Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1087 (2005) 223–228
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Enantiomeric analysis of (+)-menthol and (−)-menthol by fluorogenic derivatization and liquid chromatography

Yu-Ting Lin, Hsing-Lung Wu *, Hwang-Shang Kou, Shou-Mei Wu, Su-Hwei Chen

Graduate Institute of Pharmaceutical Sciences, Kaohsiung Medical University, Kaohsiung 807, Taiwan

Available online 5 February 2005

Abstract

A simple and sensitive liquid chromatography is described for the quantitative analysis of enantiomeric (+)-menthol and (−)-menthol that are lack of chromophore. The method is based on the derivatization of (+)-menthol and (−)-menthol with a fluorescent reagent, naproxen acyl chloride, in toluene. The resulting diastereomic derivatives were separated on a C_8 column with methanol–water–tetrahydrofuran (80:18:2, v/v) as a mobile phase; they were sensitively monitored with a fluorimetric detector (excitation 235 nm and emission 350 nm). The linear range for the quantitation of the enantiomers was 5.0–50 μM with a detection limit (signal to noise ratio = 3, injected volume 10 μl) of about 1 μM. Application of the method to the enantiomeric analysis of menthol in mint plants proved simple and feasible. Toluene was used for the extraction of menthol from the leaves of mint, and the resulting toluene extract was directly used for subsequent derivatization without solvent replacement.

Keywords: Menthol; Derivatization; Enantiomeric analysis

1. Introduction

Menthol is widely used in liqueurs, perfumery, cigarette, cough drops, and nasal inhalers [1,2] and adopted as a pharmacopeia drug [3].

Menthol is a chiral compound (Fig. 1) and there are reports that (−)-menthol has analgesic effect whereas (+)-menthol is lack of analgesic [4] and flavor [5] properties. As a consequence, chiral analysis of (+)-menthol and (−)-menthol is essential for the quality control, pharmacological study and biosynthesis observation (in plant) of the enantiomers.

A variety of methods has been established for the analysis of menthol, including achiral analysis of (+)-menthol and (−)-menthol by GC-flame ionization detection [6–8], GC–MS [9–11], LC with refractometric detection [12], LC with UV detection [13] and LC with fluorimetric detection [14], and chiral analysis of (+)-menthol and (−)-menthol by GC with substituted β-cyclodextrins [15–17] with insufficient separation, LC with polarized photometric detection [5] giving low sensitivity, and LC with circular dichroism detection [18] for configuration study.

In this work, an approach was used to label the analytes being fluorigenic and concomitantly to make the enantiomers as diastereomers, i.e., (+)-menthol and (−)-menthol were derivatized with chiral naproxen acyl chloride [19] at mild condition. The resulting diastereomic derivatives are highly fluorescent and can be separated on a conventional LC column. Application of the method to the analysis of the enantiomeric menthol in mint proved feasible.

2. Experimental

2.1. Materials and reagents

(1*S*, 2*R*, 5*S*)-(+)-Menthol [(+)-menthol] and (1*R*, 2*S*, 5*R*)-(−)-menthol [(−)-menthol] (ICN, Eschwege, Germany), α-naphthyl caprate (as internal standard, I.S.) (TCI, Tokyo, Japan), naproxen (Sigma, St. Louis, MO, USA), (±)-linalool, (±)-terpinen-4-ol and α-terpineol (Acr¯os, Morris Plains, NJ, USA), toluene and diethylamine (Tedia, Fairfied, OH, USA), thionyl chloride, methyl alcohol and tetrahydrofuran

* Corresponding author. Tel.: +886 7 3121101x2316; fax: +886 7 3159597.
E-mail address: hlwu@kmu.edu.tw (H.-L. Wu).

0021-9673/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2005.01.056

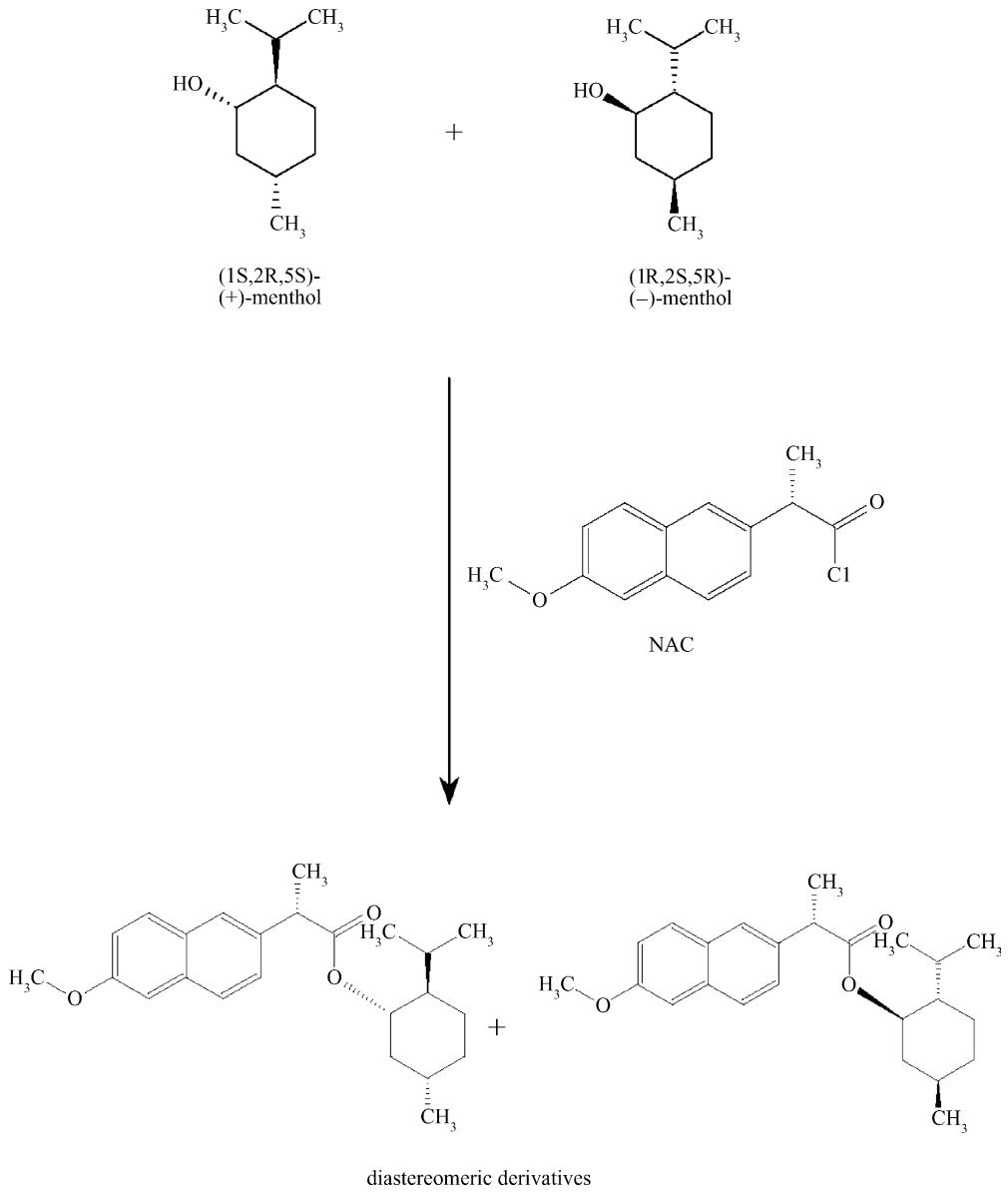

Fig. 1. Putative scheme for the derivatization of enantiomeric (+)-menthol and (−)-menthol with naproxen acyl chloride (NAC).

(E. Merck, Darmstadt, Germany) were used without further treatment. Naproxen acyl chloride was prepared as reported [19]. Other chemicals were of analytical reagent grade. Solutions of (+)-menthol, (−)-menthol, naproxen acyl chloride, and diethylamine were prepared by dissolving the appropriate amounts of the respective compounds in toluene; solution of I.S. was prepared in methanol. Distilled water purified with an ultrapure R/O water system (Millipore, MA, USA) was used for all aqueous solution.

2.2. LC conditions

A Waters LC system with a Model 515 pump, a Model 717 plus autosampler, a Model 474 scanning fluorescence detector and a Beckman Gold Nouveau data system were used. A Merck LiChrospher 60 RP-C_8 column (250 mm × 4 mm I.D., 5 μm) and a mixed solvent of methanol–water–tetrahydrofuran (80:18:2, v/v) at a flow rate of 1.2 ml/min were used. The column eluates were detected fluorimetrically (excitation at 235 nm and emission at 350 nm).

2.3. Derivatization procedure

A 100 μl aliquot of (+)-menthol and (−)-menthol solution (50 μM each in toluene) or sample solution was added to a 15 ml screw-capped test tube containing 100 μl of naproxen acyl chloride in toluene (300 mM). The reactants were mechanically shaken at 90 °C for 1 h in a thermostated shaker. After derivatization, 100 μl of diethylamine in toluene (10%,

v/v) were added and the solution was further shaken at 30 °C for 10 min to inactivate the excess acyl chloride reagent. A 100 μl aliquot of the resulting toluene solution was added with an equal volume (100 μl) of I.S. in methanol (3.00 mM) for being compatible with the mobile phase in chromatographic analysis. The resulting solution was analyzed by LC with an injection volume of 10 μl.

2.4. Sample pretreatment

Three species of mint plant including *Mentha spicata*, *Mentha piperita* 'Swiss' and *Mentha* × *piperita* 'Chocolate' were studied for the menthol content in their freshly collected leaves. Based on a preliminarily screening test, (+)-menthol and (−)-menthol can not be detected in the toluene extract of *M. spicata* (below the detection limit of this method), and the toluene extract of *M. spicata* was tentatively used as a sample blank solution for analytical calibration by spiking with various amount of (+)-menthol and (−)-menthol for real sample analysis. The detailed preparation of sample blank and sample solutions are as follows.

2.4.1. Sample blank solution

A 10.0 g aliquot of freshly collected mint leaves (*M. spicata*) was added with 90 ml of toluene in a beaker and triturated at room temperature with a homogenizer (Turrax T8, S8N-8G) (IKA, Germany) at 20,000 rpm for 15 min. The toluene extract was filtered (G2 sintered filter funnel) to a volumetric flask (100 ml). The sample residue on the homogenizer was washed with toluene (5 ml × 2) to the beaker, and the combined washings were filtered to the volumetric flask, and the final volume (100 ml) was adjusted with toluene. The resulting toluene solution tested to be in the absence of (+)-menthol and (−)-menthol was used as sample blank solution for preparing (+)-menthol and (−)-menthol spiked solution for the analytical calibration.

2.4.2. Sample solution

A 125.0 mg aliquot of freshly collected mint leaves (*M. piperita* 'Swiss' or *Mentha* × *piperita* 'Chocolate') was added with 3.0 ml of toluene in a 25 ml beaker and triturated as indicated in Section 2.4.1 at 20,000 rpm for 15 min. The toluene extract was filtered to a 5 ml volumetric flask. The sample residue on the homogenizer was washed with toluene (1 ml × 2) to the volumetric flask and the volume (5.0 ml) was adjusted with toluene (further dilution is made if required). The resulting sample solutions were subjected to subsequent derivatization (Section 2.3) for quantitation of the analytes.

3. Results and discussion

In order to optimize the derivatization conditions for (+)-menthol and (−)-menthol, several parameters affecting the derivatization of the analytes were studied, including reaction temperature and reaction time, and amount of naproxen acyl chloride. The amount of (+)-menthol and (−)-menthol each used for the study was 5 nmol (50 μM × 100 μl). The effects of the parameters on the formation of the (+)-menthol and (−)-menthol derivatives were evaluated and optimized based on the peak-area ratios of the resulting derivatives to the I.S.

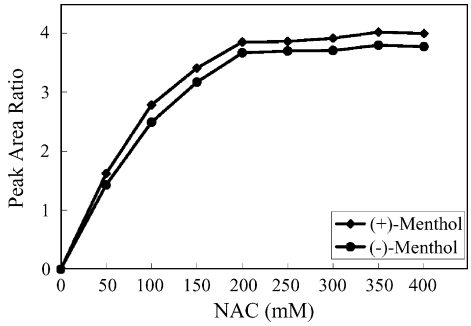

Fig. 2. Effect of naproxen acyl chloride concentration on the formation of the (+)-menthol and (−)-menthol derivatives ($n = 3$).

3.1. Optimization of the derivatization

For adopting a solvent that is miscible with the analyte menthol and capable of separating the analyte from aqueous biosample, we used water insoluble toluene as the derivatization solvent.

The main parameters in the derivatization procedure (Section 2.3) were varied to evaluate their effects on the derivatization of (+)-menthol and (−)-menthol in toluene. The effects of naproxen acyl chloride at varied concentrations (0–400 mM) on the derivatization of (+)-menthol and (−)-menthol indicate that plateau formation of the derivatives is attainable using naproxen acyl chloride at concentration ≥200 mM (Fig. 2). Considering the derivatization of (+)-menthol and (−)-menthol in a real sample covering additional nucleophic matrices, we used naproxen acyl chloride at 300 mM for the derivatization.

Derivatization of the enantiomeric menthol at 90 °C gave better yield than that derivatization at 70 °C (Fig. 3). Plateau formation of the derivatives of the menthol is attainable at 90 °C for 1.5 h, but derivatization of the analytes at 90 °C for a shorter time of 1.0 h was selected because it can give

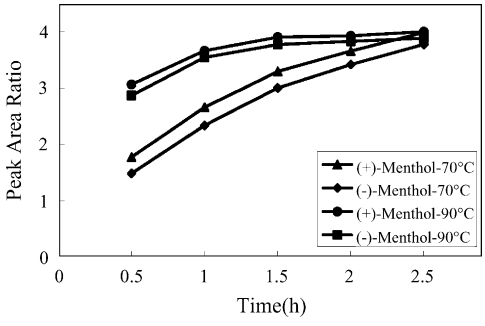

Fig. 3. Effects of reaction temperature and reaction time on the formation of the (+)-menthol and (−)-menthol derivatives ($n = 3$).

about 95% of the yield as compared to that at the plateau formation.

3.2. Stability of the derivatives

The stabilities of (+)-menthol and (−)-menthol derivatives at room temperature after completing the derivatization procedure were studied over a period of 24 h. No significant changes in the peak-area ratios of (+)-menthol or (−)-menthol derivative to the I.S. were found, indicating that the derivatives are sufficiently stable for the time required for their analysis.

3.3. Selectivity of the method

The selectivity of the method to the analysis of (+)-menthol and (−)-menthol was tested on the separation of a standard mixture of menthol related hydroxy compounds (each 50 μM in toluene) commonly found in mint plants [17], including (+)-menthol and (−)-menthol, (±)-linalool, (±)-terpinen-4-ol and α-terpineol (Fig. 4). The standard mixture was derivatized by the procedure for (+)-menthol and (−)-menthol (Section 2.3). The results indicate that no significant peaks appeared in the chromatogram with the same locations (retention times) of the I.S., (+)-menthol and (−)-menthol (data not shown).

Fig. 4 shows that (±)-linalool, (±)-terpinen-4-ol and α-terpineol are tertiary alcohols that usually bear higher steric hindrance than secondary alcohols ((+)-menthol and (−)-menthol) to a nucleophilic derivatization. As a consequence, no detectable derivatives found under present derivatization conditions, but at higher concentration of the tertiary alcohols (each at about 580 μM), minor peaks of compounds **III**, **IV** and **V** were found (compounds **III**, **IV** and **V** at that high concentration seem unable to exist with (+)-menthol and (−)-menthol in the mint plant). This suggests that the present method is selective.

3.4. Mass spectral analysis of the derivatives

The derivatives of (+)-menthol and (−)-menthol were synthesized by scaling up the amount (6.4 mmol) of (+)-menthol or (−)-menthol with similar procedure to that indicated in Section 2.3 without adding the I.S. The purified derivatives were examined by GC–MS with the following conditions: ThermoFinnigan GC with Finnigan PorisQ MS using an HP-5MS column (25 m, 0.2 mm I.D., 0.33 μm d_f); carrier gas: He with the flow rate at 1.0 ml/min; injector, column and interface temperatures respectively as 200, 230 and 275 °C, and the ionization mode of EI with an acceleration energy of 70 eV. The mass spectrum obtained from the (+)-menthol or (−)-menthol derivative each exhibited a molecular peak at *m*/*z* 368, and a base ion peak at *m*/*z* 185, corresponding to the methoxy naphthyl ethylidene fragment ($CH_3OC_{10}H_6CHCH_3$). There are no mass spectrum differences between the derivatives of (+)-menthol and (−)-menthol under present GC–MS conditions studied.

3.5. Application

3.5.1. Quantitative applicability

Based on the optimized conditions, quantitative applicability of the method for the determination of (+)-menthol and (−)-menthol in reference solution was first evaluated over the range 5–50 μM. Calibration graphs were established with y for the peak-area ratios of each derivative to the I.S., and x for the concentration (μM) of the analyte. The linear regression equations obtained are $y = (0.0692 \pm 0.0010)x + (0.0172 \pm 0.0118)$ with a correlation coefficient $r = 0.999$ ($n = 5$) for (+)-menthol and $y = (0.0672 \pm 0.0016)x + (0.0283 \pm 0.0306)$ with a correlation coefficient $r = 0.999$ ($n = 5$) for (−)-menthol. A typical chromatogram is shown in Fig. 5.

This suggests that quantitative enantiomeric analysis of the analytes in reference solution is feasible. Further application of the method to the analysis of (+)-menthol and (−)-menthol in real sample solutions was preformed.

3.5.2. Analysis of (+)-menthol and (−)-menthol in real sample

(+)-Menthol and (−)-menthol spiked in sample blank solution (Section 2.4.1) for calibration was prepared at five levels of (+)-menthol and (−)-menthol (5–50 μM each). Calibration graphs were established as indicated in Section 3.5.1. The linear regression equations obtained are $y = (0.0535 \pm 0.0013)x + (0.0878 \pm 0.0508)$ with a cor-

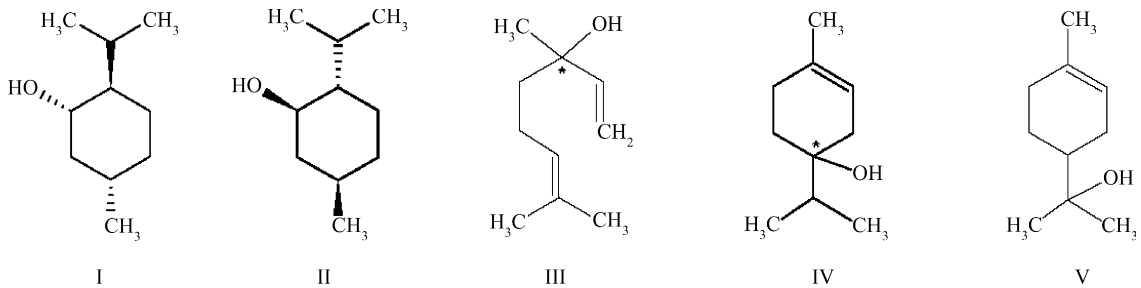

Fig. 4. Structures of menthol related compounds with an alcohol function. Compounds **I**, (+)-menthol; **II**, (−)-menthol; **III**, (±)-linalool; **IV**, (±)-terpinen-4-ol and **V**, α-terpineol.

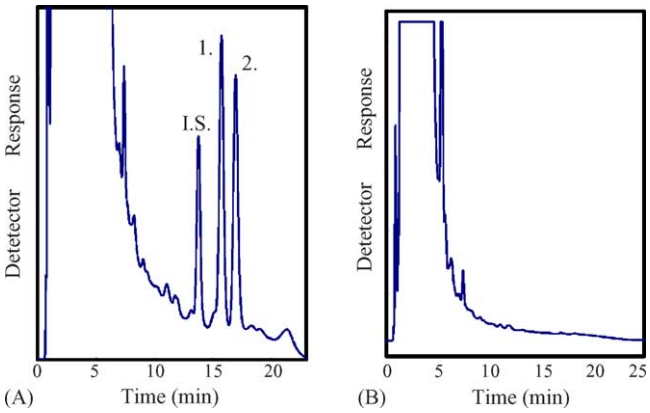

Fig. 5. HPLC chromatograms for (A) (+)-menthol and (−)-menthol (25 μM each) derivatized with naproxen acyl chloride and (B) reagent blank. Peaks 1: (+)-menthol; 2: (−)-menthol derivatives and I.S. for internal standard. HPLC conditions: column, RP-C_8 (250 mm × 4 mm I.D.; 5 μm); mobile phase: methanol:water:THF, 80:18:2 (v/v); flow rate, 1.2 ml/min; injection volume, 10 μl; fluorescence detection: λ_{ex}: 235 nm, λ_{em}: 350 nm.

relation coefficient $r = 0.999$ ($n = 5$) for (+)-menthol and $y = (0.0526 \pm 0.0012)x + (0.0909 \pm 0.0254)$ with a correlation coefficient $r = 0.999$ ($n = 5$) for (−)-menthol. The detection limit (S/N = 3, sample size, 10 μl) of each analyte is about 1 μM.

The intra- and inter-day precisions and accuracy of the method were studied based on the peak-area ratios for the analysis of (+)-menthol and (−)-menthol spiked at 10, 30 and 45 μM. The results (Table 1) indicate that the relative standard deviations (RSD) for the intra-day ($n = 5$) and inter-day ($n = 5$) analyses are all below 3.5 and 4.1, respectively and the relative errors (RE) for the intra-day ($n = 5$) and inter-day ($n = 5$) analyses are all below 4.3 and 3.6, respectively for (+)-menthol and (−)-menthol analyses.

This method was used to the analysis of the menthol in *M. piperita* 'Swiss' and *Mentha* × *piperita* 'Chocolate'. The results indicate that the (−)-menthol contents

Table 1
Precision and accuracy for the analysis of (+)-menthol and (−)-menthol

Concentration known (μM)	Concentration found (μM)	RSD (%)	RE (%)
(+)-Menthol			
Intra-day ($n = 5$)			
10	10.16 ± 0.31	3.02	1.65
30	30.16 ± 1.04	3.46	0.55
45	43.81 ± 0.76	1.74	−2.64
Inter-day ($n = 5$)			
10	10.12 ± 0.28	2.77	1.21
30	30.18 ± 1.20	3.98	0.39
45	44.72 ± 1.08	2.41	−0.62
(−)-Menthol			
Intra-day ($n = 5$)			
10	10.43 ± 0.32	3.05	4.29
30	31.28 ± 0.59	1.88	4.28
45	44.16 ± 0.78	1.77	−1.86
Inter-day ($n = 5$)			
10	10.35 ± 0.33	3.18	3.55
30	30.51 ± 1.24	4.07	1.71
45	44.76 ± 1.48	3.30	−0.54

in *M. piperita* 'Swiss' and *Mentha* × *piperita* 'Chocolate' are 0.101 ± 0.007 ($n = 3$) and 0.685 ± 0.023 ($n = 3$) mg/g of freshly collected leaves.

Fig. 6A gives the chromatogram for the analysis of the sample blank solution (Section 2.4) without observing the presence of (+)-menthol or (−)-menthol. Fig. 6B and C respectively show the typical chromatograms for the analysis of (−)-menthol in *M. piperita* 'Swiss' and *Mentha* × *piperita* 'Chocolate'. A minor peak appears at the retention time (t_R) of about 15.9 min equivalent to that of (+)-menthol derivative (Fig. 6B and C). This was further checked by spiking the reference (+)-menthol derivative to the derivatized sample solutions of both plants each leading to the increment of the target minor peak. No report is available for (+)-menthol existed in a menthol plant. Further confirmation of the tar-

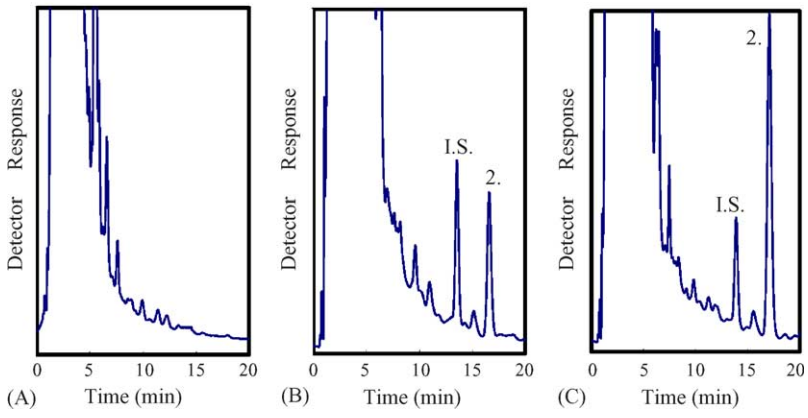

Fig. 6. HPLC chromatograms for the analysis of (A) the toluene extract of *Mentha spicata* leaves (used as sample blank) derivatized with naproxen acyl chloride, (B) the toluene extract of *Mentha piperita* 'Swiss' leaves, and (C) the toluene extract of *Mentha* × *piperita* 'Chocolate' leaves. Peak 2: (−)-menthol and I.S. for internal standard. HPLC conditions: column, RP-C_8 (250 mm × 4 mm I.D.; 5 μm); mobile phase: methanol:water:THF, 80:18:2 (v/v); flow rate, 1.2 ml/min; injection volume, 10 μl; fluorescence detection: λ_{ex}: 235 nm, λ_{em}: 350 nm.

get minor peak related to (+)-menthol was briefly studied by GC–MS. A 200.0 mg aliquot of freshly collected mint leaves from each menthol plant was extracted with 4.0 ml of toluene (see Section 2.4.2). The filtered toluene extract (3.0 ml) was subjected to the derivatization (Section 2.3) with excess naproxen acyl chloride (1.0 M × 0.9 ml). After concentrating (by rotary evaporator) to about 1 ml, the derivatized sample solution was separated on a preparative TLC plate (Merck silica gel 60 F_{254}, 20 cm × 20 cm, 0.5 mm) with a mobile phase of dichloromethane. The separated band with an R_f value of about 0.7 (equivalent to (+)-menthol and (−)-menthol derivatives used as control) was collected in a beaker and extracted with methanol (2 ml). The concentrated methanol solution (about 0.4 ml) was analyzed by GC–MS (1 μl injected) with the conditions: ThermoFinnigan GC with Finnigan PorisQ MS using an Rtx-5MS column (30 m, 0.25 mm I.D., 0.25 μm d_f); carrier gas: He with the flow rate at 1.0 ml/min; operating temperatures of injector (250 °C), interface (275 °C) and column (step-wise programming initially at 100 °C for 2 min → 50 °C/min for 3 min → 250 °C for 5 min → 25 °C/min for 2 min and then held at 300 °C), and the ionization mode of EI with an acceleration energy of 70 eV. The mass chromatograms from the samples of *M. piperita* 'Swiss' and *Mentha* × *piperita* 'Chocolate' each showed a minor peak with a t_R of 12.04 min (corresponding to that of the (+)-menthol derivative used for comparison) and a major peak with a t_R of 12.15 min (corresponding to that of the (−)-menthol derivative used). The mass spectra for the minor and major peaks from both samples each exhibited the diagnostic peaks of *m*/*z* 368 (molecular ion) and *m*/*z* 185 as mentioned in Section 3.4. This suggests that the samples from freshly collected mint leaves of *M. piperita* 'Swiss' and *Mentha* × *piperita* 'Chocolate' each may contain (+)-menthol if the conformational conversion of (−)-menthol to (+)-menthol does not occur in the preparation and analysis of the sample. The contents (mg/g of mint leaves) of (+)-menthol in the *M. piperita* 'Swiss' and *Mentha* × *piperita* 'Chocolate' were 0.015 ± 0.001 ($n = 3$) and 0.047 ± 0.002 ($n = 3$) respectively, as analyzed by the method stated in Section 2.4.2.

In conclusion, a simple and sensitive fluorimetric HPLC was developed for the quantitative enantiomeric analysis of (+)-menthol and (−)-menthol in mint. The method is based on labeling the analytes with a chiral fluorophore for sensitive detection and also renders the enantiomeric analytes resolvable as diastereomeric derivatives on a conventional C_8 column. Toluene is used as a derivatization solvent as well as an extraction solvent that favorably cuts the analytical time. The method is simple and selective for the analysis of (+)-menthol and (−)-menthol. Further application of the method to the pharmacological and biosynthesis studies of (+)-menthol and (−)-menthol could be interesting.

Acknowledgement

H.-L. Wu is grateful to the National Science Council (Taiwan) for financial support of this work (NSC93-2320-B-037-008).

References

[1] The Merck Index, 13th ed., Merck, Whitehouse Station, NJ, 2001, pp. 1043–1044.
[2] R. Eccles, J. Pharm. Pharmacol. 46 (1994) 618.
[3] USP 25/NF 20, United States Pharmacopeial Convention, Rockville, MD, 2002, p.1072.
[4] N. Galeotti, L.D.C. Mannelli, G. Mazzanti, A. Bartolini, C. Ghelardini, Neurosci. Lett. 322 (2002) 145.
[5] K. Hamasaki, K. Kato, T. Watanabe, Y. Yoshimurs, H. Nakazawa, A. Yamamoto, A. Matsunaga, J. Pharm. Biomed. Anal. 16 (1998) 1275.
[6] M. Ligor, B. Buszewski, J. Chromatogr. A 847 (1999) 161.
[7] L. Karuza, K. Folivarski, J. Pharm. Biomed. Anal. 15 (1996) 419.
[8] J.S. Valdez, D.K. Martin, M. Mayersohn, J. Chromatogr. B 729 (1999) 163.
[9] J. Rohloff, J. Agric. Food Chem. 47 (1999) 3782.
[10] R. Lin, J. Tian, G. Huang, T. Li, F. Li, Biomed. Chromatogr. 16 (2002) 229.
[11] J. Rohloff, J. Agric. Food Chem. 50 (2002) 1543.
[12] S.A. Haut, M.T. Core, J. Liq. Chromatogr. 4 (10) (1981) 1869.
[13] I. Caraballo, M. Fernandez-Arevalo, M.-A. Holgado, M.-T. Vela, A.-M. Rabasco, J. Pharm. Sci. 83 (8) (1994) 1147.
[14] Y. Tsuruta, Y. Date, K. Kobashi, Anal. Sci. 7 (1991) 411.
[15] C. Bicchi, G. Artuffo, A. D'Amato, V. Manzin, J. High Resolut. Chromatogr. 16 (1993) 209.
[16] B. Faber, A. Dietrich, A. Mosandl, J. Chromatogr. A 666 (1994) 161.
[17] C. Bicchi, A. D'Amato, P. Rubiolo, J. Chromatogr. A 843 (1999) 99.
[18] Y. Kasai, M. Watanabe, N. Harada, Chirality 15 (2003) 295.
[19] E.J.F. Franssen, J. Koiter, C.A.M. Kuipers, A.P. Bruins, F. Moolenaar, Dick de Zeeuw, W.H. Kruizinga, R.M. Kellogg, D.K.F. Meijer, J. Med. Chem. 35 (1992) 1246.