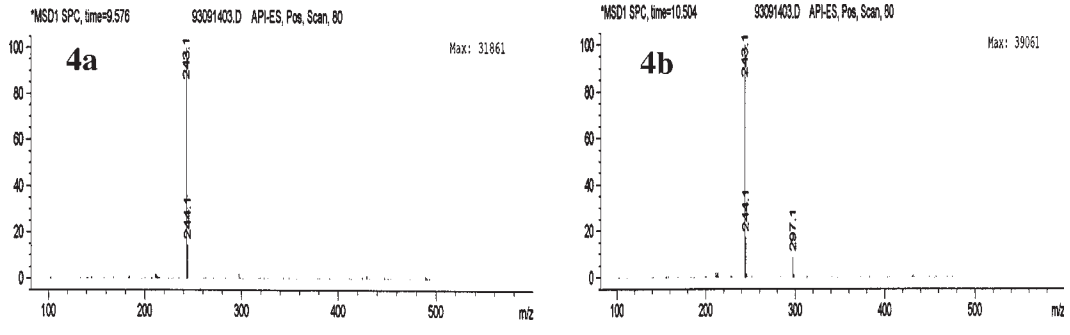
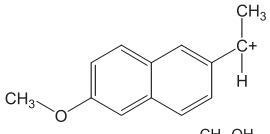
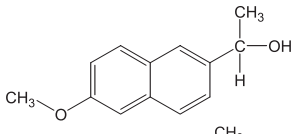
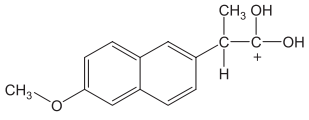
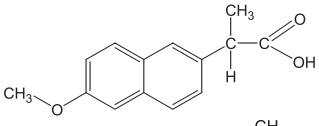
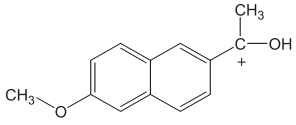
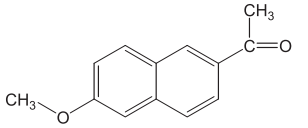
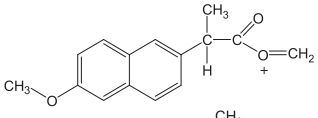
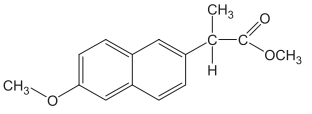
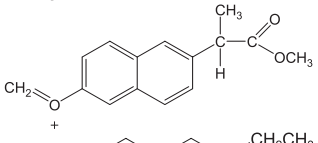
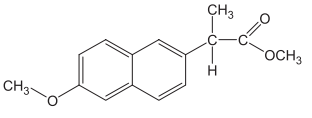
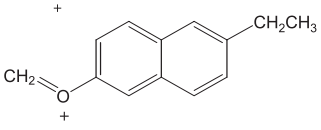
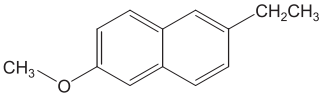


Figure 3. Mass spectra of the photoproducts **4a** and **4b**.

Acknowledgments

This study was sponsored by the Shin Kong Wu Ho-Su Memorial Hospital (SKH-TMU-93-09). The authors also thank Cheng's Foundation for Pharmaceutical Sciences and the Tajen University for partial financial support. Mr Po-Yi Wang at National Laboratories of Foods and Drugs, Department of Health, Executive Yuan for taking LC-ESIMS is also acknowledged.

Table 6. Structure elucidation of naproxen photoproducts by LC-ESIMS

Compound	Retention time (min)	Quasimolecular ion *m/z*	Charged species	Chemical structure[a]
1	5.03	$[MH]^+$: 185.1 fragmentation: 170.1		
NAP	6.21	$[MH]^+$: 231.1 fragmentation: 199.1, 185.1		
2	7.52	$[MH]^+$: 201.1 fragmentation: 159.1		
4a	9.58	$[MH]^+$: 243.1		
4b	10.50	$[MH]^+$: 243.1		
3	12.91	$[MH]^+$: 185.1 fragmentation: 170.1		

[a] IUPAC names (molecular weight in g/mol): **1**, 1-(6-methoxy-naphthalen-2-yl)-ethanol (202); **NAP**, 2-(6-methoxy-naphthalen-2-yl)-propanoic acid (230); **2**, 1-(6-methoxy-naphthalen-2-yl)-ethanone (200); **4a** and **4b**, methyl 2-(6-methoxy-naphthalen-2-yl)-propanoate (244); **3**, 2-ethyl-6-methoxy-naphthalene (186).

REFERENCES

Anderson JV and Hansen SH. Simultaneous quantitative determination of naproxen, its metabolite 6-O-desmethylnaproxen and their five conjugates in plasma and urine samples by high-performance liquid chromatography on dynamically modified silica. *Journal of Chromatography: Biomedical Applications* 1992; **115**: 525.

Chen CY, Chen FA, Chen CJ, Wu KH and Wu AB. Stability-indicating HPLC assay method of zomepirac. *Journal of Food and Drug Analysis* 2003a; **11**(4): 87.

Chen FA, Chen CY, Chen CJ and Wu AB. Quantitation of tolmetin by high-performance liquid chromatography and method validation. *Journal of Chromatographic Science* 2003b; **41**(7): 381.

Ekpe A, Tong JH and Rodriguez L. High-performance liquid chromatographic method development and validation for the simultaneous quantitation of naproxen sodium and pseudoephedrine hydrochloride impurities. *Journal of Chromatographic Science* 2001; **39**(3): 81.

Harrison IT, Lewis B, Nelson P, Rooks W, Roszkowski A, Tomolonis A and Fried JH. Nonsteroidal anti-inflammatory agents. I. 6-Substituted 2-naphthylacetic acids. *Journal of Medicinal Chemistry* 1970; **13**: 203.

Ho HT, Liou YB, Lin PY, Wang PY, Lin DZL and Wu AB. Photolysis of NSAIDs. V. Photoproducts of naproxen in alcoholic solvents. Sixth Tetrahedron Symposium. *Challenges in Organic Chemistry*, Bordeaux, France, poster abstract no. 58, 2005; 140.

Hsu HC and Chen CS. Validation of analytical methods: a simple model for HPLC assay methods. *Journal of Food and Drug Analysis* 1994; **2**(3): 161.

Kao YH, Kuo SC and Jia SW. Analysis of drug consumption and prescribing dose in NHI, using non-steroidal anti-inflammatory drugs and serum lipid reducing agents as models, Research Project (DOH90-NH-018) Report, Department of Health, Executive Yuan, Taipei, Taiwan, 2003.

Mikami E, Goto T, Ohno T, Matsumoto H and Nishida M. Simultaneous analysis of naproxen, nabumetone and its major metabolite 6-methoxy-2-naphthylacetic acid in pharmaceuticals and human urine by high-performance liquid chromatography. *Journal of Pharmaceutical and Biomedical Analysis* 2000; **23**(5): 917.

Moir DB, Beaulieu N, Curran NM and Lovering EG. Liquid chromatographic determination of naproxen and related compounds in raw materials. *Journal of the Association of Officical Analytical Chemistry* 1990; **73**(6): 902.

Monser L and Darghouth F. Simultaneous determination of naproxen and related compounds by HPLC using porous graphitic carbon column. *Journal of Pharmaceutical and Biomedical Analysis* 2003; **32**(4–5): 1087.

Peswani KS and Lalla JK. Naproxen parenteral formulation studies. *Journal of Parenteral Science Technology* 1990; **44**(6): 336.

Sidelman UG, Bjornsdottir I, Schockcor JP, Hansen SH, Lindon JC and Nicholson JK. Direct coupled HPLC-NMR and HPLC-MS approaches for the rapid characterization of drug metablites in urine: application to the human metabolism of naproxen. *Journal of Pharmaceutical and Biomedical Analysis* 2001; **24**(4): 569.

Streete PJ. Rapid high-performance liquid chromatographic methods for the determination of overdose concentrations of some non-steroidal anti-inflammatory drugs in plasma or serum. *Journal of Chromatography: Biomedical Applications* 1989; **495**: 179.

Tashtoush BM and Al-Taani BM. HPLC determination of naproxen in plasma. *Pharmazie* 2003; **58**(9): 614.

van Loenhout JWA, van Ginneken CAM, Ketelaars HCJ, Kimenai PM, Tan Y and Gribnau FWJ. A high-performance liquid chromatographic method for the quantitative determination of naproxen and des-methyl-naproxen in biological samples. *Journal of Liquid Chromatography* 1982; **5**(3): 549.

Wan SH and Matin SB. Quantitative gas-liquid chromatographic analysis of naproxen, 6-*O*-desmethyl-naproxen and their conjugates in urine. *Journal of Chromatography* 1979; **170**(2): 473.

Wu AB, Chen CY, Chu SD, Tsai YC and Chen FA. Stability-indicating HPLC assay method and photostability of carprofen. *Journal of Chromatographic Science* 2001; **39**(1): 7.