Journal of Pharmaceutical and Biomedical Analysis 51 (2010) 439–442

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Isolation and analysis of bioactive diterpenoids in *Salvia* species (*Salvia chionantha* and *Salvia kronenburgiii*) by micellar electrokinetic capillary chromatography

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ARTICLE INFO

Article history:
Received 15 January 2009
Received in revised form 7 April 2009
Accepted 13 April 2009
Available online 19 April 2009

Keywords:
Capillary electrophoresis
MEKC
Diterpenoids
Salvia
Plant
Horminone
7-O-acetylhorminone

ABSTRACT

In the present work, we isolated two bioactive diterpenoids, horminone and 7-O-acetylhorminone and developed a micellar electrokinetic chromatography (MEKC) method for the simultaneous quantitative analysis of them in Turkish *Salvia* species. The optimal separation electrolyte was 50 mmol/L SDS and 25% methanol at pH 11.5. The limits of detection (S/N = 3) were 3.269 and 4.518 μg/mL for horminone and 7-O-acetylhorminone, respectively. The method has been applied successfully to analyze these two components in *Salvia chionantha* and *Salvia kronenburgii* acetone extracts.

1. Introduction

Plants contain a large amount of structurally diverse chemical compounds. Medicinal plants have important source to invent potential drugs and safe antioxidant compounds. Many novel bioactive compounds have been isolated and identified from plant materials. However, isolation and purification step from plants is usually difficult, time consuming and expensive process. There has been a good number of research work on the identification of novel compounds from different plants, however the research work on the quantitative analysis of especially novel bioactive compounds are relatively few, mainly because of the difficulties in finding standard samples. The yield of purified secondary metabolites of plants depends on many factors, which cover growing, collection, drying conditions and extraction methods, etc. Many pure plant metabolites cannot easily found commercially and they are very expensive, in general. Their synthesis is not possible always or contains many steps, or overall yield is limited. Moreover, synthesis of them is not reasonable or efficient way without screening their bioactivity.

One of the bioactive compound is an abietane diterpene horminone and its derivatives isolated from many Labiatae (Lamiaceae) family plants, particularly *Salvia* [1], *Plectranthus* [2,3] and *Coleus* [4] species which are rich in abietane diterpenoids, as well as some other family plants. The Lamiaceae family comprises 200 genera and 3000 species [5]. From this family, 28 genera are widely distributed and over 240 species are endemic to Turkey [6], some species belong to *Mentha*, *Origanum*, *Thymus*, *Thymbra*, *Rosmarinus*, *Salvia*, etc. genera have been used as culinary herbs [7] with antioxidant and antiseptic properties. Biological activity of diterpenoids isolated from Turkish Lamiaceae plants, are reviewed lately by Topçu and Gören [8].

One of the largest genera of the mint family is *Salvia* with over 900 species in the world distributed throughout, especially in the countries near to Equator zone from America to China [9] while 90 species grown in Turkey, half of them being endemic. *Salvia* species are also important medicinal plants and have been used in the treatment of many disorders, such as tuberculosis, hemorrhage, hepatitis, and menstrual disorders. They possess antiseptic, antibacterial [10], cardioactive [11], antioxidant, antidementia [12], cytotoxic [13], antiviral [14], carminative, diuretic, hypoglycemic [15], hemostatic, wound healing, spasmolytic, stomachic, tranquilizer and sedative effects. Identification of Turkish *Salvia* diterpenoids has been studied with various biological activity by Ulubelen and Topçu for over 30 years [16].

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doi:10.1016/j.jpba.2009.04.012

Horminone and 7-O-acetylhorminone and other horminone derivatives have been isolated from many Turkish *Salvia* species [1,16–20] investigated for diterpenoids. Most of abietane diterpenoids including horminone derivatives which have aromatic or quinoid C ring exhibit antioxidant properties. The main problem in their isolation is to separate them from each other. Because, they have been oftenly isolated together from plant extracts, although there is a difference with a presence of an acetyl group in 7-O-acetylhorminone (=7-acetoxyroyleanone) compared to horminone (7,12-dihydroxyabieta-8,12-diene-11,14-dione).

Abietane quinoid diterpenes including horminone and 7-O-acetylhorminone showed many biological activities, namely cytotoxic, antiviral, antioxidant, antibacterial, particularly antituberculous activities. Horminone and its derivative 12-methyl-5-dehydrohorminone besides several abietane diterpenes, isolated from an Anatolian endemic species, *S. multicaulis*, have been investigated for antituberculous activity [21], and horminone showed a good activity, and particularly 12-methyl-5-dehydrohorminone and 12-demethylmulticauline showed noticeable activity against *Mycobacterium tuberculosis* which exhibited equal or better activity than clinically used standard tuberculostatic agents streptomycin, kanamycin, rifampicin, isonicotinic acid hydrazide, etc. In a very recent study carried out by our group on antioxidant activity of 7-O-acetylhorminone and a group of isolated abietane diterpenoids from *Salvia barrelieri*, 7-O-acetylhorminone showed the highest activity which is almost equal to that of L-ascorbic acid, the well known antioxidant, even it exhibited more potent activity than to those of BHT (butylated hydroxytoluen) and tocopherol (Vit. E) [22]. Cytotoxic activity of horminone type abietanes was first investigated by Kupchan et al. [23] by testing royleanone (7-dehydroderivative of horminone), taxodione, taxoquinone, taxodone, sugiol and Δ^5-dehydrosugiol against the Walker carcinosarcoma in rats, and royleanone has not exhibited meaningful cytotoxic activity. However, cytotoxic activity studies are going on royleanone, horminone and their derivatives (abietane quinones) with different cell lines [24]. In a study, reported by Slamenova et al. [25] royleanone, horminone and acetylhorminone were tested for their cytotoxic and DNA-damaging activity in human carcinoma cells Caco-2 and human hepatoma cells HepG2 cultured *in vitro*. Horminone derivatives have been also screened by Topçu et al. [26] as well as other scientists against different cancer cell lines [27].

Antimicrobial activity of royleanone, horminone and 7-O-acetylhorminone were reported against gram positive bacteria *Staphylococcus aureus*, isolated from *Salvia officinalis* [25]. In another study on a Turkish *Salvia* species, *S. blaepharochelana*, horminone was found to be active against several bacteria [10].

The capillary electrophoretic methods are particularly suitable in the analysis of complex natural matrices, owing to their higher resolving power. The advantage of the CE methods is the considerable diminutions in the sample preparation and analysis times, as well as in the sample and reagent consumption. The successful applications of capillary electrophoretic methods to the analysis of plant ingredients have been increased lately [28–32]. Ganzela evaluated the importance of CE for quality control of herbal medicinals and reviewed the very recent CE applications on medicinal plants or herbal products [33].To our knowledge, there is not any quantitative analysis report on horminone and/or 7-O-acetylhorminone in the literature, except a TLC analysis report on horminone in *Sphacele chamaedryoides* [34]. The purpose of this work is to separate two bioactive plant ingredients that are structurally very close to each other, using the powerful MEKC separation method, and thereby to determine their quantities within the plant.

2. Experimental

2.1. Materials

Aerial parts of *Salvia chionantha* Boiss. were collected from South-Western Turkey (Tefenni-Burdur) in July 2006. Aerial parts of *Salvia kronenburgiii* Rech. Fil. were collected from Eastern Turkey (Van, Kurubaş Geçidi, 1800 m) in July 2005. The voucher specimens were deposited in the Herbarium of Van Yüzüncü Yıl University (VAN F12936) for *S. kronenburgiii* Rech. Fil. and (TSP 1002) in the Herbarium of Muğla University, Chemistry Dept. for *S. chionantha.* Silicagel 60 was used for column chromatography and Kieselgel 60F_{254} (E. Merck) for prep. TLC as precoated plates.

All solvents used were in analytical grade. Stock solutions of both were prepared in methanol. All solutions were stored at 4 °C prior to use.

2.2. Isolation and purification of horminone and 7-O-acetylhorminone from plant material

Dried and powdered aerial parts of two *Salvia* species were extracted with acetone at room temperature. The acetone extracts were evaporated to dryness *in vacuo*. The residue of acetone extract was fractionated on a silica gel column eluting with first petrol ether, a gradient of dichloromethane and then acetone. The last fractions were eluted with 100% acetone. Similar fractions were combined, six main fractions were obtained. The third fraction rechromatographed on a silica gel column eluting with hexane and a gradient of dichloromethane up to 100%, and elution was ended with acetone–dichloromethane (1:1). Fraction obtained during the elution by dichloromethane–acetone (9:1) yielded horminone and 7-O-acetylhorminone, together. Two compounds were finally purified by repeated preparative TLC plates developing on silica gel plates using toluene as solvent.

2.3. Identification of diterpenoids

The identification of them was verified by ^{1}H and ^{13}C NMR, MS and IR spectral analyses as well as some physical properties, and they were compared with literature data [3].

Horminone: m.p.: 178 °C; $[\alpha]_D$ −130° ($CHCl_3$, c = 0.1); UV λ^{MeOH} (nm) ($\log\varepsilon$): 400 (2.0), 272 (4.2), 218 (3.4); IR $\gamma_{max}^{CHCl_3}$ (cm^{-1}): 3358, 2926, 1727, 1646, 1624, 1600, 1459, 1393, 1374, 1327, 1251, 1161, 1127, 1059, 1022, 980, 947, 902, 860; EI-MS: 332 $[M]^+$ (12) for $C_{20}H_{28}O_4$, 314 $[M\text{-}H_2O]^+$ (8), 299 $[314\text{-}Me]^+$ (9), 261 (10), 231 (8), 219 (6), 195 (27), 167 (38), 149 (100), 123 (13), 113 (14), 97 (17), 83 (35), 71 (42), 69 (45), 57 (86); ^{1}H NMR ($CDCl_3$, δ, 500 MHz): 0.92 (3H, s, H-19), 0.98 (3H, s, H-18), 1.23 (6H, d, J = 7.0 Hz, H-16 and H-17), 1.24 (3H, s, H-20), 2.70 (1H, dt, J = 3.0, 3.2, 13.0 Hz, H-1β), 3.17 (1H, septet, J = 7.0 Hz, H-15), 4.72 (1H, d, J = 3.0 Hz, H-7(), 7.22 (1H, brs, 12-OH). ^{13}C NMR ($CDCl_3$, δ, 125 MHz): 35.4 (C-1), 18.8 (C-2), 41.9 (C-3), 33.4 (C-4), 44.3 (C-5), 25.5 (C-6), 63.2 (C-7), 142.2 (C-8), 147.5 (C-9), 38.4 (C-10), 182.7 (C-11), 152.4 (C-12), 124.8 (C-13), 188.8 (C-14), 24.3 (C-15), 19.2 (C-16), 19.4 (C-17), 33.6 (C-18), 22.0 (C-19), 18.9 (C-20).

7-O-acetylhorminone: m.p.: 213 °C; $[\alpha]^D$ −14° ($CHCl_3$, c = 0.1); UV λ^{MeOH} (nm) ($\log\varepsilon$): 402 (3.0), 272 (4.5), 216 (3.4); IR $\gamma_{max}^{CHCl_3}$ (cm^{-1}): 3290, 2959, 2871, 1715, 1652, 1634, 1604, 1455, 1424, 1392, 1377, 1340, 1310, 1263, 1240, 1208, 1153, 1107, 1075, 1056, 1040, 1023, 1007, 983, 963, 946, 899, 877, 855, 843, 821, 759, 687, 648; EI-MS: 374 $[M]^+$ (4) for $C_{22}H_{30}O_5$, 332 $[M-COCH_3+H]^+$ (78), 314 $[332\text{-}H_2O]$+ (88), 299 $[314\text{-}Me]^+$ (51), 281 (10), 271 (23), 187 (21), 167 (26), 149 (68), 119 (23), 109 (29), 91 (33), 83 (52), 69 (75), 55 (100); ^{1}H NMR ($CDCl_3$, δ, 500 MHz): 0.87 (6H,s, H-18 and H-19), 1.22 (6H, d, J = 7.0 Hz, H-16 and H-17), 1.24 (3H, s, H-20), 2.02 (3H, s, 7-OAc),

2.72 (1H, dt, J = 3.0, 3.2, 13.1 Hz, H-1β), 3.15 (1H, septet, J = 7.0 Hz, H-15), 5.92 (1H, d, J = 3.0 Hz, H-7(), 7.15 (1H, brs, 12-OH).

^{13}C NMR ($CDCl_3$, δ, 125 MHz): 35.9 (C-1), 18.6 (C-2), 41.6 (C-3), 33.7 (C-4), 45.8 (C-5), 24.9 (C-6), 65.0 (C-7), 140.2 (C-8), 148.5 (C-9), 39.7 (C-10), 183.5 (C-11), 151.0 (C-12), 124.9 (C-13), 186.0 (C-14), 24.7 (C-15), 19.8 (C-16), 19.9 (C-17), 33.3 (C-18), 21.9 (C-19), 18.8 (C-20), 21.7 ($CO\underline{C}H_3$), 169.8 ($\underline{C}OCH_3$).

2.4. Preparation of plant extracts for injection to capillary column

Dried and powdered of the aerial parts of *S. chionantha* (root) and *S. kronenburgiii* (20 g of each) were extracted with acetone. Eluents evaporated to dryness *in vacuo* and dissolved in 5 mL methanol. This solution was diluted 1:10 with methanol, filtered using a microfilter with a pore size of 0.20 μm, and injected to the capillary column.

2.5. Apparatus and operating conditions

Separations were performed with an Agilent capillary electrophoresis system equipped with a diode-array detector. The data processing was carried out with the Agilent ChemStation software. The wavelength was set at 230 nm. The separation was performed at 28 kV. Injections were made at 5×10^{-3} MPa for 4 s.

The fused silica capillaries used for separation experiments were 75 μm I.D. and were obtained from Polymicro Technologies (Phoenix, AZ, USA). The total length of the capillary was 68 cm and the length to the detector was 60 cm. The fused silica capillary was conditioned prior to use by rinsing with 1 mol/L NaOH for 30 min, water for 10 min, and buffer with 10 min. The capillary was washed with 0.1 mol/L NaOH solution for 3 min and with buffer for 2 min between the runs.

The spectra were recorded with the following instruments; IR: PerkinElmer 1615 in $CHCl_3$; NMR: Bruker AC-200 L, 200 MHz and 50.32 MHz for ^{1}H- and ^{13}C-NMR and Varian-Innova-500 'Defne', 500 MHz and 125 MHz for ^{1}H- and ^{13}C-NMR, respectively, in $CDCl_3$; MS: VG ZabSpec HRMS and EI-MS; Melting point: Reichert-Kofler.

3. Results and discussion

The structures of horminone and 7-O-acetylhorminone shown in Fig. 1 suggest that they can be separated by MEKC. Thus, a micellar electrokinetic capillary chromatographic method was set up using sodium dodecyl sulfate (SDS) as the micelle forming surfactant in borate buffer. Optimization experiments with SDS, organic solvent and buffer concentrations showed that a separation electrolyte containing 50 mmol/L SDS, and 25% methanol at pH 11.5 could separate the two ingredients in the plant extract completely.

Fig. 2 shows the separation obtained for a *S. chionantha* root extracts. Peaks numbered as 1 and 2 correspond to horminone and 7-O-acetylhorminone, respectively. Peak identification was performed by spiking the samples with standard compounds and by UV spectral analysis. The purity of each peak in the extracts was better than 96% as calculated from the peak purity spectrum.

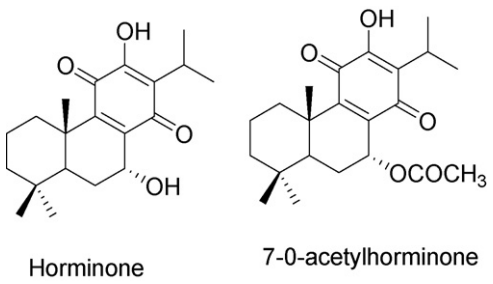

Fig. 1. Chemical structures of horminone and 7-O-acetylhorminone.

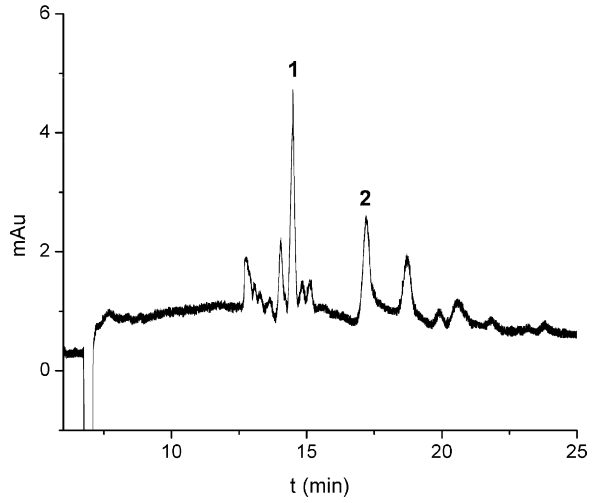

Fig. 2. Electropherogram of *Salvia chionantha* (root); separation electrolyte: 50 mM SDS, 25% metanol (pH:11.5); applied voltage 28 kV; capillary: total length 68 cm, effective length 60 cm, and internal diameter 75 μm; temperature: 25 °C; injection: 5×10^{-3} MPa for 4 s; detection: 230 nm; peaks: (1) horminone; (2) 7-O-acetylhorminone.

Fig. 3A and B shows the online peak spectra of horminone and 7-O-acetylhorminone, respectively, which were obtained by the photodiode array detector under the chromatographic conditions.

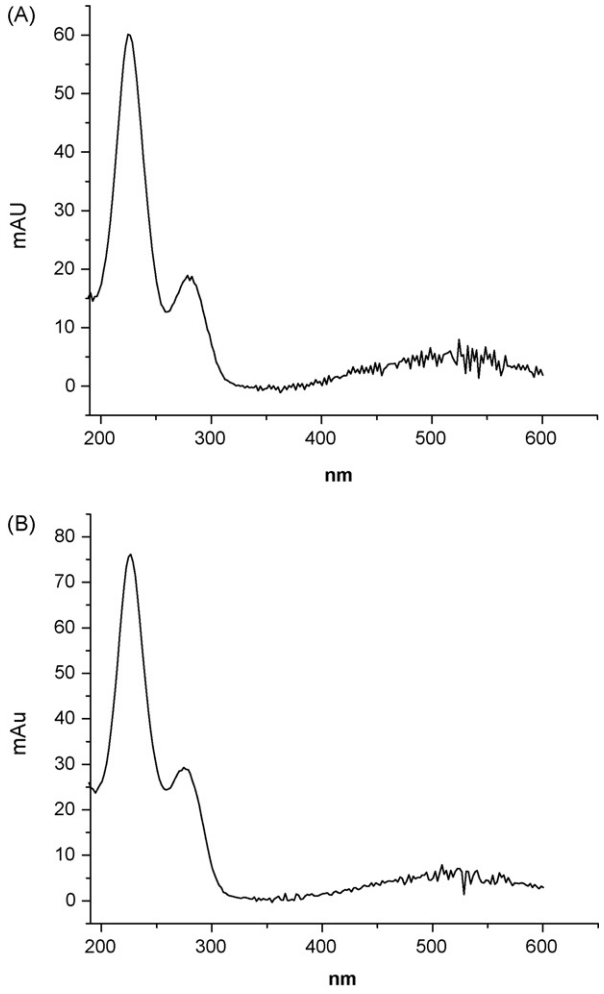

Fig. 3. Online peak spectra of (A) horminone and (B) 7-O-acetylhorminone.

The purified horminone amount was insufficient for calibration experiments. Because of the spectral similarity of two compounds, we used the calibration curve of 7-O-acetylhorminone for the quantification of both compounds.

4. Validation of the method

4.1. Linearity range

7-O-acetylhorminone purified from plant was used for quantification. The linearity for 7-O-acetylhorminone was checked using five levels of concentrations (10, 20, 30, 50, and 100 μg/mL). The regression equation for 7-O-acetylhorminone was $y = 2.13969 \times 10^{-3}x - 7.43564 \times 10^{-3}$ ($r = 0.99940$). Horminone amount in plants was calculated from calibration graph of 7-O-acetylhorminonone by making equivalent adjusment due to structural and spectral similarity.

4.2. Limits of detection

The limit of detection (LOD) was obtained as the concentrations of the horminone and 7-O-acetylhorminone that caused a peak with a height three times the baseline noise level. LOD was calculated as 3.269 and 4.518 μg/mL for horminone and 7-O-acetylhorminone, respectively.

4.3. Precision

The precision of method was calculated as the coefficient of variation (CV) of migration times and peak areas for five successive injections of standard samples. Repeatability of migration times was 1.58 and 2.86 as %R.S.D. and repeatability of peak areas was 2.86 and 4.73 as %R.S.D. for horminone and 7-O-acetylhorminone, respectively.

4.4. Quantitative amounts in Salvia samples

The developed MEKC method was applied to quantitative analysis of horminone and 7-O-acetylhorminone in *Salvia* species. The amount of horminone was found as 77.13 μg/g and 58.03 μg/g in *S. chionantha* and *S. kronenburgiii*, respectively. The amounts for 7-O-acetylhorminone in these plants were 33.23 μg/g and 11.73 μg/g, respectively.

5. Conclusion

In this study a simple and fast MEKC method was described for the qualitative and quantitative analysis of horminone and 7-O-acetylhorminone isolated from *Salvia* species. The sample matrix did not interfere with the analysis. Micelle formation contributed to increase both resolution and solubility of the ingredients in the extracts, and consequently well-resolved peaks was obtained by direct injection. For the fast and simple analysis of novel diterpenoids in plants, capillary electrophoretic methods are recommended.

Acknowledgement

We thank the TUBITAK (Grant TBA6-2312, 103T053) for financial support.

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